Ana Catarina Rodrigues Alves

Advances on anthracyclines-lipid membrane interactions: a biophysical approach

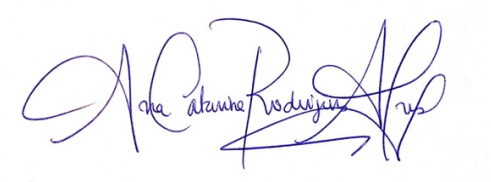
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“Learning never exhausts the mind.”

- Leonardo da Vinci

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List of Abbreviations

Abs	Absorbance
BAM	Brewster angle microscopy
Chol	Cholesterol
CL	Cardiolipin
ELS	Electrophoretic light scattering
DAN	Daunorubicin
DLS	Dynamic light scattering
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DOX	Doxorubicin
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
FWHM	Full width at half maximum
G	Gaseous phase of the monolayers
GUVs	Giant unilamellar vesicles
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IR	Infrared
L	Liquid phase (monolayers)
LC	Liquid condensed phase (monolayers)
l_d	Liquid-disordered phase
LE	Liquid expanded phase (monolayers)
l_o	Liquid-ordered phase
LUVs	Large unilamellar vesicles
MLVs	Multilamellar vesicles
MVs	Multivesicular vesicles
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PM-IRRAS	Polarization-modulation infrared absorption-reflection spectroscopy
PS	Phosphatidylserine
S	Solid phase of the monolayers
SAXS	Small-angle X-ray scattering
SUVs	Small unilamellar vesicles

TMA-DPH	1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene
UVs	Unilamellar vesicles
UV-Vis	Ultraviolet visible
WAXS	Wide-angle X-ray scattering

List of Symbols

A	Area per molecule
A_o	Limiting area per lipid molecule
C	Crystalline phase
C_s^{-1}	Elastic modulus
D	Derivative spectrum
d-spacing	Repeat distance
G	Anisotropy instrumental correction factor
H_I	Hexagonal phase
H_{II}	Inverted hexagonal phase
I	Fluorescence intensity
K_D	Stern-Volmer constant for dynamic quenching
$K_{o/w}$	Partition coefficient in an octanol/water system
K_p	Partition coefficient in a lipid/water system
K_q	Bimolecular quenching constant
K_S	Stern-Volmer constant for static quenching
K_{SV}	Stern-Volmer constant
$[L]$	Lipid concentration
L	Lamellar phase (bilayers)
L_α	Liquid-crystalline (monolayers) or fluid phase (bilayers)
L_β	Untilted solid-crystalline (monolayers) or gel phase (bilayers)
$L_{\beta'}$	Tilted solid-crystalline (monolayers) or gel phase (bilayers)
L_c	Crystalline phase (bilayers)
M	Micellar phase
$P_{\beta'}$	Ripple phase
pK_a	Logarithmic acid dissociation constant
$[Q]$	Quencher total concentration
$[Q]_m$	Effective or membrane quencher concentration
Q	Cubic phase
R	Reflectivity
r_∞	Limiting value of anisotropy
r_{ss}	Steady-state fluorescence anisotropy
T	Temperature

T_m	Main phase transition temperature
T_p	Pre-transition temperature
V_m	Membrane volume
V_φ	Lipid molar volume
γ	Surface tension
ε	Molar absorptivity
Θ	Angle of incidence in X-ray diffraction studies
λ_{max}	Wavelength of maximum absorbance
ξ	Correlation length
π	Surface pressure
τ	Fluorescence lifetime

Abstract

Anthracyclines are considered one of the most effective group of anticancer drugs ever developed. They bind with high affinity to DNA, through the intercalation of the drugs' planar anthraquinone group between the base pairs of the DNA's double helix. This interaction leads to a wide range of cytotoxic effects that includes the inhibition of DNA replication and, consequently, induction of cellular death.

Albeit the DNA has held the center stage as primary target, the mechanisms of action of anthracyclines are still the matter of considerable debate. In fact, gathering data strongly suggests the involvement of the plasma membrane in the cytotoxic action of these anticancer drugs. Even if the dominant mechanism is interaction with the nucleic acid, anthracyclines-membrane interactions are an unavoidable step on their path to the proposed target. First, the plasma membrane is the initial encountered barrier and a primary action on the physicochemical properties of the cell membrane could trigger a series of events responsible for their cytotoxic action. Second, to bind with the targets these drugs have to pass through the membranes, and thus their interactions with biomembranes are expected to play a key role in their mechanism of action. Furthermore, a number of physicochemical properties of the plasma membrane can be affected by anthracyclines that, ultimately, could modulate the location and activity of several proteins important for cell signaling. Consequently, different cellular events implicated in the signal transduction may be indirectly affected due to membrane perturbations by these drugs. The therapeutic and toxic effects of anthracyclines are thus, strongly influenced by their lipid affinity. Finally, it is reported that agarose-linked doxorubicin was able to kill cells, without penetrating them and just through its interaction with the plasma membrane. Despite all these evidences that the antitumor activity of anthracyclines can start from their interplay with the cellular membranes, there is still a lot to be elucidated. In this context, the aim of the present thesis was to investigate the interaction of anthracyclines with lipid membranes and to study their ability to bind to surface-active phospholipids, as well as their effect on the lipid dynamic properties of membrane models, once the results may help to further understand their mode of action.

The anthracyclines studied (daunorubicin and doxorubicin) were chosen because they are one of the most used drugs in cancer therapy but also due to their structural, chemical and functional differences. In order to identify and isolate the effect of different membrane parameters on the interplay of these drugs, biomembranes were mimic by the use of model membranes due to their structural similarities. The first step on the investigation of anthracyclines interactions with model membranes was the determination of

liposome/water partition coefficient (Kp) trough derivative spectroscopy techniques that allowed to assess and compare the extent of drug penetration and/or interaction with membrane lipids. Fluorescence quenching studies were used to preview the location of these drugs within the bilayer. Modifications of membrane biophysical properties (such as fluidity, molecular order and packing) induced by these drugs were also evaluated by fluorescence measurements of steady-state anisotropy and by small and wide angle X-ray scattering (SAXS and WAXS). Besides the 3D (i.e. bilayers), 2D models (i.e. Langmuir monolayer) were used to assess the influence of the anthracyclines compounds on the monolayers properties, such as the lateral pressure, surface area, phase transitions and compressibility. In addition, the morphology of lipid monolayers along with the effects produced by the anthracyclines on the fluidity and shape/size of lipid domains were observed by Brewster angle microscopy (BAM). The infrared absorption-reflection spectroscopy (IRRAS) allowed the study of the influence of anthracyclines on the structure of the chosen membrane models. Also, in order to complement the gathered information molecular dynamic simulations were employed.

The results obtained within this thesis provide an insight into anthracyclines' ability to be inserted into lipid bilayers and alter the lipid dynamics. The anthracyclines-membrane interactions are expressed as perturbing effects at the membrane level and include modifications in the fluidity, molecular order and packing. Furthermore, these interactions are intrinsically dependent on the lipid composition, the membrane´s physical state and structure that, ultimately, might favor or restrain the drug's mechanism of action and/or its side effects.

Keywords: biophysics; drug-membrane interactions; membrane models; anthracyclines; cancer.

Resumo

As antraciclinas são consideradas um dos grupos mais eficazes de fármacos anticancerígenos alguma vez desenvolvidos. Estas moléculas ligam-se com alta afinidade ao ADN, através da intercalação do seu grupo planar antraquinona entre os pares de bases da dupla hélice do DNA. Esta interação leva a uma série de efeitos citotóxicos que inclui a inibição da replicação do ADN e, consequentemente, à indução da morte celular.

Embora o ADN seja considerado principal alvo das antraciclinas, os seus mecanismos de ação ainda continuam a ser assunto de considerável debate. De facto, várias evidências sugerem fortemente o envolvimento da membrana plasmática na ação citotóxica destes fármacos. Mesmo que o mecanismo dominante seja a interação com o ácido nucleico, as interações das antraciclinas com a membrana são um passo inevitável no seu caminho para o alvo proposto. Em primeiro lugar, a membrana plasmática é a primeira barreira encontrada por estes fármacos, de forma que uma ação primária nas propriedades físico-químicas da membrana celular pode desencadear uma série de eventos responsáveis pela sua ação citotóxica. Em segundo lugar, para se ligarem com os alvos estes fármacos têm de atravessar as membranas e, nesse sentido, é esperado que as biomembranas desempenhem um papel fundamental no seu mecanismo de ação. Para além disso, as antraciclinas podem afetar uma série de propriedades físico-químicas da membrana plasmática e dessa forma modular a localização e a atividade de várias proteínas importantes na sinalização celular. Como consequência, diferentes eventos celulares implicados na transdução de sinal podem ser indiretamente afetados devido a perturbações membranares causadas por estes fármacos. Os efeitos terapêuticos e tóxicos das antraciclinas são, desta forma, fortemente influenciados pela sua afinidade lipídica. Por último, foi demonstrado que a doxorrubicina ligada a agarose, e por isso incapaz de penetrar a célula, era capaz de induzir morte celular apenas através da sua interação com a membrana plasmática. Apesar de todas essas evidências, de que a atividade antitumoral das antraciclinas pode começar a partir de sua interação com as membranas celulares, ainda há muito a ser elucidado. Neste contexto, o objetivo da presente tese foi investigar a interação de antraciclinas com membranas lipídicas e estudar sua capacidade de se ligar a fosfolípidos, bem como seu efeito sobre as propriedades dinâmicas dos modelos membranares, uma vez que os resultados podem ajudar a melhor compreender o seu modo de ação.

As antraciclinas estudadas (daunorrubicina e doxorrubicina) foram escolhidas por serem dos fármacos mais utilizadas na terapia do cancro, mas também devido às suas diferenças estruturais, químicas e funcionais. Com o intuito de identificar e isolar o efeito de diferentes parâmetros de membrana sobre a interação desses fármacos, as biomembranas foram imitadas através uso de modelos membranares devido às suas similaridades estruturais. O

primeiro passo na investigação das interações antraciclinas com modelos membranares foi a determinação do coeficiente de partição lipossoma/água (Kp) através de técnicas de espectroscopia derivada que permitiu avaliar e comparar a extensão da penetração e/ou interação com as membranas. Para além disso, estudos de desativação de fluorescência foram utilizados para inferir acerca da localização destes fármacos na bicamada. As alterações das propriedades biofísicas da membrana (tais como a fluidez, a ordem molecular e o empacotamento) induzidas por estes fármacos foram também avaliadas através de medições de fluorescência da anisotropia no estado estacionário e pela difração de raios X de pequeno e grande ângulo (SAXS e WAXS). Além dos modelos 3D (isto é, bicamadas), foram utilizados modelos 2D (i.e., monocamada de Langmuir) para avaliar a influência das antraciclinas nas propriedades das monocamadas, tais como a pressão lateral, a área de superfície, as transições de fase e a compressibilidade. Para além disso, a morfologia das monocamadas lipídicas bem como os efeitos produzidos pelas antraciclinas na fluidez e forma/tamanho dos domínios lipídicos foram observados por microscopia de ângulo de Brewster (BAM). A espectroscopia de reflexão de absorção no infravermelho (IRRAS) permitiu ainda o estudo da influência das antraciclinas na estrutura dos modelos membranares escolhidos. Por fim, no sentido de complementar as informações recolhidas através das técnicas mencionadas anteriormente, foram ainda efetuadas simulações moleculares.

Os resultados obtidos com esta tese fornecem uma visão sobre a capacidade das antraciclinas em interagirem com a membrana e alterar a dinâmica lipídica. As interações antraciclinas-membrana são expressas como efeitos perturbadores ao nível da membrana e incluem modificações na fluidez, ordem molecular e empacotamento lipídico. Além disso, estas interações são intrinsecamente dependentes da composição lipídica e do estado físico e estrutura da membrana que, em última instância, podem favorecer ou restringir o mecanismo de ação do fármaco e/ou dos seus efeitos secundários.

Palavras-chave: biofísica; interações fármaco-membrana; modelos membranares; antraciclinas; cancro.

Chapter I

Aim and scope of this thesis

Cancer, one of the leading cause of death worldwide, is a complex disease that results from the accumulation of mutations and/or environmental factors that together damage the cell's system of cell growth and death. Besides rapid proliferation and reduced cell apoptosis, cancer cells have the ability to infiltrate the tissues, establish a blood supply, metastasis from their initial location to other places in the body and cause malfunction of the affected organs.

Among the different treatments available for cancer, chemotherapy is one of the most commonly used. Nevertheless, despite all the progress in the design of cancer therapy there is still no cancer treatment that is 100% effective and able to increase the survival time and the patient' quality of life by reducing the undesired harmful side-effects. For that reason, effective disease targeting represents an enormous biomedical challenge in the treatment of cancer since the lack of selectivity of current therapeutics results in numerous side effects, including organ and tissue damage. Such problems constitute a huge limitation of current therapies once drugs must often be administered at suboptimal doses to reduce cytotoxicity. In this context, it became urgent to develop new drugs or new systems that can target specific differences between tumors and healthy cells and tissues and, thereby, reduce the anticancer drugs toxicity. In order to achieve that goal, it is important to better understand the mechanism of action of the existing drugs and explain why they induce so many side effects. Actually, there is still a lot to be explored and to be known and, in this particular case, regarding anthracyclines compounds.

Anthracyclines antibiotics, originally derived from *Streptomyces peucetius*, are anticancer compounds widely prescribed in human cancer chemotherapy. Although the mechanisms proposed to explain the activity of these compounds are still a matter of substantial controversy, their central action has been mainly attributed to the intercalation of the drugs' planar anthraquinone group between the base pairs of native DNA, which inhibit topoisomerase II enzyme and, consequently, unwinds DNA for transcription. Even if the dominant mechanism is this interaction with the nucleic acid, anthracyclines must pass through a variety of other organelles to reach the DNA. First, the drug must pass the cell boundary that is the plasma membrane and, then the nuclear membrane. Interaction with lipid membranes is, thus, an unavoidable step in these anticancer drugs activity, whatever the exact mechanism of action proves to be. Furthermore, the efficiency of the anthracyclines' interactions with membranes constitutes one of the most important

pharmacological features, playing an essential role on their biological activity, and should be accounted in order to obtain further knowledge about the full mechanism of action. In fact, in the early 1980s, the anticancer drugs anthracyclines were shown to kill cancer cells by a direct interaction with the plasma membrane (1). Agarose–doxorubicin complexes that cannot enter the cancer cells kill them by modulating the membrane lipid structure, and subsequently altering the localization and activity of important signaling proteins. It is also probable that, the existing limiting factors in the use of anthracyclines as antitumoral agents, such as chronic or acute cardiotoxicity and a spontaneous or acquired resistance, are due to an action at the membrane level. The gathered evidences underline that the role of lipid bilayers extends beyond a simple physical boundary and that biological membranes have a key role the cytotoxic action of these drugs (2).

Membranes form selective barriers that separate, communicate and define cells and their internal organelles, and they also receive and propagate important signals that control cellular behavior. Most of the activity of a cell occurs within or around membranes, and in this context, the functions of membranes do not depend exclusively on the particular proteins that they contain, but rather, they also depend on lipid composition and the lipid–protein structures of membranes. Of upmost importance, alterations to the membranes of a cell may be involved in various pathological processes, such as cancer. In fact, modifications in the lipid composition or structure of cell membranes have been associated with the development of this pathological state.

Moreover, the features of membranes both influence and participate in the specific functions associated with different cells and the overall control of a cell's physiology. Changes of the cell lipid structure induced by anticancer drugs can, thus, lead to disturbances in the activity of important proteins, which in turn might be associate with their antitumor activity. In this context, it seems necessary to deepen the knowledge of the anthracyclines drugs already available, namely exploring alternative aspects to the mere study of the direct interaction with the nucleic acids.

Regarding the abovementioned considerations, this thesis arises in order to perform studies that allow gathering physical and chemical aspects of the interaction of anthracyclines with biological membranes through a biophysical approach. Actually, the thorough knowledge of the interplay between cellular membranes and anthracyclines represent an important field of research because it is directly related their pharmacological activity. Besides, it is also crucial to comprehend the distribution parameters of these molecules in the body, both for the establishment of relations between their physicochemical and pharmacological properties and for the discovery of fundamental relationships between structure and activity. Ultimately, this may be helpful in the design of new compounds with greater efficacy and fewer undesirable side effects.

To accomplish this goal, the biophysical studies performed in the scope of this thesis seek the assessment of the interaction of several anthracyclines with simple membrane models (liposomes and lipid monolayers) that can mimic the biological membranes and, at the same time, allow to evaluate separately different parameters without the interference of other factors.

Membrane models, due to their composition and organization, in which the phospholipids, the basic unit of the cellular membranes, are arranged in monolayers, bilayers and, at higher level of organization, in spherical vesicles, can be perfectly characterized and manipulated, and constitute a precious instrument to study the drug-membrane interactions.

The methodologies used in the evaluation of anthracyclines-lipid interactions are based on the evaluation of the alteration of the physicochemical properties of the membrane itself or of an exogenous probe associated with its microenvironment. They allow the determination of the drug partition coefficient, its location in the lipid matrix, its effects on the surface charge and membrane fluidity, and also on other biophysical properties of the membrane phospholipids, such as impact on order and packing.

Structure

This thesis is organized in 5 chapters.

Chapter I: **Aim and scope of this thesis**. This chapter explain the motivation and the main goals of the thesis. A general description of the remaining chapters, including their organization and structure is also provided.

Chapter II: General Introduction. In this chapter a revision manuscript entitled "Biophysics in Cancer: The Relevance of Drug-Membrane Interaction Studies" is presented. The revision contains the main theoretical concepts that are important to understand the outcomes and conclusions described in this thesis. In this context, it also provides a description of the fundamental aspects related to biological membranes, especially the structural, biophysical and functional differences between cancer and normal cell membranes. The membrane model systems (such as liposomes and lipid monolayers) used to mimic biomembranes are also described with respect to their structure and chemical composition, physical properties and applications. Through the compilation of the knowledge achieved to date regarding the interplay between plasma membranes and anticancer drugs, from a biophysical perspective, the manuscript highlights the importance of studying these interactions.

Chapter III: **Methods to evaluate the anthracyclines-membrane interactions**. A general overview of the biophysical techniques, along with a description of the theoretical foundations underlying the experimental methods used throughout the thesis is given in this chapter. The studies summarized here include: the methods for determining the partition coefficient, the methods for predicting the membrane location and the methods used to assess the effects on the biophysical properties of the membrane.

Chapter IV: **Progress beyond the state-of-art.** The manuscripts originated from this thesis are presented in this chapter and include:

1. A biophysical approach to daunorubicin interaction with model membranes: relevance for the drug's biological activity
2. Influence of doxorubicin on model cell membrane properties: insights from *in vitro* and *in silico* studies
3. A synchrotron X-ray assessment regarding the nature of anthracyclines–lipid interactions
4. Daunorubicin and doxorubicin molecular interplay with 2D membrane models
5. The daunorubicin interplay with mimetic model membranes of cancer cells: a biophysical interpretation
6. Doxorubicin molecular interactions with cancer membrane models: *in vitro* and dynamic simulation studies
7. Synchrotron SAXS and WAXS investigation of the interactions of anthracyclines with lipid membranes

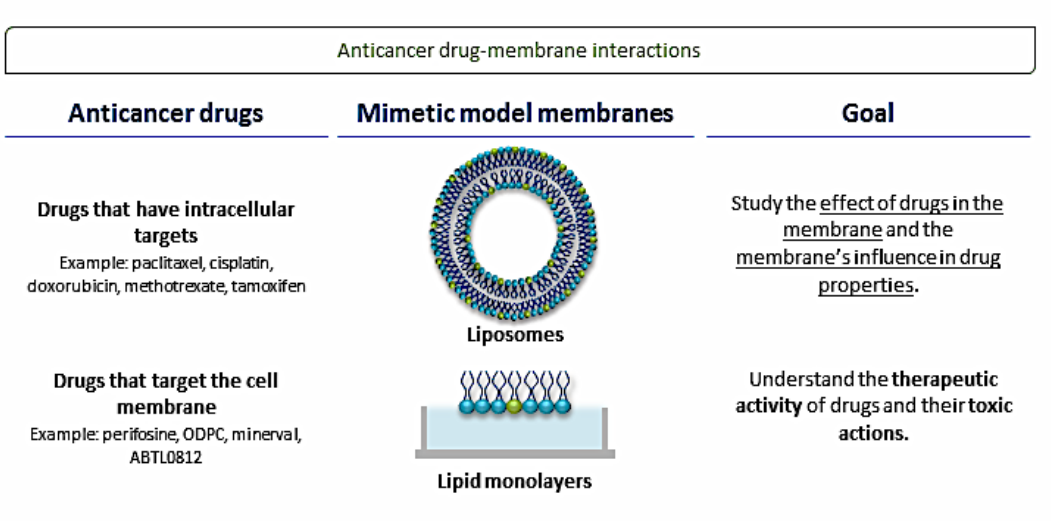
Chapter V: **Concluding Remarks.** In this final chapter, the main concluding remarks based on the findings of the studies presented in this thesis are described, as well as the future perspectives opened by the work developed.

Chapter II

General Introduction

This chapter presents a revision manuscript entitled:

Biophysics in Cancer: The Relevance of Drug-Membrane Interaction Studies



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Review

Biophysics in cancer: The relevance of drug-membrane interaction studies

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ABSTRACT

Lipidomics has been proving that membrane lipids play a crucial role in several cell functions and are involved in several pathologies, including cancer. In fact, beyond a scaffold where proteins and other components are embedded, the cell membrane can also act as a barrier or a target for anticancer drugs. From this point of view, the development of new chemotherapeutic agents should also take into account the role of the membrane in their activity. This Review aims to highlight the importance of anticancer drug-membrane interactions as a powerful strategy to improve cancer therapy. Biophysical techniques emerge, therefore, as essential tools to unveil such interactions.

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Abbreviations: DEPC, 1,2-dierucoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); DSPC, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine; CEUs, 1-aryl-3-(2-chloroethyl) ureas; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; 2OHOA, 2-hydroxyoleic acid or Minerval; ODPC, 10-(Octyloxy) decyl-2-(trimethylammonium) ethyl phosphate; APC, alkylphosphocholines; ALP, alkyllysophospholipids; AFM, atomic force microscopy; BAM, Brewster angle microscopy; CL, cardiolipin; Chol, cholesterol; CLL, chronic lymphocitic leukemia; CD, circular dichroism; DSC, differential scanning calorimetry; DLS, dynamic Light Scattering; EPC, egg L-α-phosphatidylcholine; ELS, electrophoretic light scattering; EPR, electron paramagnetic resonance spectroscopy; FTIR, Fourier transform infrared spectroscopy; L_{β}, gel phase; GUVs, giant unilamellar vesicles; HCL, hairy cell leukemia; LUVs, large unilamellar vesicles; L_{α}, liquid crystalline or fluid phase; L_o, liquid ordered phase; MDR, multi drug resistance; MLVs, multilamellar liposomes; NMR, nuclear magnetic resonance; Pgp, P-glycoprotein; T_m, phase transition temperature; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SAXS, small angle X-ray scattering; SUVs, Small unilamellar vesicles; SM, sphingomyelin; SCD1, stearoyl-CoA desaturase; SLBs, supported lipid bilayers; WAXS, wide angle X-Ray scattering.

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1. Introduction

Cellular functions in normal and pathological conditions are generally associated with the activity of specific proteins (such as channels, receptors, enzymes) and nucleic acids [1]. For that reason, most chemotherapeutic agents target those macromolecules in order to treat the related malfunction and, therefore, the disease. Anticancer drugs may act on membrane surface (in membrane proteins) or have intracellular targets (such as the DNA); therefore, through their biological journey, the interaction and/or penetration of the cell membrane becomes inevitable. Biological membranes serve as functional platforms for such proteins and are susceptible to modifications by drug-lipid interactions that, ultimately, might modulate the location and/or activity of the membrane proteins.

Even though the emergence of cancer cell may have a genetic link, evidence shows that cells, during malignant transformation, suffer alterations in their membranes' lipid profile and biophysical properties [1–3]. Moreover, changes in the type and/or amount of lipids can be associated with the different cancer states and might affect signaling cascades involved in the therapy [4]. These lipid alterations can, for example, promote resistance of cancer cells to chemotherapeutics [5], a major clinical problem that leads to therapy failure.

In this context, the interplay between anticancer drugs and the cell membrane, and its implications in the membranes' biophysical parameters, represents an important field of research. The knowledge acquired with this kind of studies may play a significant role in the design of new drugs and delivery systems, and the identification of new membrane targets that may be the base of a membrane-lipid therapy. In fact, there are already a number of anticancer drugs that exert their therapeutic effects by modulating the environment of tumor membranes [6,7].

Due to the cell membrane's complexity, many mimetic model systems and biophysical techniques have been developed and used to study anticancer drug-membrane interactions. It is important to stress that the simplification of the membranes and thus a complete control of such complex structures is critical to understand the interactions at the molecular level.

This Review aims, thereby, to explore and discuss the findings regarding the interactions between plasma membranes and anticancer drugs from a biophysical perspective. An overview of the techniques used to study drug-membrane interactions with model membranes is provided, as well as on the knowledge achieved to date.

2. Features of cancer cell membranes

Changes in membrane lipids have been described for a number of pathologies, including cancer [8]. In fact, cancer cells present diverse chemical, structural, metabolic and biophysical characteristics that are different from normal cells. Cancer is characterized by a deregulated cell proliferation in which the rapidly dividing cells require several biomolecules, including fatty acids and membrane lipids, to fulfil their needs [9]. As a consequence, tumor cells present a constitutive activation of the fatty acid biosynthesis in order to provide enough building blocks to form new membranes [10–12]. It has also been demonstrated that the phospholipid and fatty acid profiles are altered in cancers such as breast [13,14] and colorectal [15], and also in some chemoresistant cancers [16]. For example, extracellular membranes in cells from hepatocellular carcinoma contained four times more sphingomyelin (SM) and increased levels of unsaturated fatty acids, than their normal counterparts [17]. Increased levels of all phospholipids were found in human colorectal cancer cells, including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) [18]. It has also been shown that lymphocite membranes from patients with chronic lymphocitic leukemia (CLL) present a general increase in phospholipids, cholesterol (Chol), SM, glucosylceramide and lactosylceramide concentrations [19]. On the other hand, hairy cells from hairy cell leukemia (HCL) possess membranes with higher content in Chol and lower fluidity than CLL lymphocytes [20]. Furthermore, an accumulation of esterified Chol which might be related with cell cycle progression and tumor growth has been observed [21]. Additionally, evidence demonstrated that SM is reduced in a number of cancer cell membranes and that there is a relationship between its levels and tumorigenesis [22].

Cell membranes are dynamic, intricate structures where phospholipids are distributed asymmetrically across the lipid bilayer [23]. This asymmetric distribution between the two leaflets is mainly maintained by flippases, floppases and scramblases, which are proteins that differently shuttle lipids from one leaflet to another. Therefore, while flippases use ATP to move the aminophospholipids PS and PE from the plasma membrane outer monolayer to the inner monolayer, floppases use ATP to transport substrates such as PC, sphingolipids and cholesterol in the opposite direction [24]. Finally, scramblases are ATP independent and facilitate the flip-flop of lipids in a non-selective manner [24].

Normal membranes exhibit an outer leaflet that is mostly composed by zwitterionic PC and SM, while anionic PS along with most of the PE are usually comprised in the inner leaflet [23]. By contrast, a loss of this asymmetric distribution has been observed for several types of cancer [25,26], which results in the exposure of the negatively charged PS on the surface of their membranes (Fig. 1) [27,28]. Since the flippase aminophospholipid translocases maintain the distribution of both PE and PS, evidences demonstrated that PE is also located in the membrane's outer leaflet of tumor endothelial cells [29].

Apart from changes in phospholipid constitution and, consequently, in surface charge, membrane fluidity also appears to be altered in cancer cells [30,31]. In fact, alterations in membrane fluidity in cancer cells may be correlated with their malignant potential [30,32] and capability to metastasize [33]. Although no pattern has been described, such alterations can be responsible for cancer cell sensitivity or resistance to several anticancer drugs [31]. For example, some tumor cells, including lymphomas and lung carcinomas [34–36], exhibit higher membrane fluidity than their normal counterparts. Contrary, other cancer cells such as hepatoma tumor cell line present decreased membrane fluidity, as a result of lower degree of fatty acid unsaturation and increased cholesterol content [37].

Not only is the cancer cell altered but also its surrounding environment. In fact, the tumor microenvironment is hostile and largely determined by a chaotic vasculature that causes an unbalanced and heterogeneous blood supply [38]. Consequently, many regions are hypoxic and present high glucose consumption rates which results in an acidic medium [39,40]. Several studies reported that this acidic extracellular pH (pH_e) is a typical intrinsic feature of cancer phenotype and benefits the malignant cells because it promotes invasiveness [41]. This is very important because it can influence the ionization state of drugs and, consequently, be a crucial factor for their partition with membranes. In fact, the amount of ionized and non-ionized forms of a drug is determined by its ionization constant (pKa) and by the medium pH. In this context, anticancer drugs might interact differently with biological membranes according to their charge state which is closely dependent on each microenvironment pH encountered.

The structural, biophysical and functional differences between cancer and normal cell membranes can promote or inhibit drug activity and/or toxicity [42]. In fact, evidences suggest that lipids can be used as targets to overcome anticancer drug resistance [43]. In addition, the characteristics of the membrane also modulate drug penetration, conformation and/or location within membrane and, consequently, their effect on the therapeutic target [42]. Drugs will also have different effects on the membrane that include modifications in lipid conformation, surface charge, lipid domains and packing, membrane fluidity and curvature and, consequently, cell function [44]. Therefore, understanding the interactions of chemotherapeutic compounds with biological membranes is crucial, since it is directly associated with the therapeutic activity and toxicity of drugs.

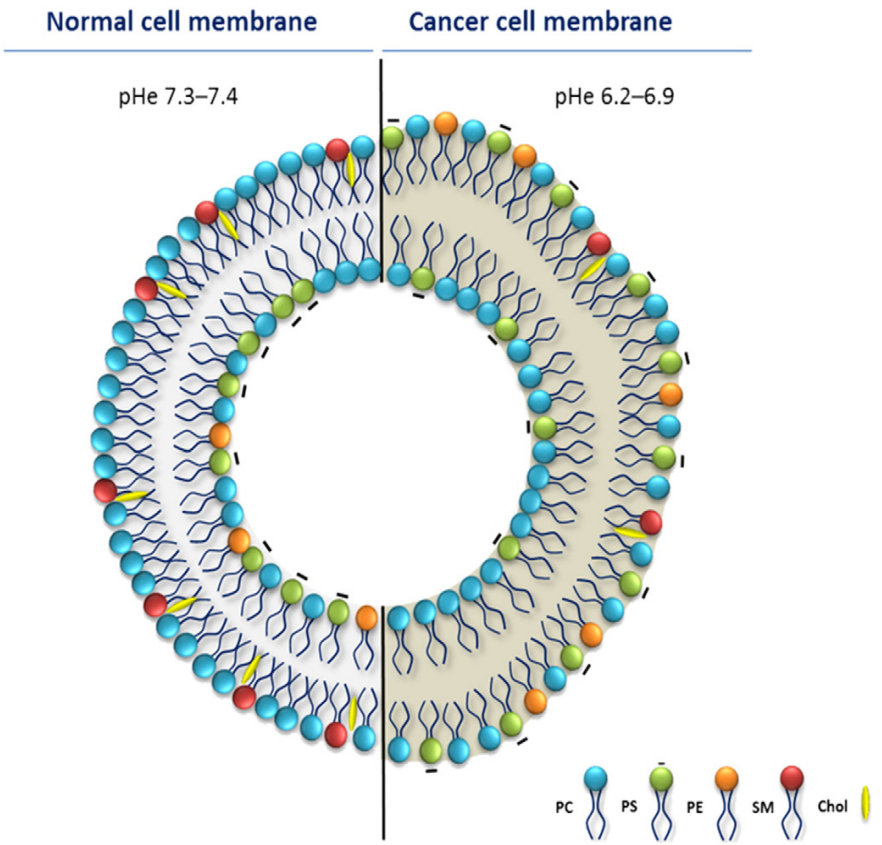


Fig. 1. Schematic representation of some features of normal and tumoral cell membranes regarding lipid composition and extracellular pH (pH_e).

3. Biophysical techniques and model membranes

The complexity and dynamic behavior of biological membranes represents a great challenge in the study of anticancer drug-membrane interactions [45,46]. In fact, membranes possess a broad lipid composition, present lipid domains and lipid asymmetry between the leaflets, that, together with proteins and carbohydrates, difficult this type of investigations. Using artificial models it is possible to develop membranes with the desired constitution and degree of complexity by just varying their composition of lipid and membrane proteins (proteoliposomes). A wide variety of membrane mimetic models exists to assess the effects of chemotherapeutic agents in the overall membrane properties. Lipid monolayers and liposomes are the most used model systems and allow the investigation of specific interactions between anticancer drugs and membrane lipids, such as with the phospholipid's headgroups and/or tails.

The emerging of simple model membranes led to the development of a wide variety of techniques that allow a molecular description of those interactions. Indeed, several techniques can be used to obtain information about a drug's effect on the membrane's physicochemical properties and about the membrane's influence in the drug's activity (Fig. 2). Nevertheless, no single method can provide all the necessary data to draw the complete scenario behind the interactions between drugs and membrane components, and reliable information can only be gathered from different measurements [47]. In addition, different model systems and specific biophysical techniques can be used depending on the type of parameter to be studied (Table 1). A brief description of the most used biomimetic models in the context of the study of anticancer drugs-membrane interactions is given below. More detailed information about these and other biomembrane models can the found in the following reviews [45,48].

Liposomes are highly suitable to assess different biophysical parameters and are by far the most used models to study anticancer drug-membrane interactions. Depending on their size and lamellarity they can be classified as giant unilamellar vesicles (GUVs), multilamellar liposomes (MLVs), small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs), having the latter a similar curvature to the cell membrane [44]. Nonetheless, the choice of the type of liposomes is dependent on the technique used. Therefore, while some techniques - such as small and wide angle X-ray diffractometry (SAXS and WAXS), differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) - can take advantage of the increased amount of sample material that is present in MLVs. Other techniques — such as microscopy-related methods — that detect structural alterations caused by the presence of anticancer drugs take advantage of GUVs' size, which is closer to the cell size.

From the gathered information depicted in Table 1, it is clear that a variety of experimental techniques have been used to study the interaction of anticancer drugs with membranes using liposomes as mimetic systems. Thus, the partition coefficient of chemotherapeutic drugs has been determined by different techniques, such as derivative spectrophotometry [49], fluorescence quenching [50] and electrophoretic light scattering (ELS) [49]. In addition, membrane drug location and binding have been assessed by NMR [51] and by fluorescence intensity measurements [52]. Finally, the determination of drug conformation and changes in phospholipids conformation order were assessed by circular dichroism (CD) [53] and by fourier transform infrared spectroscopy (FT-IR) [54], respectively.

Furthermore, the combination of several techniques with liposomes have been also providing a great amount of information about the anticancer drugs' effects on membrane properties that accounts for the assessment of membrane phase transitions, structure and surface

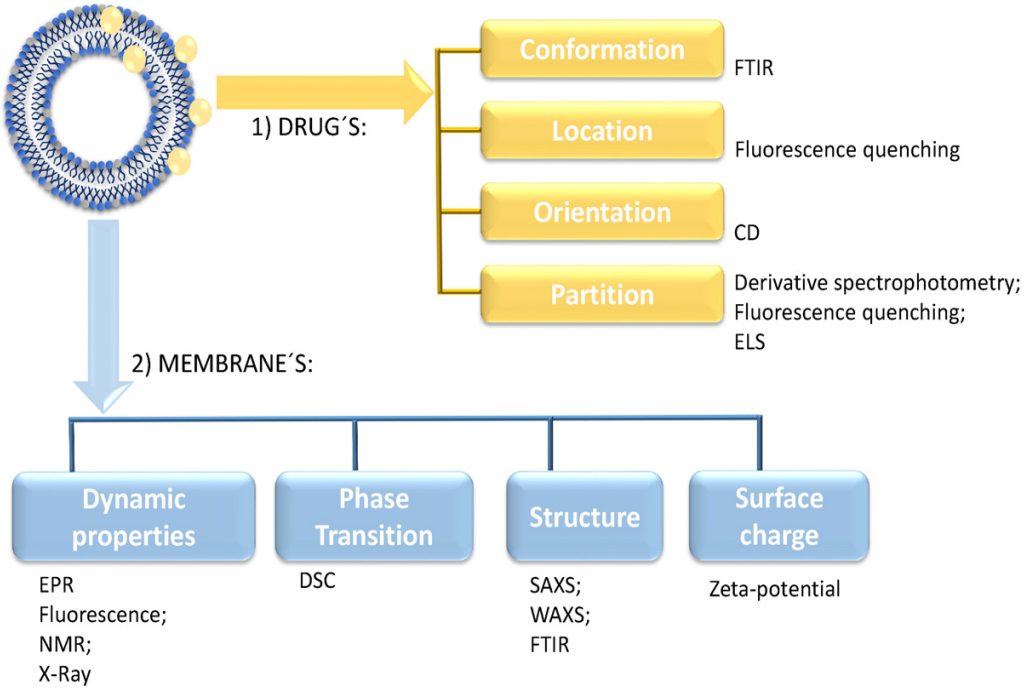


Fig. 2. Physicochemical parameters and associated techniques that can be evaluated through drug-liposomes interaction studies.

charge. Alteration in phase transition temperature (T_m) and cooperativity caused by the interaction of chemotherapeutic agents with membranes have been detected through DSC [55], NMR [56] and dynamic light scattering (DLS) [57]. Modifications in membrane fluidity and organization have been determined by electron paramagnetic resonance spectroscopy (EPR) [58]. Drug influence on membrane ordered domains was studied by phase contrast microscopy [59] and by fluorescence intensity measurements [60]. Ultimately, the disturbing effect of antineoplastic compounds on bilayer structure and dynamics has been evaluated by X-ray diffractometry [61].

Table 1
Biophysical techniques and model membranes used to study different parameters regarding anticancer drug-membrane interactions.

Technique	Model membrane	Biophysical parameter studied	Anticancer drug	Ref.
NMR	Liposomes	Interaction with the phospholipid bilayer	Cisplatin	[56,79]
	SUVs	Drug location and effect on membrane T_m	Etoposide and cytarabide	[51]
DSC	SUVs	Effect on membrane T_m and cooperativity	Etoposide and cytarabide	[51]
	MLVs		Methotrexate, paclitaxel Squalene-gemcitabine, cisplatin, tamoxifen and gemcitabine	[54,55,58,61,78,94,110]
FTIR	SUVs	Determination of changes in membrane structure and phospholipid conformation	Etoposide and cytarabide	[51]
	MLVs	Effects on lipid membrane order and dynamics	Tamoxifen	[54,55]
		Effects on the membrane T_m and phospholipid order; study of drug incorporation into the bilayer	CEUs	[105]
		Determination of changes in phospholipids conformation order		[107]
Fluorescence	SUVs	Assessment of drug membrane binding	Doxorubicin	[52]
	LUVs	Determination of drug partition coefficient and binding constants	Doxorubicin, daunorubicin and idarubicin	[50]
		Effect in the packing of ordered domains and in the global order of the membrane	2-Hydroxyoleic acid (2OHOA)	[60]
Derivative spectrophotometry	LUVs	Assessment of drug partition coefficient	Daunorubicin	[49]
EPR	MLVs	Changes in membrane fluidity	Paclitaxel	[58]
		Long range bilayer organization	Gemcitabine	[110]
SAXS and WAXS	MLVs	Effect on structural parameters	Squalene-gemcitabine and Gemcitabine	[61,110]
CD	LUVs	Assessment of drug conformation within the membranes	Doxorubicin, daunorubicin, idarubicin and idarubicinol	[53]
DLS	MLVs	Determination of the effect on T_m	Doxorubicin	[57]
	LUVs	Assessment of drug partition coefficient	Daunorubicin	[49]
Phase contrast microscopy	GUVs	Drug effect on membrane raft domains	ODPC	[59]
Langmuir isotherms	Lipid monolayers	Assessment of drug penetration into the monolayer	Doxorubicin, paclitaxel and edelfosine	[52,57,62,74,122,123,156]
BAM	Lipid monolayers	Drug effect on lipid monolayer structural properties	Edelfosine	[123,157]

Lipid monolayers are very well-defined, stable, homogeneous bidimensional systems with planar geometry. When referring to the lipid monolayer, it is inevitable to mention the Langmuir trough, where various parameters such as lipid composition, subphase, and temperature can be chosen and modified in order to mimic the biological conditions. The Langmuir trough or Langmuir balance provides information about the characteristics of a monolayer on the aqueous surface by measuring the changes in surface tension upon compressing the monolayer.

Lipid monolayers are formed at the air-water interface in a Langmuir trough and offer the possibility of using methodologies that are able to detect membrane drug penetration [62] and the drug's effect on lipid monolayer structural properties by Brewster angle microscopy (BAM) [63] (Fig. 3). More detailed information about the application of Langmuir monolayer associated techniques to the study of interactions between biomolecules and cell membranes can be found in the following reviews [64–66].

4. Anticancer drug-membrane interaction studies

4.1. Conventional anticancer drugs

Chemotherapeutic drugs are widely used for cancer treatment, targeting rapidly growing cells and promoting cellular death. However, depending on their structural features they act through different mechanisms. For example, cisplatin binds to the DNA and causes inhibition of DNA synthesis and cell division [67]. Paclitaxel compounds bind to tubulin, stabilizing them against depolymerization which results in the cell division's inhibition [68]. It is thought that anthracyclines (such as doxorubicin and daunorubicin) exhibit cytotoxic activity mainly through intercalation with DNA and alteration of topoisomerase II which, in turn, inhibits DNA and RNA synthesis and leads to apoptosis [69]. Although anticancer drugs operate through different mechanisms to promote cancer cell apoptosis, their main targets are intracellular and, therefore, they must penetrate the plasma membrane and, eventually, the nuclear membrane to exert pharmacological activity. Therefore, one way to understand the drug's pharmacological activity is to understand its interactions with the cell membrane.

Paclitaxel is a highly hydrophobic compound used in the treatment of a broad variety of cancers, including breast, ovary, small/non-small cell lung and AIDS-related Kaposi's sarcoma but it is also responsible for serious side effects. Using different model membranes, the influence of many membrane characteristics such as lipid composition (Chol and SM), the lipid chain length, unsaturation and headgroup, was assessed in different studies of paclitaxel-membrane interactions [58,70–73]. Furthermore, the drug's partition, penetration and its location within the membrane were also analyzed, along with the paclitaxel's effect in membranes.

Studies using DSC and Langmuir isotherms demonstrated that paclitaxel is inserted in the cooperative zone of the bilayer, which corresponds to the acyl chain C1–C8 region, through van der Waals or hydrophobic forces but also establishing interactions with the polar headgroups [71–73]. The drug's partition with model membranes caused alterations in the membrane's physical properties, namely the T_m and the lipid order. In fact, it was observed that the incorporation of paclitaxel into the 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayer induced a reduction in the cooperativity of the phase transition, which translates into the fluidizing effect that the compound exerts in the membrane [71]. Preetha et al. evaluated the penetration profile of paclitaxel in monolayers of normal and cancerous cervical membranes and compared it with a DPPC monolayer [74]. It was observed that due to differences in lipid composition, which in turn affect membrane fluidity, the drug's penetration profile was different between normal and cancer membrane. Thereby, both membrane composition and fluidity can influence paclitaxel penetration and, consequently, its biological action.

To assess the importance of different types of phospholipids properties, Zhao et al. studied paclitaxel penetration profiles in model membranes composed by PC with different chain lengths, i.e. of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) (C14:0), DPPC (C16:0) and 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (DSPC) (C18:0) [73]. It was shown that the lipid chain length also influences drug-membrane interactions (Fig. 4) and, while phospholipids of shorter chain length demonstrated to have a higher ability to incorporate paclitaxel, longer chains prevented the penetration process due to higher packing, therefore, acting as a physical barrier. On the other hand, it was observed that DPPC (C16:0) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (DEPC) (C22:1) have a stronger ability to incorporate paclitaxel than 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) (C16:0) and DSPC (C18:0) (Fig. 4) [72]. In fact, DPPC and DPPE present the same hydrophobic components but are different in the geometry of their polar headgroups. Therefore, the paclitaxel has greater

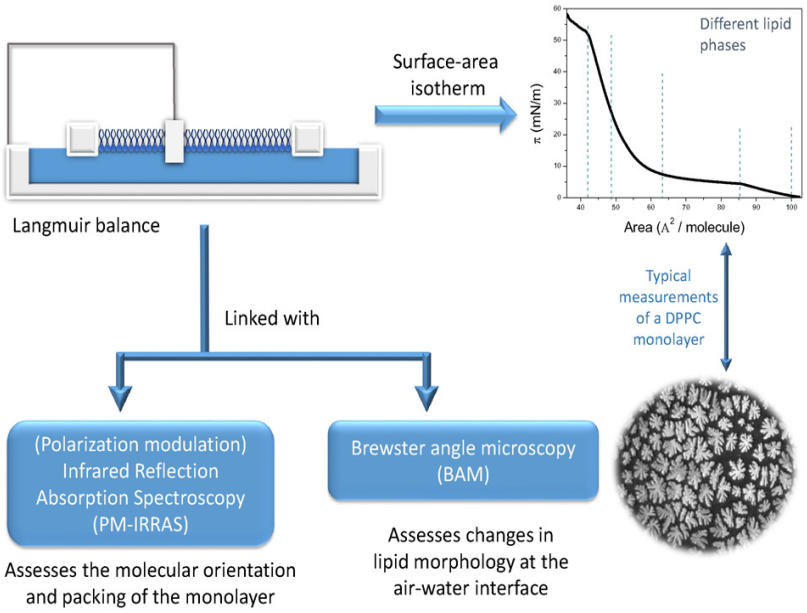


Fig. 3. Sets of studies that can be performed with monolayers to assess drug-lipid interactions.

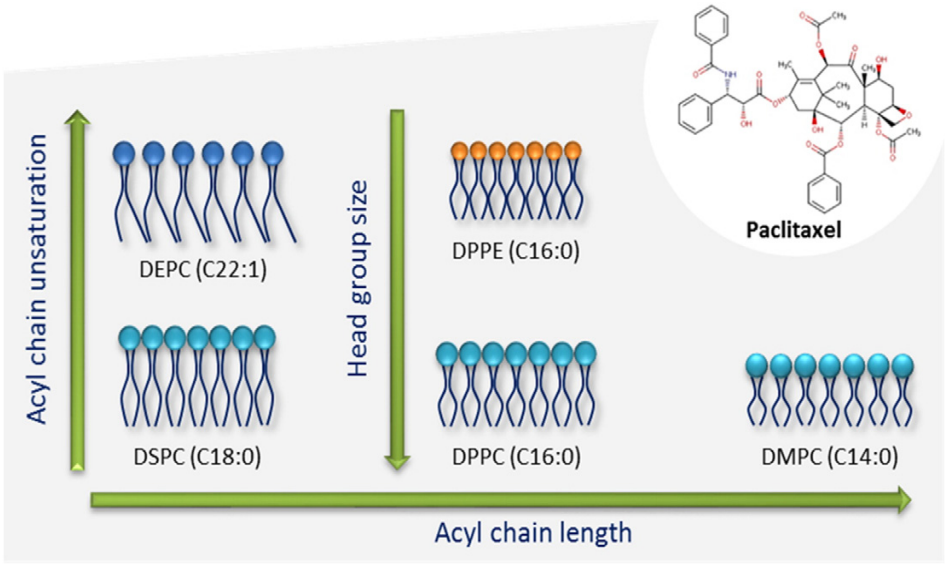


Fig. 4. Influence of phospholipid's properties (unsaturation, headgroup size and acyl chain length) in the membrane penetration of paclitaxel. The direction of each arrow indicates an increase in paclitaxel incorporation associated with the property described next to it.

affinity for lamellar-prone phospholipids than for hexagonal (H_{II})-prone phospholipids. Additionally, saturated phospholipids can pack closely together which results in a smaller incorporation of paclitaxel. These results gave new insigths to the paclitaxel-lipid interaction, since they highlighted its dependence on the molecular area of phospholipids (that depend on chain length, unsaturation and headgroup) and can be explained by intermolecular forces and geometric accommodation [72].

Zhao and colleagues also examined the effect of Chol in the paclitaxel-membrane interplay [58,75] and observed that, including Chol in a DPPC bilayer, the paclitaxel penetration decreased. Moreover, the introduction of SM, a lipid also known to be crucial to proliferation modulation in cancer [76], to a DPPC monolayer revealed a rigidifying effect which was directly proportional to the amount of SM added. Actually, Chol and SM form membrane rafts [77] and provide a barrier for drug permeability. As such, membrane fluidity changes anticancer drug's penetration into biological membranes, thereby reducing their side effects and also, improving their efficacy towards cancer cells.

The several outcomes evidence the importance of membrane lipid composition in anticancer drugs' biological activity. For that reason, paclitaxel's therapeutic action can be favored or hindered, depending on the type of lipids that cancer cells membranes contain, which in turn, depend on the type of cancer to treat.

Cisplatin is used in chemotherapy for different types of cancer. Notwithstanding, it causes severe side effects such as neurotoxicity and cellular resistance that are thought to be related to the interplay of cisplatin with biological membranes. Over the years, considerable studies regarding cisplatin interaction with model membranes have been performed [56,78–83]. Particular attention has been paid to anionic phospholipids, such as PS — as they play crucial roles in several cell functions [84] — and their influence in cisplatin-lipid interaction.

In this regard, binding assays using LUVs reported that cisplatin interacts with all negatively charged phospholipids (phosphatidic acid (PA), phosphatidylglycerol (PG), PS, PI and cardiolipin (CL)), but with none of the zwitterionic phospholipids tested (PC, SM and PE) [78]. Cisplatin seems to form a stable complex with PS [56,79,80] that is driven by electrostatic forces between the anticancer drug and phospholipid headgroup [79] (Fig. 5). Cisplatin binding to anionic phospholipids can contribute to the reduction of the chemotherapeutic agent uptake by

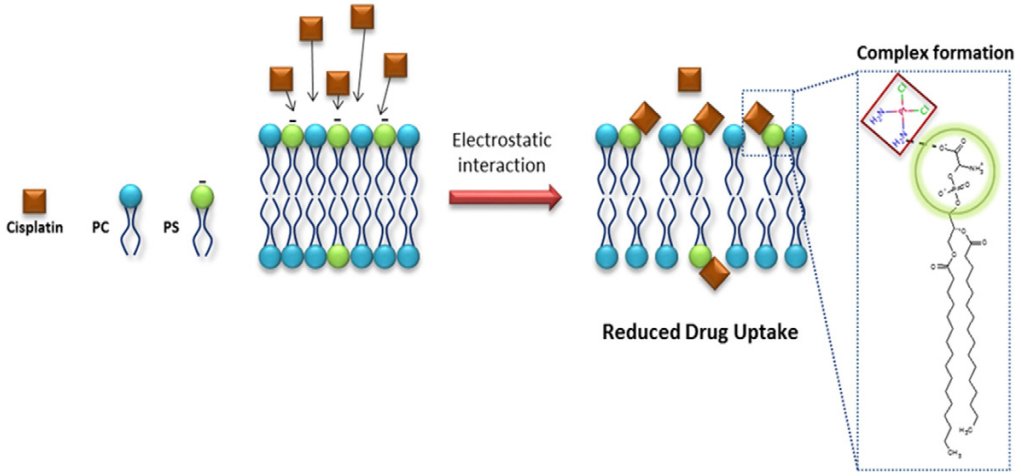


Fig. 5. Schematic representation of the interaction of cisplatin with phosphatidylcholine (PC) and the anionic phosphatidylserine (PS) on a hypothetical membrane. Cisplatin forms a stable complex with PS due to electrostatic forces between the anticancer drug and the headgroup of the phospholipid.

the cancer cells and, therefore, to the development of drug resistance. The gathered information suggests that, due to the complex formation, less amount of cisplatin is available to target the DNA, which can explain the loss of efficacy that occurs during chemotherapy. Moreover, DSC assays have shown that cisplatin caused an increase in the T_m of model membranes composed of negatively charged phospholipids and, as a result, a decrease in membrane fluidity [78]. The rigidifying effect caused by the anticancer drug can be explained by the interaction of the positively charged cisplatin that leads to a neutralization of the negatively charged PS, confirming that electrostatic interactions are involved in cisplatin-phospholipid binding [78]. Membrane fluidity plays a crucial role in the correct distribution and function of some proteins [1]. Alterations in membrane fluidity can profoundly alter functional properties of the cell and induce apoptotic pathways that can result in cell death [31]. In fact, studies using several cancer cell lines demonstrated that cisplatin induces apoptosis through the translocation and/or activation of the Fas receptor in cellular lipid rafts [85].

Anthracyclines are among the most used drugs in cancer treatments. Understanding the details of anthracycline-membrane interactions becomes crucial to unveil the mechanisms of passive diffusion and drug transport through the membrane and the process of multidrug-resistance commonly associated with this class of antineoplastic drugs. The compounds comprised in this group (such as doxorubicin, daunorubicin, epirubicin, idarubicin and pirarubicin) are amphiphilic molecules that display a dihydroxyanthraquinone ring bound by a glycosidic bond to the amino sugar [69]. The amino sugar bears the positive charge through the protonated amino nitrogen and is responsible for the electrostatic interactions that take place between these drugs and membranes. As a result, their lipophilicity depends on the substituents that will determine their ability to be incorporated within the bilayer and, consequently, its passive diffusion (Fig. 6a). The slight structural differences found between these compounds causes significant consequences on their spectrum of activity and pharmacokinetics [69].

In addition to intracellular targets, strong evidence suggests that the cell membrane can itself be a target for this type of agents. In fact, Triton et al. demonstrated that the sole interaction of anthracyclines with the membrane induced cancer cell death without entering them [86]. Later on, Escriba et al. showed that daunorubicin reduced the formation of the nonlamellar hexagonal (H_{II}) phase of liposomes composed by (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) POPE and stabilized the bilayer structure just by interacting with membrane phospholipids. The repercussion of such interaction is an alteration of various cellular events involved in signal transduction, in which these lipid phases are involved [87].

Peetla et al. isolated the lipids from doxorubicin-sensitive and –resistant breast cancer cells in order to understand how the membrane biophysical properties, such as lipid packing and fluidity, can affect drug uptake and, consequently, drug efficacy [5] (Fig. 6b). Furthermore, it appears that acquisition of resistance to anthracyclines by some cancer cells is related to alterations of membrane lipid composition [88]. In this context, the binding of anthracyclines to both cancer cell membranes and liposomes can explain, in part, the antitumor effect of these compounds [16]. In fact, Escriba et al. described that different lipid content can determine how daunorubicin binds to membranes. While the presence of phosphatidylserine greatly increased both the drug's affinity and stoichiometry of binding to model lipid membranes, highlighting once more the importance of electrostatic interactions in this process; the presence of cholesterol caused a reverse effect [16]. A strong affinity of daunorubicin to cardiolipin, a lipid abundant in heart tissue has also been described [89]. As a consequence of electrostatic interactions, a complex is formed that is thought to be involved in the cardiotoxicity of this drug [89]. Finally, it was reported that daunorubicin-triggered cell death is mediated by a signaling pathway that starts with an early production of sphingomyelin-derived ceramide [90], which emphasizes the importance of the cell membrane's lipid content for this anticancer drug's activity. Recent studies have also shown that daunorubicin induces apoptosis via signaling through plasma membrane lipid rafts, where the death receptor pathway is involved [91,92]. The process is initiated by the activation of a sphingomyelinase, which in turn causes the hydrolysis of SM and generates ceramide. Later on, ceramide is responsible for triggering different signaling pathways among which a stress-activated protein kinase cascade plays a crucial role. Overall, the gathered results evidence the affinity that these drugs have for membranes and explain the anticancer mechanism of action based on anthracycline-membrane interactions.

Biophysical studies demonstrated, once more, that understanding the membrane lipids' role in drug therapy is crucial to overcome cancer drug resistance and to develop new approaches to improve drug efficacy. More details regarding this class of chemotherapeutic compounds can be found in a recent review by Bourgaux et al. [46].

Methotrexate is an antimetabolite often used in combination with other drugs for the treatment of several types of cancer, such as leukemia, non-Hodgkin's lymphoma, osteosarcoma and solid tumours of the head, neck, breast and lungs. Even though it is rather effective, it also causes severe side effects that include neuro and hepatotoxicity. This chemotherapeutic agent has been studied in regards to its ability to penetrate and affect membrane fluidity [93]. The results showed that methotrexate caused an increase in membrane fluidity explained by its interaction with the phospholipids polar heads. Consequently, the anticancer drug fluidizes the hydrophobic center of the bilayer by creating defects that disorganize the packing of the lipids, which can ultimately result in the disintegration of the membrane [93]. The gathered information can be correlated with the anticancer drug's therapeutic activity but also with the adverse neuronal side effects, that will depend on the degree of membrane damage caused by the drug [93].

In order to improve methotrexate's pharmacokinetic properties, several derivatives and prodrugs have been developed. Pignatello and his team went further to uncover the effect of shortening the alkyl side chain of methotrexate-aminoacid conjugates and the effect of modulating their lipophilicity by adding glucose residues [94]. The studies were conducted through DSC using MLVs of DMPC and it was found that longer side chains allowed the drug to interact at a deeper level with the membranes, causing a decrease in the T_m and the transition enthalpy. In consequence, the addition of the glucose residue seemed to be detrimental for the drug's therapeutic effect [94]. Furthermore, Fry et al. proved that positively charged phospholipid content maximizes methotrexate's ability to cross biomembranes [95].

Tamoxifen is an antagonist of estrogen and mainly prescribed for the management of breast cancer. The assessment of the influence of lipid composition, length of the acyl chains and charge of the lipid constituents in tamoxifen-membrane interactions has been performed through an array of biophysical techniques [54,55,96–99].

Bilge et al. investigated the effect of acyl chain length on structural parameters (such as lipid order or the strength of hydrogen bonding between lipids) and the effect of lipid charge on tamoxifen-membrane interaction using MLVs composed of zwitterionic (DPPC and DMPC) and anionic (1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG)) phospholipids [54]. DSC assays showed that tamoxifen led to a decrease in the T_m regardless of lipid constitution. Through FT-IR it was demonstrated that the interaction of tamoxifen with the lipid membranes is dependent on surface charge but not on acyl chain length [54]. These outcomes are important since cancer cells present a higher negative surface charge when compared with normal cells.

Other assays employed the same techniques to study the effects of this drug on the gel (L_{β}) and liquid crystalline (L_{α}) phases of DSPC MLVs [55,96]. FT-IR results showed that tamoxifen causes a disordering effect in both L_{β} and L_{α} phases, which seems to imply that tamoxifen penetrates the bilayer and locates at the hydrophobic core [55,96]. DSC experiments support the idea that tamoxifen penetrates and fluidizes the membrane [55]. Assessments made through fluorescence polarization, DSC and turbidity are concurrent with the findings referred above, indicating that tamoxifen causes higher acyl chain disorder

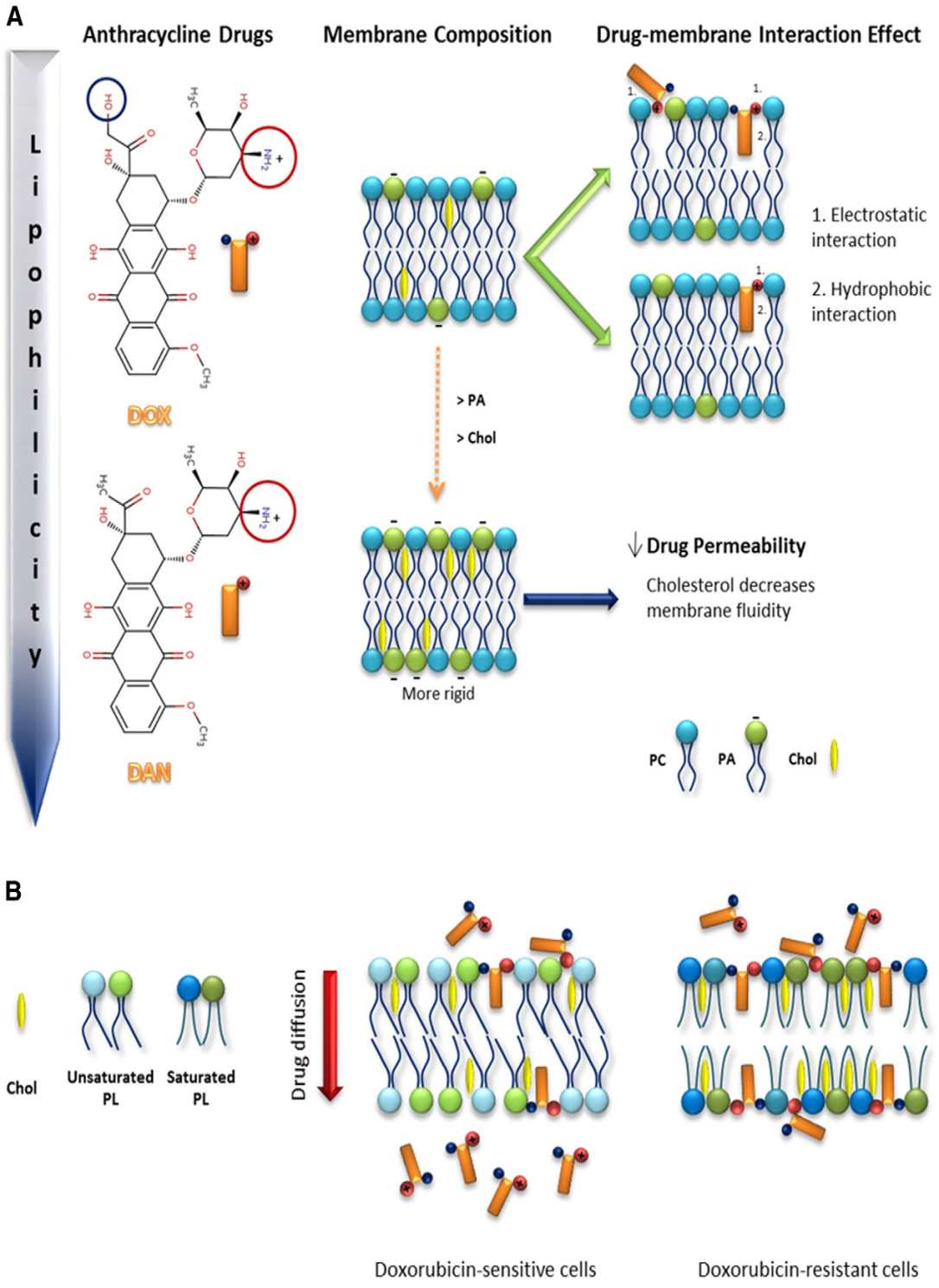


Fig. 6. a Representation of the effects of drugs with different lipophilicity (daunorubicin, DAN, and doxorubicin, DOX) on model membranes of phosphatidylcholine (PC) with different levels of phosphatidic acid (PA) and cholesterol (Chol). Studies with doxorubicin and daunorubicin demonstrated that these drugs establish two types of interactions with model membranes: electrostatic (1) between the positively charged amino sugar and the negatively charged moiety of phospholipids; hydrophobic (2) with the phospholipids acyl chains. In the case of doxorubicin two situations can happen regarding the position of the dihydroxyanthraquinone ring: for low molar ratio of phospholipid/drug, it remains outside the bilayer, in the aqueous phase; and for high molar ratio of phospholipid/doxorubicin, the ring is embedded in the bilayer, intercalated between the lipid acyl chains. In the case of daunorubicin, which is more hydrophobic than doxorubicin, the dihydroxyanthraquinone moiety remains embedded in the bilayer even when increasing the molar ratio of phospholipid/drug [50,53]. **b:** Interaction of doxorubicin with the phospholipid (PL) and cholesterol (Chol)-composed cell membranes of sensitive and resistant cancer cells. It was found that lipids from resistant cell membrane formed more condensed monolayers than the ones from the sensitive cells. Doxorubicin interactions with those models showed that this anticancer drug established a strong hydrophobic interaction with resistant cell membrane lipids when comparing with sensitive ones [5]. These results confirm previous studies suggesting that doxorubicin presents higher affinity to membrane lipid domains [159]. As a result of such affinity, doxorubicin appears to be trapped in the resistant lipid bilayer and, consequently, it is observed a decrease in intracellular drug transport through diffusion [5].

and, therefore, increased membrane fluidity in DMPC, DPPC and DSPC model membranes [97–99].

4.2. Hybrid anticancer drugs

1-aryl-3-(2-chloroethyl) ureas (**CEUs**) are a class of antineoplastic agents, synthetized from the combination of aromatic nitrogen mustards and aliphatic nitrosoureas, two very potent types of anticancer drugs [100,101]. There are a wide variety of these compounds and the difference in their properties resides in the composition and structure of their substituent (R) group, which influence its cytotoxic activity (Table 2). CEUs present increased cytotoxicity against several cancer cell lines [102–104], but did not present signs of systemic toxicity [105]. However, their mechanism of action is not fully understood and studies of CEU-membrane interactions arise to better explain such mechanisms.

Saint-Laurent et al. have explored the interaction between CEU derivatives with different cytotoxic activities and DMPC bilayers by NMR [106]. It has been shown that several characteristics regarding the position and nature (degree of ramification, length of the alkyl chain, presence of a sulfur atom) of the R substituent on the aromatic ring cause distinct effects in anticancer drug-membrane interactions that are related to their cytotoxic activity, opening a path to the understanding of the mechanism of action of this type of drugs. In fact, the most cytotoxic drugs are the ones that present a bulky branched R substituent in the position 4 and a short alkyl chain (such as 4-*sec*-butyl CEU). As a consequence of their structural features, these compounds are responsible for the smallest ordering effect at the interfacial region of the membrane and for the greatest disordering effect on the lipid acyl chains. Contrarily, CEUs with long chains (such as 4-*n*-hexadecyl CEU) and CEUs substituted at the position 2 (such as 2-isopropyl CEU) cause a smaller disordering effect on the lipid acyl chains and present a weak cytotoxic activity [106]. These findings are also in agreement with studies further developed by the same authors using FTIR spectroscopy [105]. In this sense, it is possible to establish a correlation between structural characteristics of CEU derivatives and their anticancer activities.

Cronier et al., using different model membranes, evidenced the importance of the membrane lipid composition, particularly the mitochondrial membrane, in the effects of CEUs [100]. The obtained results show that the incorporation of different CEU derivatives had distinct repercussions on the head and acyl chains of phospholipids depending on their structure. Furthermore, it was observed that increased amount of cardiolipin in the mitochondrial inner membrane generates a more fluid system and, consequently, the rigidifying effect caused by the presence of CEUs was more pronounced in this model when compared with the mitochondrial outer and endoplasmic reticulum membranes.

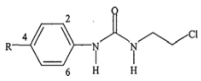
The membrane-interaction of 4-*n*-butyl CEU and 4-*sec*-butyl CEU, which differ on the R substituents, were assessed with mimetic models composed of DMPC and a mixture of DMPC and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [107]. The studied compounds had different effects on the order of the lipid acyl chains that are related with their respective cytotoxicity [107]. In fact, 4-*sec*-butyl CEU is more cytotoxic than 4-*n*-butyl CEU, causing a higher conformational disordering effect on the lipid acyl chains, as previously shown.

Gicquaud et al. have studied the interaction of one CEU derivative, 4-*tert*-butyl-[3-(2-chloroethyl) ureido] benzene (tBCEU), with bilayers composed of DSPC [101]. Results obtained with DSC and FTIR demonstrated that tBCEU is incorporated in the lipid bilayer with its hydrophobic portion intercalating between the phospholipid acyl chains and its hydrophilic region near the phospholipid headgroups [101]. This proposed location is supported by Cronier et al. [100]. Such incorporation caused a decrease in the T_m of the lipid and an increase in the number of gauche conformers in the L_α phase which means that it has a bilayer-fluidizing effect. These outcomes were confirmed by another study where the interaction between several CEU derivatives with lipids with different properties, such as charge and degree of unsaturation (DMPC, 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG) and a mixture of DMPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)), were assessed by FTIR [105]. In addition, the presence of CEUs in DMPC bilayers containing Chol markedly decreased the cooperativity of the lipid T_m and increased the conformational disorder of the lipid acyl chains [105]. The presented facts suggest that CEU derivatives cause similar disordering effects in membranes, but in a degree that is dependent on the nature and the position of the R substituent and, consequently, is associated with the drug's cytotoxicity.

Gemcitabine has been coupled with squalene, a natural lipid precursor of Chol biosynthesis [61]. Apparently this hybrid is much more effective than the gemcitabine drug on its own [108]. Studies using DSC, SAXS and WAXS techniques have demonstrated that gemcitabine-squalene is able to penetrate the bilayer [61,109], contrarily to gemcitabine, which, at neutral pH, does not disturb the thermal and structural properties of DPPC, locating mainly in the aqueous phase [110]. Studies have also shown that the hybrid molecules seemed to be inserted between the lipid acyl chains with their polar head group anchored at the aqueous interface [111]. Furthermore, the gemcitabine-squalene anticancer drug induced the formation of an unusual inverse bicontinuous cubic phase over time, partly similar to the liquid ordered phase (L_o) formed in saturated mixtures of PC or SM with Chol [109]. The combination of the several results obtained can help to clarify some of the mechanisms involved in this hybrid's cytotoxic activity. In fact, the gemcitabine "squalenoylation" protects it from rapid deamination and leads to an amphiphilic prodrug that has the ability to insert into the bilayer and disturb its curvature [109]. Furthermore, it appears that the intracellular gemcitabine-squalene uptake takes place through passive transport and, consequently, the drug is capable of overcoming different aspects of resistance [109]. The hybrid has also the ability to induce the formation of non-lamellar structures, which can contribute to cell death by compromising the integrity of the membrane [109]. This has therapeutic implications, because perturbation of membrane curvature and lipid phase can cause changes in protein conformation, modulating their activities and the mechanisms of signaling associated with them [1] and, therefore, inducing apoptosis or altering cell susceptibility to apoptosis.

Propofol-docosahexaenoate (**propofol-DHA**) and related compounds are novel hybrid drugs with anticancer action [112]. Propofol-DHA hybrid is composed by propofol, a well-tolerated and non-toxic anesthetic molecule, covalently bound to a fatty acid (DHA). The strategy was based on the fact that both propofol and DHA molecules have interesting anticancer features, and to develop a compound with enhanced effect against breast tumor cell lines. Recent evidences observed that DHA analogues also present potent anticancer properties, especially in the treatment of breast cancer [113]. It is though that these conjugates might influence cell-signaling pathways, since it has been demonstrated that the DHA interactions with cells leads to its incorporation into the membrane phospholipids with consequently reorganization and formation of microdomains [114]. Besides, the conjugate probably helps propofol to be retained in the membrane for a longer time and, thus, increasing its anticancer activity.

Table 2
Structures and cytotoxic values (IC_{50}) of some CEUs.

CEUs	Name	R substituent	IC_{50} (μM)[a]
	4-*sec*-butyl CEU	$CH_3–CH_2–CH(CH_3)–$	2
	4-*tert*-butyl CEU	$C(CH_3)_3–$	4
	4-*n*-butyl CEU	$CH_3–(CH_2)_3–$	14
	4-*n*-ethyl CEU	$CH_3–CH_2–$	5
	4-*n*-hexadecyl CEU	$CH_3–(CH_2)_{15}–$	>200
	4-methyl CEU	$CH_3–$	25
	4-thiomethyl CEU	$CH_3–S–$	12
	4-isopropyl CEU	$CH(CH_3)_2–$	2
	2-isopropyl CEU	$CH(CH_3)_2–$	150

[a] Cytotoxic values obtained using a breast cancer cell line (MDA-MB-231), from [158].

The overall results suggest that the therapeutic, along with the toxic, effects of anticancer drugs seem to be also related to their ability to trigger several alterations in membrane properties, such as in membrane fluidity, membrane microdomain distribution and features, membrane order and surface packing. In this sense, further anticancer drug-membrane interaction studies can be crucial to provide information about their biological activity that could help the development of drugs with better selectivity and reduced unwanted effects. The abovementioned evidence suggests that membrane-lipid therapy might be a novel therapeutic approach. In fact, anticancer drugs to target the cell membrane have already been developed.

4.3. Anticancer drugs to target the cell membrane

Membrane lipids are much more than just a scaffold in which proteins and other molecules are embedded, being important elements that regulate several cell functions [84]. For that reason, it is conceivable that compounds interacting with membrane lipids and influencing lipid organization, may produce alterations in the overall membrane properties, protein function or gene expression. In fact, two classes of synthetic phospholipid analogues, the alkyllysophospholipids (ALP) and the alkylphosphocholines (APC), have been identified as novel anticancer drugs that target cellular membranes and present great antitumoral effects in cell culture and animal models [115]. Contrary to conventional chemotherapeutic compounds that target the DNA and proteins, APC and ALP interact with biological membranes through the modulation of membrane permeability and fluidity, lipid composition, phospholipid metabolism and proliferation of signal transduction, and, consequently, induce selective apoptosis in cancer cells [115]. For example, different studies have demonstrated that edelfosine becomes entrapped in lipid rafts [116–118] and induces apoptotic cell death by redistributing the lipid rafts' protein composition [119–121].

Edelfosine has been mainly studied using Langmuir monolayer techniques and it has been found that it incorporates directly into monolayers constituted by PC [122,123]. A recent review comprising studies performed in monolayers evidence a key role of cholesterol and membrane rafts in the mechanism of action of this compound [124]. This compound has the ability to induce modifications in the membrane lipid raft structure, which results in the induction of cancer cell apoptosis [125]. However, it is unlikely that it will be used in humans due to its toxicity and modest efficacy [4]. In contrast, the edelfosine analogues miltefosine and perifosine are currently in clinical trials for the treatment of cutaneous leishmaniasis and cancer, respectively. **Perifosine** induces cell cycle arrest and apoptosis in human carcinoma cell lines [126]. It has also been shown that this compound caused the recruitment of the death receptor Fas/CD95 into lipid rafts, leading to death-inducing signaling complex (DISC) formation and selective apoptosis in multiple myeloma cells [120]. Furthermore, evidence demonstrated that perifosine accumulated in lipid rafts and was then internalized in S49 mouse lymphoma cells through these domains [127], which highlights the role of lipid domains in the drug's mechanism of action.

10-(Octyloxy) decyl-2-(trimethylammonium) ethyl phosphate (**ODPC**) is another ALP that can interact with cell membranes due to its amphiphilic character [128]. Studies conducted by Santos et al. using DSC measurements demonstrated that the incorporation of ODPC into DPPC and DPPC:Chol bilayers induced a decrease in the T_m and enthalpy in a concentration dependent way, showing that the compound has the ability to destabilize the membrane [128]. Furthermore, it was observed that the effect on the enthalpy in the transition was more pronounced in models with increased amount of Chol and might indicate that the ODPC must be inside the hydrophobic part of the phospholipid. It was also demonstrated by Thomé et al. that this alkylphospholipid targets high cholesterol domains in model membranes and induces apoptosis in leukemia cells but not in normal hematopoietic and epithelial cells [129].

In order to assess the role of lipid rafts in the mechanism of action of ODPC, GUVs composed of 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC), SM and Chol were used [59]. Experiments showed that models containing SM and Chol in homogeneous fluid lipid bilayers were protected from disruption caused by ODPC. Additionally, in models composed of a homogeneous mixture of DOPC:Chol:SM, which display liquid ordered (Lo)–liquid disordered (Ld) phase coexistence, the lipid domains were disrupted in the presence of ODPC [59]. Such information is in accordance with the previously described experiments and has clinical relevance, since lipid rafts play key roles in several cell functions such as cell signal transduction [130] and provide signaling platforms able to activate both pro-apoptotic and anti-apoptotic signaling pathways [131]. The integrity of lipid rafts is also required for cellular homeostasis. In fact, disruption of lipid raft integrity disrupts homeostasis by altering cellular survival and apoptotic pathways and, consequently, can lead to cell death [132].

The lipid raft disruption induced by both ODPC and perifosine, and probably other ALPs, may be correlated with their toxicity towards cancer cells, because the selective (dis)association of crucial proteins within such microdomains must take place in the plasma membrane [59].

Another antitumor drug developed was the synthetic fatty acid 2-hydroxyoleic acid (2OHOA, **Minerval**), a first-in-class hydroxylated fatty acid, currently in phase I/II clinical trials for glioma. This compound was shown to cause membrane lipid modifications, which lead to important signaling alterations and, ultimately, cell death [133]. In fact, studies revealed that it can recover the SM levels that are reported to be reduced in cancer cells [134]. Martin et al. have also studied the effect of 2OHOA on the cancer cell membrane and observed that it increased the packing of ordered domains, once it can incorporate into the membrane [60]. It was also shown that Minerval induced a rearrangement of membrane fatty acid composition, where stearoyl-CoA desaturase (SCD1) inhibition appears to be crucial due to the constitutive activation of fatty acid biosynthesis presented on cancer [10]. Such alterations influence the properties of cell membrane microdomains that are important for the regulation of crucial signaling proteins (such as PKC and Ras). Furthermore, it has been demonstrated that 2OHOA regulates sphingomyelin synthase 1 (SMS1) producing dramatic changes in the PE:SM ratio in cancer cells, which in turn are associated with the translocation of proliferation-associated proteins from the membrane to the cytosol [6]. This change appears to be critical because brain cancer (glioma) patients with lower expression of SMS1 have a median survival of 2 and 5-years that is much lower than patients with normal SMS1 expression [6]. In fact, 2OHOA has a double mechanism of action that includes binding and regulation of SMS1 and binding to membrane [135]. In a recent review by Lladó et al. the mechanisms behind the regulation of the cancer cell membrane lipid composition by 2OHOA are summarized [6].

The compound 2-hydroxy-linoleic acid (ABTL0812) is a novel first-in-class hydroxylated fatty acid and a small molecule, similarly to 2OHOA, with antiproliferative effects on tumor cells that is also currently in clinical trials [136].

The results obtained can thereby support the knowledge that, due to the lipid membrane changes which occur specifically in cancer but not in normal cells, it becomes important to develop compounds able to modulate membrane structure in order to improve cancer treatment.

5. Proteoliposomes as tools to study MDR: the case of Pgp

Membrane model systems can also comprise proteins in their composition (proteoliposomes), enabling the understanding of protein-lipid as well as protein-drug interactions. In the case of the study of anticancer drugs, many proteoliposomes have been developed with the P-glycoprotein (Pgp). Pgp, a member of the ATP-binding cassette family of membrane transporters, acts as an efflux pump, transporting a wide range of xenobiotics out of the cell at the apical membrane of many secretory cell types [137]. However, its overexpression is linked to multidrug resistance (MDR) in tumours, since it modifies the absorption and

distribution of many anticancer drugs in tissues and, thereby, represents a significant barrier to the efficacy of cancer treatments. Pgp in tumours also appears to provide resistance to apoptosis induced by different stimuli, including serum starvation, Fas, TNF and UVB- and γ-irradiation [138,139]. The mechanism whereby Pgp inhibits apoptosis is still unclear and controversial. Nevertheless, the mechanism by which Pgp performs its efflux pump function seems to be highly related to the lipid membrane environment. The most accepted model is the hydrophobic vacuum cleaner model, which suggests that Pgp removes hydrophobic molecules that are within the membrane by a "vacuum cleaner device" [140–142]. Since the substrates that are embedded in the membrane are the ones that gain access to Pgp, a drug's lipid–water partition coefficient (Kp) is a key property, since it can modulate the interactions with the transporter [143].

Most of the studies regarding the transport activity of Pgp were performed in plasma membrane vesicles and reconstituted proteoliposomes, as it has been proven to be difficult to characterize the transport properties of Pgp in complex intact cell systems. Most Pgp proteoliposomes used until now include Pgp purified from Chinese hamster, mouse, and human from drug-selected MDR cell lines - or cells that express recombinant proteins [144] - and defined phospholipids, allowing detailed assessment of how Pgp function is modulated by membrane properties [145,146].

Pgp-mediated drug transport is highly affected by the fluidity of the membrane. Increase in the fluidity of canalicular membrane vesicles by benzyl alcohol, a known membrane fluidizer, reduced Pgp-mediated transport of daunorubicin and vinblastine two- to four-fold [147]. Pgp inhibition as a consequence of an increase in membrane fluidity has also been verified in models in which Pgp was reconstituted into proteoliposomes formed of two synthetic PCs with phase transition temperatures [148]. This study showed a high rate of transport in the rigid L_β (where the maximum transport rate was observed at the melting temperature of the bilayer) and a lower transport rate in the fluid L_α [147]. Although drug partition is generally smaller in more rigid phases, their transport by Pgp is enhanced. These studies suggest that membrane fluidizers may revert MDR by interfering with the ability of Pgp to efflux drug and thus generate a drug concentration gradient [144]. In fact, other studies demonstrated that while polyoxyethylene surfactants reversed MDR and also decreased lipid fluidity in plasma membrane vesicles from MDR cells, surfactants that did not reverse MDR did not influence membrane fluidity [149]. Moreover, low concentrations of Pluronic 61 were found to greatly increase the rates of phospholipid flip–flop and transbilayer movement of doxorubicin in lipid bilayers [150].

Other lipid membrane features seem to modulate Pgp activity by, for instance modifying its insertion into lipid rafts. In fact, it has been shown that Pgp becomes fully functional after reconstitution into liquid-ordered membranes [151]. However, other studies demonstrate that Pgp is not associated with lipid rafts at all [152]. Consequently, any proposed role for these domains in functional modulation of Pgp should be cell type-specific. Moreover, there are dissonant studies regarding the binding of drugs to Pgp in environments enriched in Chol. It has been suggested that the presence of Chol in reconstituted proteoliposomes increased Pgp's binding affinity for drugs with a small molecular mass [153]. However, in a fluorescence quenching study, no relationship between drug size and the effect of Chol on binding affinity was found. In fact, vinblastine showed a large reduction in binding affinity at high levels of Chol in the bilayer, whereas daunorubicin (a "small" drug) showed little change in affinity [154]. A recent and very comprehensive review by Sharom on the role of the membrane in modulating Pgp activity addressed this and other related issues in a very detailed manner [144].

6. Concluding remarks

The lipidomics era led scientists from different fields to look at the membrane lipids through a completely new perspective, where these play crucial roles in different cellular functions and are involved in several pathologies, including cancer [155]. In this sense, anticancer drug-membrane interaction studies arise as important tools to better understand the therapeutic activity of drugs and their toxic actions, once these processes could be related with the effect of antineoplastic agents at a membrane level. The emerging of several biomimetic model membranes and biophysical techniques to study those interactions allowed to obtain new insights regarding the effect of drugs in the membrane (structure and phase behavior) and the membrane's influence in drug properties (partition, location and conformation within membranes). Such knowledge becomes extremely important when dealing with cancer cells that are characterized by distinctive features when comparing with normal cells, providing rational basis to improve cancer treatment. Through biophysical studies, different variables can be isolated in order to find out the basis of drug-membrane interactions and, therefore, unveil crucial details about drug biological actions. In fact, model membranes present several advantages when compared with cell lines. These systems can be used under conditions that cells could not bear to maintain their viability in. Beyond that, model membranes can be powerful tools to predict, in a fast and inexpensive way, the activity, selectivity and efficacy of drugs.

The latest works in cancer research point towards an interesting novel direction - the membrane-lipid therapy - with the membrane taking a central role as a target for novel drugs that are able to modulate the lipid composition and/or structure of the membrane. Furthermore, a synergetic effect could be obtained through the use of drugs able to alter membrane fluidity and conventional cytotoxic drugs used in chemotherapy. It becomes evident that several important properties of cancer cell membrane should be highlighted in order to improve drug development: membrane charge, membrane surface packing, non-lamellar phase propensity, membrane lipid composition, membrane asymmetry, membrane sugars, lipid rafts and fluidity [4]. Based on the gathered results, the future of anticancer drug discovery may include developing compounds that target the negatively charged cancer membrane, modulate the membrane permeability and fluidity and induce pro-apoptotic signals. Naturally, further progress on this approach requires a deep knowledge of the cell membranes' lipid composition as well as biophysical properties for both cancer and healthy cells from the various tissues. Such knowledge will allow the development of model membranes, such as novel lipid-protein models systems with functioning proteins that present some of the properties of biological membranes and could better mimic the intricacy of normal and cancer cell membranes.

The field of research of new cancer therapies using membrane biophysics through innovative perspectives could be a path to overcoming the problems found in the clinical practice. Finally, a great effort from experts of different fields, such as biophysicists, pharmacists and cell biologists is imperative to understand the implications of anticancer drug-membrane interactions and, therefore, improve cancer therapy.

Transparency document

The Transparency document associated with this article can be found, in online version.

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References

[1] P.V. Escriba, Membrane-lipid therapy: a new approach in molecular medicine, Trends Mol. Med. 12 (2006) 34–43.
[2] E.O. Aboagye, Z.M. Bhujwalla, Malignant transformation alters membrane choline phospholipid metabolism of human mammary epithelial cells, Cancer Res. 59 (1999) 80–84.
[3] I. Dobrzynska, B. Szachowicz-Petelska, S. Sulkowski, Z. Figaszewski, Changes in electric charge and phospholipids composition in human colorectal cancer cells, Mol. Cell. Biochem. 276 (2005) 113–119.
[4] P.V. Escriba, et al., Membrane lipid therapy: modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment, Prog. Lipid Res. 59 (2015) 38–53.
[5] C. Peetla, et al., Drug resistance in breast cancer cells: biophysical characterization of and doxorubicin interactions with membrane lipids, Mol. Pharm. 7 (2010) 2334–2348.
[6] V. Llado, et al., Regulation of the cancer cell membrane lipid composition by NaCHOleate: effects on cell signaling and therapeutical relevance in glioma, Biochim. Biophys. Acta 1838 (2014) 1619–1627.
[7] B. Holohan, et al., Perifosine as a potential novel anti-telomerase therapy, Oncotarget 6 (2015) 21816–21826.
[8] A. Preetha, R. Banerjee, N. Huilgol, Surface activity, lipid profiles and their implications in cervical cancer, J. Cancer Res. Ther. 1 (2005) 180–186.
[9] P.P. Hsu, D.M. Sabatini, Cancer cell metabolism: Warburg and beyond, Cell 134 (2008) 703–707.
[10] C.R. Santos, A. Schulze, Lipid metabolism in cancer, FEBS J. 279 (2012) 2610–2623.
[11] J.V. Swinnen, et al., Selective activation of the fatty acid synthesis pathway in human prostate cancer, Int. J. Cancer 88 (2000) 176–179.
[12] F.P. Kuhajda, Fatty acid synthase and cancer: new application of an old pathway, Cancer Res. 66 (2006) 5977–5980.
[13] N. Azordegan, et al., Carcinogenesis alters fatty acid profile in breast tissue, Mol. Cell. Biochem. 374 (2013) 223–232.
[14] T.E. Merchant, et al., Malignant breast tumor phospholipid profiles using (31)P magnetic resonance, Cancer Lett. 176 (2002) 159–167.
[15] T.E. Merchant, et al., Phospholipid profiles of human colon cancer using 31P magnetic resonance spectroscopy, Int. J. Color. Dis. 6 (1991) 121–126.
[16] P.V. Escriba, A.V. Ferrer-Montiel, J.A. Ferragut, J.M. Gonzalez-Ros, Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug-resistance phenomena, Biochemistry 29 (1990) 7275–7282.
[17] J.K. Selkirk, J.C. Elwood, H.P. Morris, Study on the proposed role of phospholipid in tumor cell membrane, Cancer Res. 31 (1971) 27–31.
[18] L. Baro, et al., Abnormalities in plasma and red blood cell fatty acid profiles of patients with colorectal cancer, Br. J. Cancer 77 (1998) 1978–1983.
[19] J. Hildebrand, D. Marique, J. Vanhouche, Lipid composition of plasma membranes from human leukemic lymphocytes, J. Lipid Res. 16 (1975) 195–199.
[20] L.F. Liebes, E. Pelle, D. Zucker-Franklin, R. Silber, Comparison of lipid composition and 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization measurements of hairy cells with monocytes and lymphocytes from normal subjects and patients with chronic lymphocytic leukemia, Cancer Res. 41 (1981) 4050–4056.
[21] M.F. Mulas, et al., Cholesterol esterification during differentiation of mouse erythroleukemia (Friend) cells, Hematol. Rep. 3 (2011), e19.
[22] G. Barceló-Coblijn, et al., Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy, Proc. Natl. Acad. Sci. 108 (2011) 19569–19574.
[23] A. Yamaji-Hasegawa, M. Tsujimoto, Asymmetric distribution of phospholipids in biomembranes, Biol. Pharm. Bull. 29 (2006) 1547–1553.
[24] M.R. Clark, Flippin' lipids, Nat. Immunol. 12 (2011) 373–375.
[25] S. Ran, A. Downes, P.E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels, Cancer Res. 62 (2002) 6132–6140.
[26] S. Ran, P.E. Thorpe, Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy, Int. J. Radiat. Oncol. Biol. Phys. 54 (2002) 1479–1484.
[27] S. Riedl, et al., In search of a novel target — phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy, Biochim. Biophys. Acta 1808 (2011) 2638–2645.
[28] R.F. Zwaal, P. Comfurius, E.M. Bevers, Surface exposure of phosphatidylserine in pathological cells, Cell. Mol. Life Sci. 62 (2005) 971–988.
[29] J.H. Stafford, P.E. Thorpe, Increased exposure of phosphatidylethanolamine on the surface of tumor vascular endothelium, Neoplasia 13 (2011) 299–308.
[30] G. Deliconstantinos, Physiological aspects of membrane lipid fluidity in malignancy, Anticancer Res. 7 (1987) 1011–1021.
[31] S. Baritaki, et al., Reversal of tumor resistance to apoptotic stimuli by alteration of membrane fluidity: therapeutic implications, Adv. Cancer Res. 98 (2007) 149–190.
[32] M. Shinitzky, Membrane fluidity in malignancy adversative and recuperative, Biochim. Biophys. Acta Rev. Cancer 738 (1984) 251–261.
[33] I. Nakazawa, M. Iwaizumi, A role of the cancer cell membrane fluidity in the cancer metastases: an ESR study, Tohoku J. Exp. Med. 157 (1989) 193–198.
[34] G.V. Sherbet, Membrane fluidity and cancer metastasis, Exp. Cell Biol. 57 (1989) 198–205.
[35] M. Sok, et al., Cell membrane fluidity and prognosis of lung cancer, Ann. Thorac. Surg. 73 (2002) 1567–1571.
[36] R. Campanella, Membrane lipids modifications in human gliomas of different degree of malignancy, J. Neurosurg. Sci. 36 (1992) 11–25.
[37] T. Galeotti, S. Borrello, G. Minotti, L. Masotti, Membrane alterations in cancer cells: the role of oxy radicals, Ann. N. Y. Acad. Sci. 488 (1986) 468–480.
[38] R.J. Gillies, N. Raghunand, G.S. Karczmar, Z.M. Bhujwalla, MRI of the tumor microenvironment, J. Magn. Reson. Imaging 16 (2002) 430–450.
[39] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis? Nat. Rev. Cancer 4 (2004) 891–899.
[40] S.K. Parks, J. Chiche, J. Pouyssegur, pH control mechanisms of tumor survival and growth, J. Cell. Physiol. 226 (2011) 299–308.
[41] R.A. Cardone, V. Casavola, S.J. Reshkin, The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis, Nat. Rev. Cancer 5 (2005) 786–795.
[42] J.K. Seydel, M. Wiese, Drug-membrane interactions: analysis, Drug Distribution, Modeling, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2002.
[43] A.B. Hendrich, K. Michalak, Lipids as a target for drugs modulating multidrug resistance of cancer cells, Curr. Drug Targets 4 (2003) 23–30.
[44] M. Lucio, J.L. Lima, S. Reis, Drug-membrane interactions: significance for medicinal chemistry, Curr. Med. Chem. 17 (2010) 1795–1809.
[45] C. Peetla, A. Stine, V. Labhasetwar, Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery, Mol. Pharm. 6 (2009) 1264–1276.
[46] C. Bourgaux, P. Couvreur, Interactions of anticancer drugs with biomembranes: what can we learn from model membranes? J. Control. Release 190 (2014) 127–138.
[47] A.M. Seddon, et al., Drug interactions with lipid membranes, Chem. Soc. Rev. 38 (2009) 2509–2519.
[48] M. Deleu, J.M. Crowet, M.N. Nasir, L. Lins, Complementary biophysical tools to investigate lipid specificity in the interaction between bioactive molecules and the plasma membrane: a review, Biochim. Biophys. Acta 1838 (2014) 3171–3190.
[49] C. Matos, C. Moutinho, P. Lobao, Liposomes as a model for the biological membrane: studies on daunorubicin bilayer interaction, J. Membr. Biol. 245 (2012) 69–75.
[50] L. Gallois, et al., The overall partitioning of anthracyclines into phosphatidyl-containing model membranes depends neither on the drug charge nor the presence of anionic phospholipids, Eur. J. Biochem. FEBS 241 (1996) 879–887.
[51] D. Pentak, Physicochemical properties of liposomes as potential anticancer drugs carriers. Interaction of etoposide and cytarabine with the membrane: spectroscopic studies, Spectrochim. Acta A Mol. Biomol. Spectrosc. 122 (2014) 451–460.
[52] F.A. de Wolf, et al., Characterization of the interaction of doxorubicin with (poly)phosphoinositides in model systems. Evidence for specific interaction with phosphatidylinositol-monophosphate and -diphosphate, FEBS Lett. 288 (1991) 237–240.
[53] L. Gallois, M. Fiallo, A. Garnier-Suillerot, Comparison of the interaction of doxorubicin, daunorubicin, idarubicin and idarubicinol with large unilamellar vesicles. Circular dichroism study, Biochim. Biophys. Acta 1370 (1998) 31–40.
[54] D. Bilge, N. Kazanci, F. Severcan, Acyl chain length and charge effect on tamoxifen–lipid model membrane interactions, J. Mol. Struct. 1040 (2013) 75–82.
[55] D. Bilge, I. Sahin, N. Kazanci, F. Severcan, Interactions of tamoxifen with distearoyl phosphatidylcholine multilamellar vesicles: FTIR and DSC studies, Spectrochim. Acta A Mol. Biomol. Spectrosc. 130 (2014) 250–256.
[56] M. Jensen, M. Bjerring, N.C. Nielsen, W. Nerdal, Cisplatin interaction with phosphatidylserine bilayer studied by solid-state NMR spectroscopy, J. Biol. Inorg. Chem. 15 (2010) 213–223.
[57] M.H. Gaber, M.M. Ghannam, S.A. Ali, W.A. Khalil, Interaction of doxorubicin with phospholipid monolayer and liposomes, Biophys. Chem. 70 (1998) 223–229.
[58] L. Zhao, S.S. Feng, N. Kocherginsky, I. Kostetski, DSC and EPR investigations on effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within lipid bilayer membrane, Int. J. Pharm. 338 (2007) 258–266.
[59] A.B. Gomide, et al., Disrupting membrane raft domains by alkylphospholipids, Biochim. Biophys. Acta 1828 (2013) 1384–1389.
[60] M.L. Martin, et al., The role of membrane fatty acid remodeling in the antitumor mechanism of action of 2-hydroxyoleic acid, Biochim. Biophys. Acta 1828 (2013) 1405–1413.
[61] L. Bildstein, et al., Interaction of an amphiphilic squalenoyl prodrug of gemcitabine with cellular membranes, Eur. J. Pharm. Biopharm. 79 (2011) 612–620.
[62] D. Nieciecka, A. Królikowska, A. Joniec, P. Krysinski, Partitioning of doxorubicin into Langmuir and Langmuir–Blodgett biomimetic mixed monolayers: electrochemical and spectroscopic studies, J. Electroanal. Chem. 710 (2013) 59–69.
[63] A. Więcek, et al., Interactions between an anticancer drug – edelfosine – and cholesterol in Langmuir monolayers, Thin Solid Films 516 (2008) 8829–8833.
[64] T.M. Nobre, et al., Interactions of bioactive molecules & nanomaterials with Langmuir monolayers as cell membrane models, Thin Solid Films 593 (2015) 158–188.
[65] C. Stefaniu, G. Brezesinski, H. Möhwald, Langmuir monolayers as models to study processes at membrane surfaces, Adv. Colloid Interf. Sci. 208 (2014) 197–213.
[66] J.J. Giner-Casares, G. Brezesinski, H. Möhwald, Langmuir monolayers as unique physical models, Curr. Opin. Colloid Interface Sci. 19 (2014) 176–182.
[67] M.A. Fuertes, J. Castilla, C. Alonso, J.M. Prez, Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways, Curr. Med. Chem. 10 (2003) 257–266.
[68] S.B. Horwitz, Taxol (paclitaxel): mechanisms of action, Ann. Oncol. 5 (Suppl. 6) (1994) S3–S6.
[69] G. Minotti, et al., Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity, Pharmacol. Rev. 56 (2004) 185–229.
[70] C. Bernsdorff, R. Reszka, R. Winter, Interaction of the anticancer agent Taxol (paclitaxel) with phospholipid bilayers, J. Biomed. Mater. Res. 46 (1999) 141–149.
[71] L. Zhao, S.S. Feng, M.L. Go, Investigation of molecular interactions between paclitaxel and DPPC by Langmuir film balance and differential scanning calorimetry, J. Pharm. Sci. 93 (2004) 86–98.
[72] L. Zhao, S.S. Feng, Effects of lipid chain unsaturation and headgroup type on molecular interactions between paclitaxel and phospholipid within model biomembrane, J. Colloid Interface Sci. 285 (2005) 326–335.
[73] L. Zhao, S.S. Feng, Effects of lipid chain length on molecular interactions between paclitaxel and phospholipid within model biomembranes, J. Colloid Interface Sci. 274 (2004) 55–68.

[74] A. Preetha, N. Huilgol, R. Banerjee, Comparison of paclitaxel penetration in normal and cancerous cervical model monolayer membranes, Colloids Surf. B Biointerfaces 53 (2006) 179–186.
[75] L. Zhao, S.-S. Feng, Effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within the lipid monolayer at the air–water interface, J. Colloid Interface Sci. 300 (2006) 314–326.
[76] C.P. Reynolds, B.J. Maurer, R.N. Kolesnick, Ceramide synthesis and metabolism as a target for cancer therapy, Cancer Lett. 206 (2004) 169–180.
[77] Y. Barenholz, Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications, Subcell. Biochem. 37 (2004) 167–215.
[78] G. Speelmans, et al., The interaction of the anti-cancer drug cisplatin with phospholipids is specific for negatively charged phospholipids and takes place at low chloride ion concentration, Biochim. Biophys. Acta 1283 (1996) 60–66.
[79] M. Jensen, W. Nerdal, Anticancer cisplatin interactions with bilayers of total lipid extract from pig brain: a 13C, 31P and 15N solid-state NMR study, Eur. J. Pharm. Sci. 34 (2008) 140–148.
[80] G. Speelmans, et al., Cisplatin complexes with phosphatidylserine in membranes, Biochemistry 36 (1997) 10545–10550.
[81] K.N. Burger, R.W. Staffhorst, B. De Kruijff, Interaction of the anti-cancer drug cisplatin with phosphatidylserine in intact and semi-intact cells, Biochim. Biophys. Acta 1419 (1999) 43–54.
[82] L. Nierzwicki, et al., Interaction of cisplatin and two potential antitumoral platinum(II) complexes with a model lipid membrane: a combined NMR and MD study, Phys. Chem. Chem. Phys. 17 (2015) 1458–1468.
[83] Q. Liu, Y. Qu, R. Van Antwerpen, N. Farrell, Mechanism of the membrane interaction of polynuclear platinum anticancer agents. Implications for cellular uptake, Biochemistry 45 (2006) 4248–4256.
[84] P.V. Escriba, et al., Membranes: a meeting point for lipids, proteins and therapies, J. Cell. Mol. Med. 12 (2008) 829–875.
[85] S. Lacour, et al., Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells, Cancer Res. 64 (2004) 3593–3598.
[86] T.R. Triton, G. Yee, The anticancer agent adriamycin can be actively cytotoxic without entering cells, Science (New York, N.Y.) 217 (1982) 248–250.
[87] P.V. Escribá, M. Sastre, J.A. García-Sevilla, Disruption of cellular signaling pathways by daunomycin through destabilization of nonlamellar membrane structures, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 7595–7599.
[88] C. Peetla, S. Vijayaraghavalu, V. Labhasetwar, Biophysics of cell membrane lipids in cancer drug resistance: implications for drug transport and drug delivery with nanoparticles, Adv. Drug Deliv. Rev. 65 (2013) 1686–1698.
[89] H.S. Schwartz, P.M. Kanter, Chemical interactions of cardiolipin with daunorubicin and other intercalating agents, Eur. J. Cancer 15 (1979) (1965) 923–928.
[90] J.P. Jaffrézou, et al., Daunorubicin-induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis, EMBO J. 15 (1996) 2417–2424.
[91] S. Grazide, et al., Ara-C- and daunorubicin-induced recruitment of Lyn in sphingomyelinase-enriched membrane rafts, FASEB J. 16 (2002) 1685–1687.
[92] M.T. Dimanche-Boitrel, O. Meurette, A. Rebillard, S. Lacour, Role of early plasma membrane events in chemotherapy-induced cell death, Drug Resist. Updat. 8 (2005) 5–14.
[93] K.U. Maheswari, Lipid bilayer-methotrexate interactions: a basis for methotrexate neurotoxicity, Curr. Sci. 81 (2001) 571–573.
[94] R. Pignatello, L. Di Guardo, A. Puleo, G. Puglisi, Lipophilic conjugates of methotrexate with glucosyl-lipoamino acids: calorimetric study of the interaction with a biomembrane model, Thermochim. Acta 426 (2005) 163–171.
[95] D.W. Fry, J.C. White, I.D. Goldman, Alterations of the carrier-mediated transport of an anionic solute, methotrexate, by charged liposomes in Ehrlich ascites tumor cells, J. Membr. Biol. 50 (1979) 123–140.
[96] H. Boyar, F. Severcan, Tamoxifen-model membrane interactions: an FT-IR study, J. Mol. Struct. 408–409 (1997) 265–268.
[97] J.B. Custodio, L.M. Almeida, V.M. Madeira, The anticancer drug tamoxifen induces changes in the physical properties of model and native membranes, Biochim. Biophys. Acta 1150 (1993) 123–129.
[98] F. Severcan, N. Kazanci, F. Zorlu, Tamoxifen increases membrane fluidity at high concentrations, Biosci. Rep. 20 (2000) 177–184.
[99] N. Kazanci, F. Severcan, Concentration dependent different action of tamoxifen on membrane fluidity, Biosci. Rep. 27 (2007) 247–255.
[100] F. Cronier, A. Patenaude, R. C.G., M. Auger, Membrane composition modulates the interaction between a new class of antineoplastic agents deriving from aromatic 2-chloroethylureas and lipid bilayers: a solid-state NMR study, Chem. Phys. Lipids 146 (2007) 125–135.
[101] C. Gicquaud, et al., Interaction of 4-*tert*-butyl-[3-(2-chloroethyl) ureido] benzene with phosphatidylcholine bilayers: a differential scanning calorimetry and infrared spectroscopy study, Arch. Biochem. Biophys. 334 (1996) 193–199.
[102] J. Legault, et al., Microtubule disruption induced in vivo by alkylation of β-tubulin by 1-aryl-3-(2-chloroethyl)ureas, a novel class of soft alkylating agents, Cancer Res. 60 (2000) 985–992.
[103] E. Mounetou, J. Legault, J. Lacroix, R. C-Gaudreault, Antimitotic antitumor agents: synthesis, structure–activity relationships, and biological characterization of N-aryl-N′-(2-chloroethyl)ureas as new selective alkylating agents, J. Med. Chem. 44 (2001) 694–702.
[104] E. Mounetou, J. Legault, J. Lacroix, R.C. Gaudreault, A new generation of N-aryl-N′-(1-alkyl-2-chloroethyl)ureas as microtubule disrupters: synthesis, antiproliferative activity, and β-tubulin alkylation kinetics, J. Med. Chem. 46 (2003) 5055–5063.
[105] A. Saint-Laurent, et al., Membrane interactions of a new class of anticancer agents derived from arylchloroethylurea: a FTIR spectroscopic study, Chem. Phys. Lipids 111 (2001) 163–175.
[106] A. Saint-Laurent, et al., Interaction between lipid bilayers and a new class of antineoplastic agents derived from arylchloroethylurea: a 2H solid-state NMR study, Biochemistry and cell biology = Biochimie et biologie cellulaire 76 (1998) 465–471.
[107] J.F. Labbe, F. Cronier, R. C.G., M. Auger, Spectroscopic characterization of DMPC/DOTAP cationic liposomes and their interactions with DNA and drugs, Chem. Phys. Lipids 158 (2009) 91–101.
[108] P. Couvreur, et al., Squalenoyl nanomedicines as potential therapeutics, Nano Lett. 6 (2006) 2544–2548.
[109] B. Pili, et al., Interaction of a new anticancer prodrug, gemcitabine-squalene, with a model membrane: coupled DSC and XRD study, Biochim. Biophys. Acta 1798 (2010) 1522–1532.
[110] B. Pili, et al., Interaction of an anticancer drug, gemcitabine, with phospholipid bilayers, J. Therm. Anal. Calorim. 98 (2009) 19–28.
[111] F. Castelli, et al., Enhancement of gemcitabine affinity for biomembranes by conjugation with squalene: differential scanning calorimetry and Langmuir-Blodgett studies using biomembrane models, J. Colloid Interface Sci. 316 (2007) 43–52.
[112] R.A. Siddiqui, et al., Anticancer properties of propofol-docosahexaenoate and propofol-eicosapentaenoate on breast cancer cells, Breast Cancer Res. 7 (2005) R645–R654.
[113] K.A. Harvey, et al., Characterization of anticancer properties of 2,6-diisopropylphenol–docosahexaenoate and analogues in breast cancer cells, Bioorg. Med. Chem. 18 (2010) 1866–1874.
[114] W. Stillwell, S.R. Wassall, Docosahexaenoic acid: membrane properties of a unique fatty acid, Chem. Phys. Lipids 126 (2003) 1–27.
[115] V. Jendrossek, R. Handrick, Membrane targeted anticancer drugs: potent inducers of apoptosis and putative radiosensitisers, Curr. Med. Chem.: Anti-Cancer Agents 3 (2003) 343–353.
[116] C. Gajate, et al., Intracellular triggering of Fas aggregation and recruitment of apoptotic molecules into Fas-enriched rafts in selective tumor cell apoptosis, J. Exp. Med. 200 (2004) 353–365.
[117] T. Nieto-Miguel, C. Gajate, F. Mollinedo, Differential targets and subcellular localization of antitumor alkyl-lysophospholipid in leukemic versus solid tumor cells, J. Biol. Chem. 281 (2006) 14833–14840.
[118] A.H. van der Luit, et al., Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis, J. Biol. Chem. 277 (2002) 39541–39547.
[119] C. Gajate, F. Mollinedo, The antitumor ether lipid ET-18-OCH3 induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells, Blood 98 (2001) 3860–3863.
[120] C. Gajate, F. Mollinedo, Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts, Blood 109 (2007) 711–719.
[121] V. Zaremberg, et al., Cytotoxicity of an anti-cancer lysophospholipid through selective modification of lipid raft composition, J. Biol. Chem. 280 (2005) 38047–38058.
[122] J.V. Busto, et al., Surface-active properties of the antitumour ether lipid 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (edelfosine), Biochim. Biophys. Acta Biomembr. 1768 (2007) 1855–1860.
[123] A. Więcek, et al., Interactions between an anticancer drug – edelfosine – and DPPC in Langmuir monolayers, Colloids Surf. A Physicochem. Eng. Asp. 321 (2008) 201–205.
[124] P. Dynarowicz-Latka, K. Hac-Wydro, Edelfosine in membrane environment - the Langmuir monolayer studies, Anti Cancer Agents Med. Chem. 14 (2014) 499–508.
[125] F. Mollinedo, et al., Lipid raft-targeted therapy in multiple myeloma, Oncogene 29 (2010) 3748–3757.
[126] H.R. Fei, G. Chen, J.M. Wang, F.Z. Wang, Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation, Cytotechnology 62 (2010) 449–460.
[127] S.R. Vink, et al., Lipid rafts and metabolic energy differentially determine uptake of anti-cancer alkylphospholipids in lymphoma versus carcinoma cells, Biochem. Pharmacol. 74 (2007) 1456–1465.
[128] G.A. dos Santos, et al., Interaction of 10-(octyloxy) decyl-2-(trimethylammonium) ethyl phosphate with mimetic membranes and cytotoxic effect on leukemic cells, Biochim. Biophys. Acta 1798 (2010) 1714–1723.
[129] C.H. Thome, et al., Linker for activation of T-cell family member2 (LAT2) a lipid raft adaptor protein for AKT signaling, is an early mediator of alkylphospholipid antileukemic activity, Mol. Cell. Proteomics 11 (2012) 1898–1912.
[130] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[131] K.S. George, S. Wu, Lipid raft: a floating island of death or survival, Toxicol. Appl. Pharmacol. 259 (2012) 311–319.
[132] K. Simons, M.J. Gerl, Revitalizing membrane rafts: new tools and insights, Nat. Rev. Mol. Cell Biol. 11 (2010) 688–699.
[133] S. Teres, et al., 2-Hydroxyoleate, a nontoxic membrane binding anticancer drug, induces glioma cell differentiation and autophagy, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 8489–8494.
[134] G. Barcelo-Coblijn, et al., Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 19569–19574.
[135] S. Piotto, et al., Differential effect of 2-hydroxyoleic acid enantiomers on protein (sphingomyelin synthase) and lipid (membrane) targets, Biochim. Biophys. Acta 1838 (2014) 1628–1637.
[136] T. Erazo, et al., The new antitumor drug ABTL0812 inhibits the Akt/mTORC1 Axis by upregulating tribbles-3 pseudokinase, Clin. Cancer Res. (2015).
[137] D. Lopez, S. Martinez-Luis, Marine natural products with P-glycoprotein inhibitor properties, Mar. Drugs 12 (2014) 525–546.

[138] Z. Binkhathlan, A. Lavasanifar, P-Glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives, Curr. Cancer Drug Targets 13 (2013) 326–346.
[139] R.H. Ho, R.B. Kim, Transporters and drug therapy: implications for drug disposition and disease, Clin. Pharmacol. Ther. 78 (2005) 260–277.
[140] C.F. Higgins, M.M. Gottesman, Is the multidrug transporter a flippase? Trends Biochem. Sci. 17 (1992) 18–21.
[141] R.J. Ferreira, M.-J.U. Ferreira, D.J.V.A. dos Santos, Molecular docking characterizes substrate-binding sites and efflux modulation mechanisms within P-glycoprotein, J. Chem. Inf. Model. 53 (2013) 1747–1760.
[142] C. Rauch, S.W. Paine, P. Littlewood, Can long range mechanical interaction between drugs and membrane proteins define the notion of molecular promiscuity? Application to P-glycoprotein-mediated multidrug resistance (MDR), Biochim. Biophys. Acta Gen. Subj. 1830 (2013) 5112–5118.
[143] A.T. Clay, F.J. Sharom, Lipid bilayer properties control membrane partitioning, binding, and transport of P-glycoprotein substrates, Biochemistry 52 (2013) 343–354.
[144] F.J. Sharom, Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: its role in modulating protein function, Front. Oncol. 4 (2014) 41.
[145] F.J. Sharom, R. Liu, B. Vinepal, Fluorescence studies of drug binding and translocation by membrane transporters, Methods Mol. Biol. 637 (2010) 133–148.
[146] M. Dong, F. Penin, L.G. Baggetto, Efficient purification and reconstitution of P-glycoprotein for functional and structural studies, J. Biol. Chem. 271 (1996) 28875–28883.
[147] F.A. Sinicrope, et al., Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles, J. Biol. Chem. 267 (1992) 24995–25002.
[148] P. Lu, R. Liu, F.J. Sharom, Drug transport by reconstituted P-glycoprotein in proteoliposomes. Effect of substrates and modulators, and dependence on bilayer phase state, Eur. J. Biochem. FEBS 268 (2001) 1687–1697.
[149] P.K. Dudeja, et al., Reversal of multidrug resistance phenotype by surfactants: relationship to membrane lipid fluidity, Arch. Biochem. Biophys. 319 (1995) 309–315.
[150] O.O. Krylova, et al., Pluronic L61 accelerates flip-flop and transbilayer doxorubicin permeation, Chemistry 9 (2003) 3930–3936.
[151] S. Modok, C. Heyward, R. Callaghan, P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment, J. Lipid Res. 45 (2004) 1910–1918.
[152] P. Reungpatthanaphong, et al., Decrease of P-glycoprotein activity in K562/ADR cells by MbetaCD and filipin and lack of effect induced by cholesterol oxidase indicate that this transporter is not located in rafts, J. Bioenerg. Biomembr. 36 (2004) 533–543.
[153] Y. Kimura, et al., Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol, Biochem. J. 401 (2007) 597–605.
[154] P.D. Eckford, F.J. Sharom, Interaction of the P-glycoprotein multidrug efflux pump with cholesterol: effects on ATPase activity, drug binding and transport, Biochemistry 47 (2008) 13686–13698.
[155] M.R. Wenk, The emerging field of lipidomics, Nat. Rev. Drug Discov. 4 (2005) 594–610.
[156] K. Hąc-Wydro, P. Dynarowicz-Łątka, P. Wydro, K. Bąk, Edelfosine disturbs the sphingomyelin–cholesterol model membrane system in a cholesterol-dependent way – the Langmuir monolayer study, Colloids Surf. B: Biointerfaces 88 (2011) 635–640.
[157] K. Hac-Wydro, P. Dynarowicz-Latka, P. Wydro, K. Bak, Edelfosine disturbs the sphingomyelin-cholesterol model membrane system in a cholesterol-dependent way - the Langmuir monolayer study, Colloids Surf. B: Biointerfaces 88 (2011) 635–640.
[158] P. Béchard, J. Lacroix, P. Poyet, R. C-Gaudreault, Synthesis and cytotoxic activity of new alkyl[3-(2-chloroethyl)ureido]benzene derivatives, Eur. J. Med. Chem. 29 (1994) 963–966.
[159] M. Duarte-Karim, J.M. Ruysschaert, J. Hildebrand, Affinity of adriamycin to phospholipids. A possible explanation for cardiac mitochondrial lesions, Biochem. Biophys. Res. Commun. 71 (1976) 658–663.

Chapter III

Methods to assess the anthracyclines-membrane interactions

In this chapter are presented all the techniques used during this work with the goal of investigating the interaction of anthracyclines with membranes.

1. Biomembranes and model systems

1.1. The cell membrane

The evolution of cellular life required the presence of a boundary to define its dimension but also to separate the interior of the cell from the outside environment - a need fulfilled by biological membranes (Figure 1). Biomembranes are essential structural and functional elements of all biological cells. These very complex and highly dynamic structures protect the cell from external xenobiotics, act as selectively permeable barriers and participate in a variety of cellular processes such as cell adhesion and cell signaling.

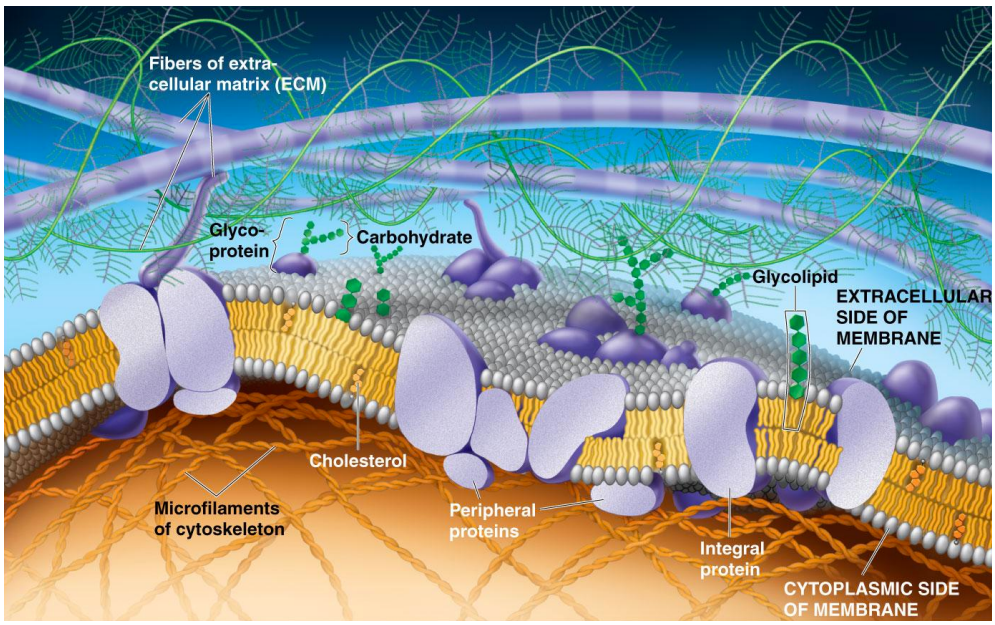


Figure 1: The general molecular structure of biological membranes is a continuous phospholipid bilayer in which proteins and carbohydrates are embedded.

When a pharmacological active compound arrives at a cell in the body, the first structure that encounters will be the plasma membrane. The properties of this membrane will determine whether and how the cell will react to the compound. Will it cross the cell boundary and enter? What determines whether it crosses the membrane? How can the compound interactions with the membrane components induce alterations in cell function? It becomes obvious that the understanding of membranes structure, organization and composition is imperative when studying drug-membrane interactions. In fact, drug-membrane interactions conditions the partitioning, orientation and conformation of drugs and, thereby, highly dictate their pharmacokinetic properties (e.g. drug transport, distribution, accumulation, efficacy and resistance) (3).

1.1.1. Membrane lipid composition

Biological membranes present a high variability in their composition and physical properties, depending of the cell and even within the cells (e.g. according to the organelle). Although the plasma membrane contains proteins and carbohydrates, amphiphilic lipids, which are made up of polar head groups and hydrophobic tails (acyl chains), constitute its structural basic unit. Due to their chemical structure, lipids spontaneously self-assemble in an aqueous environment into membrane structures such as bilayers, with the fatty acid tails of the two layers facing each other, and the polar heads interacting with the water (Figure 2).

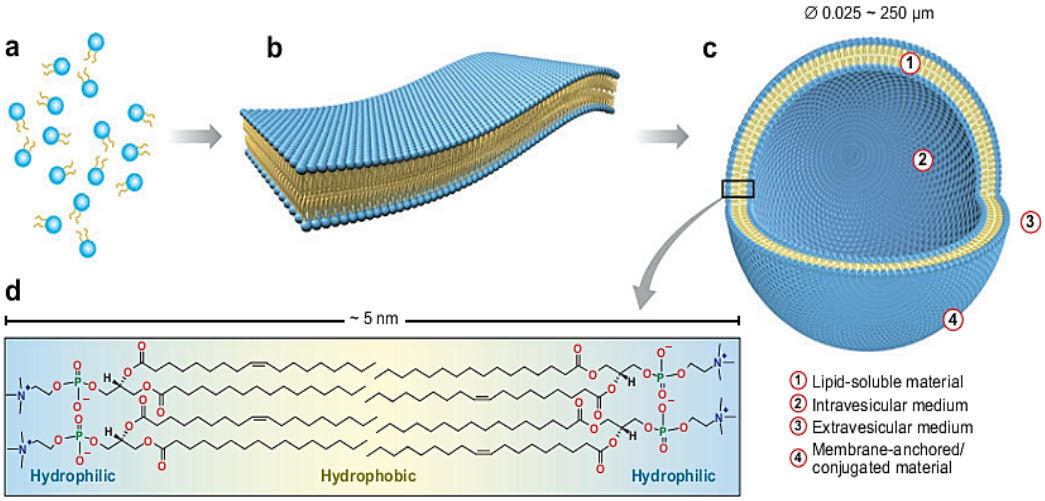


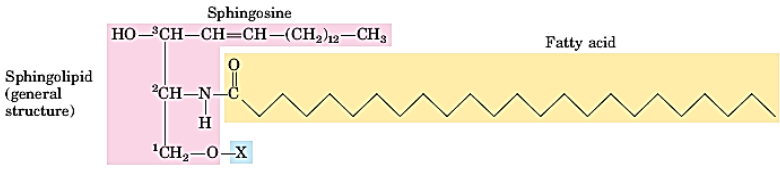
Figure 2: Schematic illustration of the fundamental self-assembly process from individual phospholipid molecules (a) to bilayer membrane leaflets (b), followed by transformation into liposomes (c). From (4).

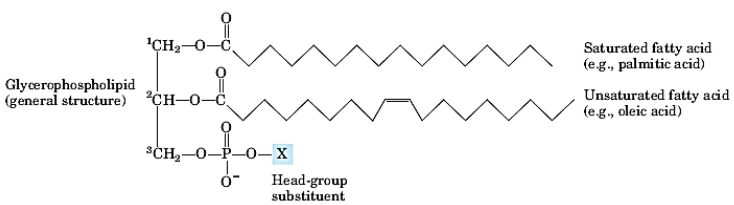
Recent advances in Lipidomics have provided new perspectives from which the roles of membrane lipids in cells can be evaluated, having evolved from a simple physical barrier to a critical component in membrane fluidity regulation, cell signaling and other cellular processes (5). Eukaryotic cell membranes are composed of a large variety of different lipid species that include phospholipids, glycolipids, and sterols (among which cholesterol is the most important). In order to focus on the scope of this study, only the phospholipids will be discussed in more detail.

Phospholipids are the most abundant constituents of membranes and, although biological membranes all share a similar structure, there are many kinds of different phospholipids and membranes from different cells or organelles may differ greatly in their lipid composition. The phospholipids can be of two types: sphingolipids or glycerophospholipids (Table 1).

Table 1: The two types of phospholipids and their structural composition.

Phospholipids type	*Characteristics*
Sphingolipids	Have a sphingosine base linked by an amide bond to a saturated fatty acid with a long chain (up to 24 carbon atoms). These lipids impart greater hydrophobicity to the core of the lipid bilayer.
Glycerophospholipids	Are composed of glycerol, a phosphate group, and two fatty acid chains. Glycerol is a three-carbon molecule that functions as the backbone of these membrane lipids. Within an individual glycerophospholipid, fatty acids are attached to the first and second carbons, and the phosphate group is attached to the third carbon of the glycerol backbone. Variable head groups are attached to the phosphate.

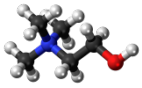
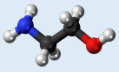
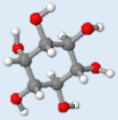
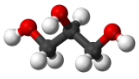




Phospholipids can differ in terms of fatty acid chain length (number of carbon atoms), degree of unsaturation (number of double bonds) and the kinds of polar groups present. The most common saturated fatty acids in membranes are the palmitic acid (C16), stearic acid (C18), and myristic acid (C14), while oleic acid (C18:1) represents the most important unsaturated fatty acid. Saturated fatty acids allow close packing of phospholipids in the bilayer, whereas the "kinks" in the unsaturated fatty acids provide a less dense, more fluid packing.

The glycerophospholipids are the main type of lipid comprised in biomembranes and, depending on the alcohol group, can be divided into different groups, based on their hydrophilic headgroup (Table 2).

Table 2: Phospholipid classification according to their hydrophilic headgroups.

Alcohol group	*Phospholipid name (Abb.)*	*Structure of the head group*
----	Phosphatidic acid (PA)	- H
Choline	Phosphatidylcholine (PC)	
Ethanolamine	Phosphatidylethanolamine (PE)	
Serine	Phosphatidylserine (PS)	
Inositol	Phosphatidylinositol (PI)	
Glycerol	Cardiolipin (CL)	

Phospholipids containing choline, often called lecithins, are the most abundant in nature. These molecules are zwitterionic at physiological pH (7.4), due to the quaternary ammonium group which is positively charged and to the phosphate group which is deprotonated, and thus negatively charged. With the exception of PC and PE, all other phospholipids are negatively charged.

Cholesterol (Figure 3) is also a major component of the animal cell membrane (up to 20-30 % of the lipid content) being fundamental for the maintenance of membrane integrity (3). This sterol molecule contains a hydroxyl group that interacts with the phosphate head of phospholipids, whereas the bulky steroid region interacts with phospholipid acyl chains. Among other important physical properties of membranes, these interactions regulate membrane fluidity, membrane packing, non-lamellar phase propensity and the formation of microdomains (5). In fact, cholesterol has a key role in maintaining a cell membrane that has high fluidity (fast lateral diffusion) since it confers rigidity and impermeability. This sterol molecule acts as a "fluidity buffer" due to its ability to orientationally order chains of fluid phase-lipids, which leads to increased bilayer thickness, and decreased permeability (6). Moreover it has the ability to cause positional/translational disordering of gel-phase lipids while still maintaining a high degree of orientational ordering (6).

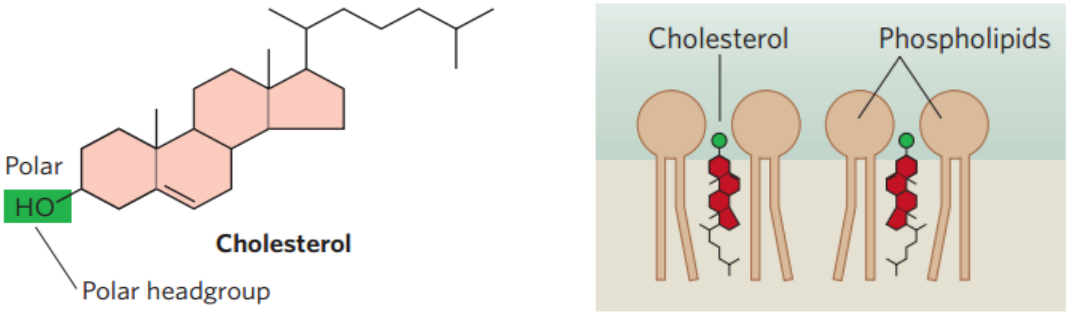


Figure 3: Cholesterol in biological structure.

1.1.2. Membrane lipid structure

Membrane lipids have different physical properties (sometimes even within the same lipid class) in terms of phase transition temperatures, cross-sectional area, electric charge, molecular weight and nonlamellar-phase propensity. These molecules are invariably polymorphic, which means that they can present a variety of different kinds of organized structures, especially when hydrated.

Lipids in aqueous environment can form lamellar structures or non-lamellar phases, such as micellar (M), hexagonal (H_I), inverted hexagonal (H_{II}), and cubic (Q) phases (Figure 4). The polymorphic form depends not only on the structure of the lipid molecule itself and on its degree of hydration, but also on other parameters, such as temperature, pressure, ionic strength, and pH.

The most common and relevant phase, from the biological point of view, is the lamellar liquid-crystalline phase or fluid phase (L_α) (8, 9) (Figure 5). When temperature decreases,

the lipid bilayer suffers a phase transition to a more ordered phase in which the lipids are more tightly packed and the acyl chains are ordered, forming a solid-crystalline lamellar phase or gel phase, which can be tilted ($L_{\beta'}$) or untilted (L_{β}) (10). The transition between fluid phase and gel phase occurs at the main phase transition temperature (T_m) (10). For some lipids, such as DMPC and DPPC, an intermediate phase between fluid and gel phases also occurs at the pre-transition temperature (T_p), and is called ripple phase ($P_{\beta'}$). With further temperature decrease, a highly ordered lamellar crystalline phase (L_c) is formed.

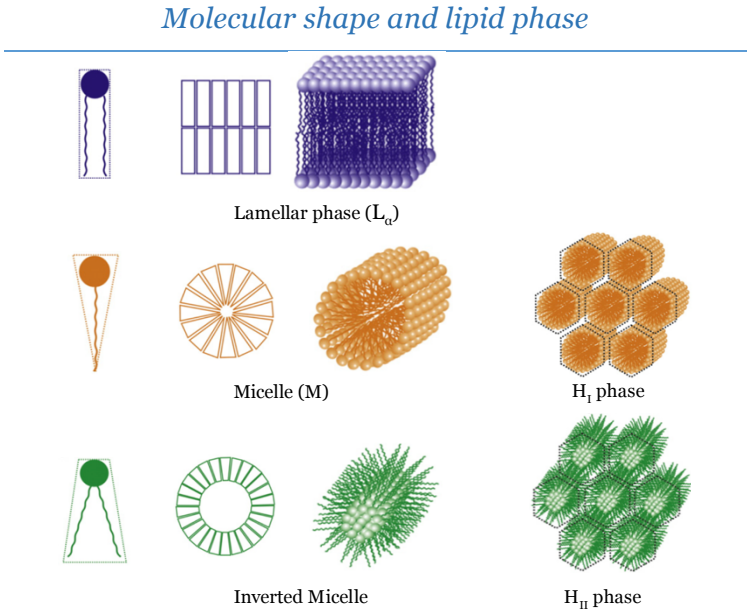


Figure 4: Membrane lipid structure: examples of lipid shapes and their influence on membrane structure. Adapted from (7).

The so-called liquid-ordered phase (L_o) is formed by defined domains enriched in saturated lipids, is cholesterol dependent and shares features with lipid raft domains in cell membranes (11) (12). In the L_o phase, the acyl chains of lipids are extended and tightly packed as in the gel phase, but they have a higher degree of lateral mobility as in the fluid phase.

The described lipid phases can be altered not only by the abovementioned factors (temperature, hydration, type of lipid), but also by pressure, lipid-lipid and lipid-peptide interactions that occur in the membrane, or even by the disturbance created when a foreign molecule (e.g. drug) interacts with the lipid membrane (13-16). Thus, the drug-membrane interaction can be studied in terms of how the membrane biophysical properties are modified as a result of this interaction, which may have physiological consequences related to the drug mechanism of action (14). For example, the incorporation of a drug in the membrane lipid environment can alter membrane microviscosity and, indirectly, alter the

functioning of enzymes that normally, to be effective, require very specific biophysical environment at the membrane interface (17-19). Moreover, the inclusion of a drug in the membrane can change its curvature (14), and the curvature of membranes is an indicator of the internal stress of the lipid bilayer being also an important factor in the functioning of proteins.

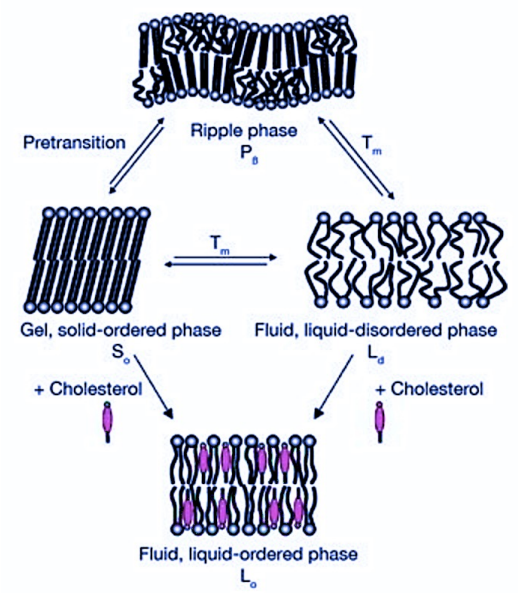


Figure 5: Representation of the lamellar phase transitions.

1.1.3. Membrane lipid organization

In 1972, Singer and Nicholson (20) proposed the fluid-mosaic model to explain the membrane organization, where lipids and the enclosed proteins show a rapid lateral diffusion without any specific order. Since then, biochemical and biophysical findings have provided a detailed model of the composition and structure of membranes, which includes levels of dynamic organization both across the lipid bilayer (lipid asymmetry) and in the lateral dimension (lipid domains) of membranes (21).

In this context, most biological membranes are asymmetrical (Figure 6), both laterally and in cross-section. The two layers of a membrane are characterized by an asymmetrical distribution of their constituents: the cytoplasmic leaflet is generally enriched in PE and PS, while the exoplasmic leaflet presents mainly PC and SM. Additionally, minor phospholipids, such as phosphatidic acid and PI, are also found on the cytoplasmic face.

Nevertheless, the organization of specific domains is a crucial aspect of membrane structure, which has been receiving increasing attention in the last past years. Because lipids

have significant lateral mobility, it might be expected their homogenous distribution along the majority of membranes. However, both model and biological membranes exhibit non-ideal mixing in systems containing two or more elements (5). Although various different domains are known to exist, research has focused on lipid rafts for many years (5).

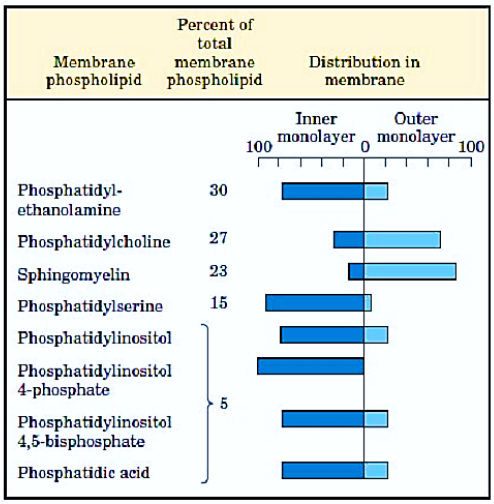


Figure 6: Membrane phospholipids distribution between the two leaflets. Adapted from (22).

The raft (Figure 7) hypothesis postulates that the membrane is able to laterally segregate its constituents (dynamic liquid-liquid immiscibility of regions enriched in cholesterol and sphingolipids (11)) creating nanometer-sized domains which over time can change size, composition and lateral position. These membrane domains are supposed to act as platforms for membrane signaling and trafficking (11). Notwithstanding, despite the huge investigation performed on lipid rafts, there is still remains some controversy about their existence in the cellular plasma membrane, their size, and function (23).

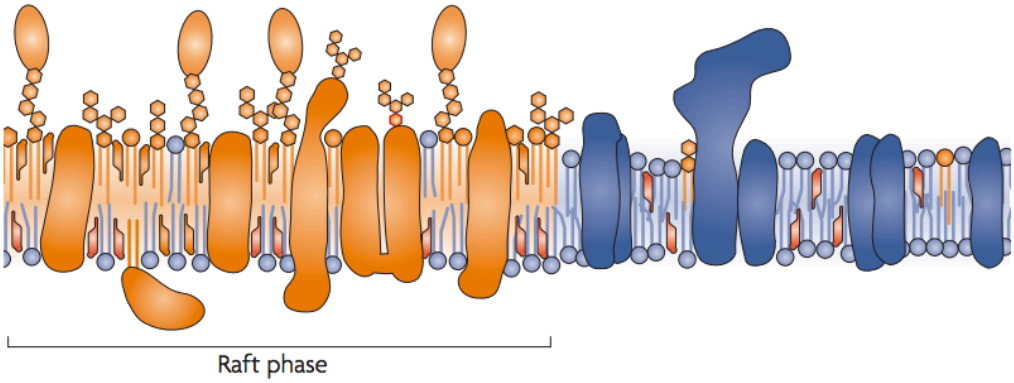


Figure 7: Schematic representation of lipid raft enriched in cholesterol and sphingolipids, forming platforms for membrane signaling and trafficking. Adapted from (11).

1.2. Liposomes

The intricate biomembrane' structure along with the highly dynamic nature of lipid-lipid and lipid-protein interactions difficult the study of biophysical interactions between drugs and biological membranes. In this context, membrane model systems have been developed to provide a simplified basis for analyzing the bilayer structure and properties (24-26). Most of the current knowledge of the chemistry of membrane structures has been achieved through the investigation of membrane mimetic models (24, 27). However, the perfect model does not exist and no single model can faithfully mimic all structural and functional aspects of biomembranes. Concerning the experimental science, there is no need to always reproduce nature in such a detail, since the simplification often helps the interpretation of isolated phenomena.
Among the several available models of biomembranes, liposomes and lipid monolayers were selected for the studies performed in the ambit of this thesis.

Liposomes are spherical vesicles based on (phospho)lipid bilayers with an internal aqueous compartment. They present remarkable structural, chemical and anisotropic resemblance with biological membranes and can, therefore, provide a simple model to mimic the highly complex cell membranes. Morphologically liposomes are classified according to their size and lamellarity, i.e. number of membrane bilayers (Figure 8).

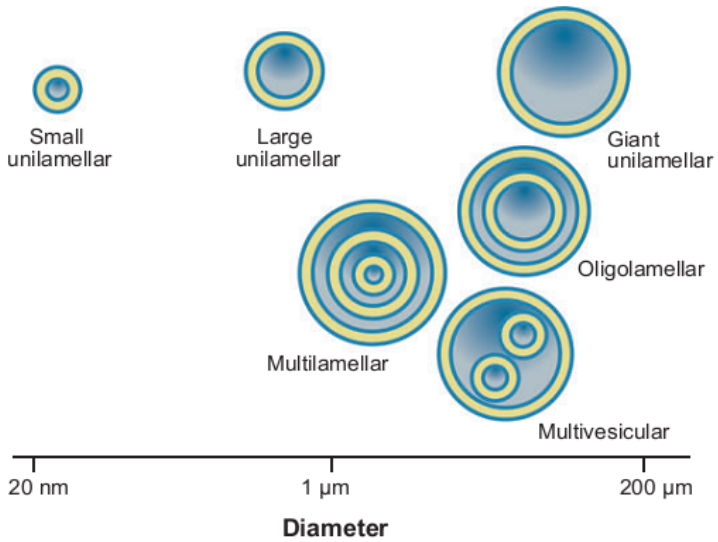


Figure 8: Schematic representation of the commonly applied classification for liposomes. Adapted from (4).

These systems can be easily prepared and characterized, providing accurate and reproducible results. In this thesis, MLVs were prepared according to the thin film-hydration method and LUVs were produced by extrusion (28, 29) (Figure 9). Firstly, lipids

are dissolved in organic solvents, followed by rotary evaporation to form a thin lipid film. After that, the film is hydrated by adding an aqueous buffer and the mechanical agitation allows the spontaneous formation of MLVs. Extrusion is a technique suitable for generating unilamellar liposomes of well-defined sizes, where the MLVs are forced to pass through a narrow-pore membrane filter under pressure at a temperature above the main phase transition temperature (T_m) of the lipid (30). In this thesis, polycarbonate membranes of sequentially decreasing pore sizes (200 and 100 nm) have been used, and vesicles were extruded ten times through each membrane until the formation of the desired LUVs with 100 nm.

Among the wide range of natural and synthetic phospholipids available on the market, phosphatidylcholines have been chosen as the main component of the models, since they are the most abundant phospholipids of natural plasma membranes [24]. Moreover, these phospholipids can be easily characterized in terms of phase transition temperature and thermodynamic parameters (29).

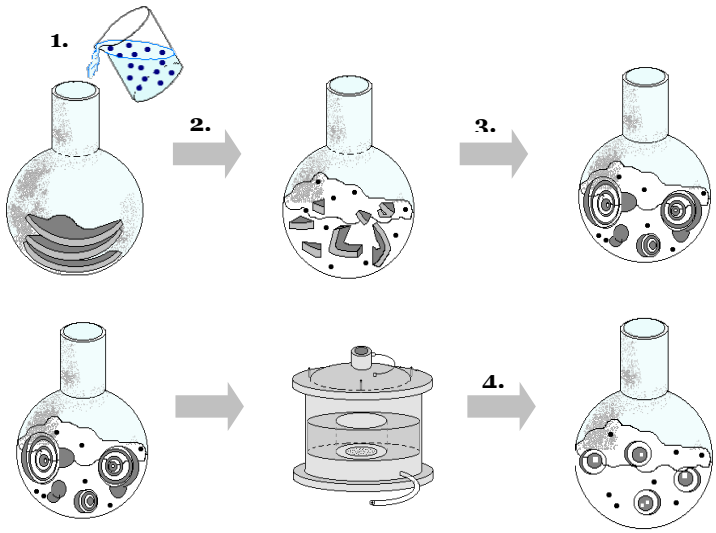


Figure 9: Schematic representation of the film-hydration method: 1) hydration of the lipid film; 2) mechanical agitation allows the lipid film to detach; 3) aggregates of lipids form liposome – MLVs (large multilamellar vesicles); 4) LUVs (small multilamellar vesicles) can be obtained by the extrusion of MLVs.

1.3. Lipid monolayers

Monolayers are known considerably longer than liposomes; in fact, they started to be studied in 1773 by Benjamin Franklin. Since then, many advances have been made and it is now known that most of the substances that form a monolayer are amphipathic molecules containing an alkyl chain length of 12 or more carbon atoms. The amphiphilic nature of the substances dictates the orientation of the molecules at the interface (air/water) (Figure 10). Therefore, while the hydrophilic heads are soaked in water and are responsible for spreading the film on the water surface, due to its greater interaction with the aqueous subphase; the hydrocarbon chains decrease the solubility of the molecules in the aqueous subphase and come in contact with air.

Lipid monolayers are very well-defined, stable, homogeneous bidimensional systems with planar geometry (31). When referring to the lipid monolayer, it is inevitable to mention the Langmuir trough, where various parameters such as lipid composition, subphase, and temperature can be chosen and modified in order to mimic the biological conditions (31). It was actually Langmuir who reported in 1917 the method whereby such studies could be carried-out by using the apparatus called Langmuir trough or Langmuir balance (32). The Langmuir trough (Figure 10) provides information about characteristics of a monolayer on the water surface by measuring the changes in surface tension upon compressing the monolayer. The reduction of surface tension by the addition of the lipid molecules on the air/water interface is known as the surface pressure.

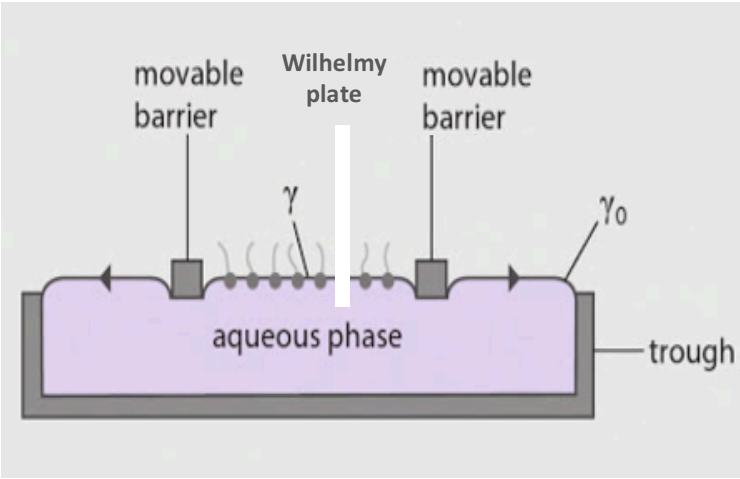


Figure 10: Schematic representation of a Langmuir trough with a monolayer. Adapted from (33).

The surface pressure (π) is defined as the difference between the surface tension of the bare subphase (γ_o) and the surface tension of the subphase covered by amphiphiles (γ):

$$\pi = \gamma_0 - \gamma \qquad \textbf{Equation 1}$$

The film pressure is measured by the Wilhelmy plate method. Usually the Wilhelmy plate is a piece of absorbent paper hanging into the water subphase. The force acting on it is proportional to the surface tension.
Usually water or an aqueous buffer is used as the subphase. The lipids are dissolved in a solvent (normally chloroform) which is volatile and not miscible with the subphase. Drops of the lipid solution are placed on the liquid surface and after evaporation of the solvent a lipid film remains, a process that is called "spreading". Via movable barriers the film balance allows to manipulate the density of molecules on the liquid surface by compressing or expanding the lipid film. Compression of the monolayer leads to a decrease of the area per molecule (A).

Generally, using the Langmuir trough the interaction between drugs and lipids can be investigated in two ways (31):
i) Keeping the film area constant. Changes in surface pressure are recorded upon addition of drugs to the subphase. In practical terms the lipid monolayer on the water/buffer surface is compressed to the wanted surface pressure by applying lateral pressure. Usually the chosen surface pressure is 30 $mN.m^{-1}$ once at this surface pressure, the lipid packing density of a monolayer is similar to that of a cell membrane (31).
ii) Surface-area isotherms. The lipid is spread over a subphase containing the studied drug at the desired concentration. Upon spreading, if the drug adsorbs into the monolayer the surface pressure will increase. When the pressure stops to increase, the monolayer is then compressed, and comparisons are made between the surface-area isotherms of the pure lipid or of the lipid with drug.

2. Membrane partitioning

A pharmaceutical compound, in order to exert its pharmacological action, comes inevitably in contact with biological membranes (34). In fact, such lipid structures can serve either as a target or barrier that drugs must overcome on their path to the action site (3). Furthermore, the drugs physicochemical properties such as lipophilicity play a pivotal role in drug-membrane interactions and, consequently, can determine the ability of these molecules to achieve the desired target (35) (Figure 11). How drugs interplay and are distributed on the membrane and its aqueous phase influences the extents of their penetration/permeation into or across the membrane to access the site of action (36) and, thus, their bioavailability (37).

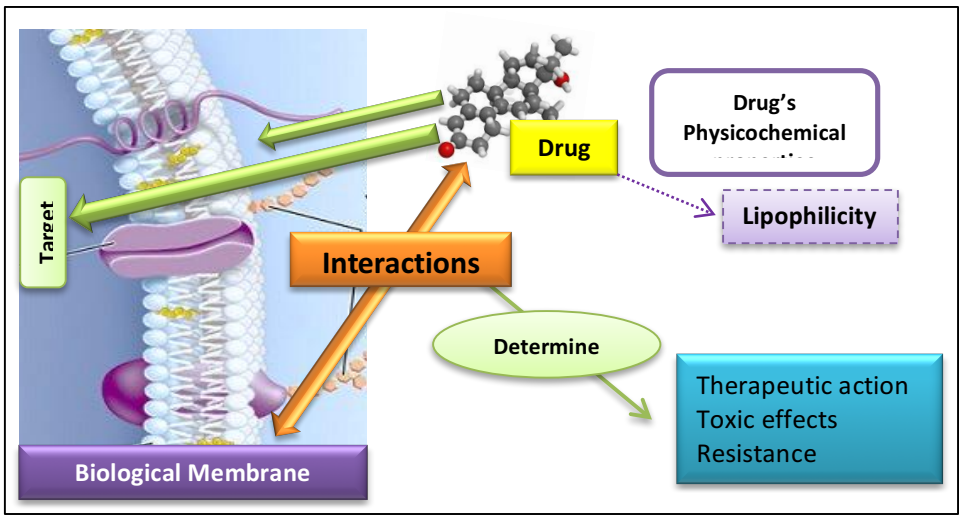


Figure 11: Importance of the drug-membrane interaction studies.

In this context, the assessment of the partition coefficient (P), a measure of the drug lipophilicity, is of vital importance to understand and explain drugs pharmacological activity and also to predict its possible side effects (38). This parameter is defined by the ratio of drug concentrations between the lipid and aqueous phases at equilibrium (35) and should be the first step in studies of drug-membrane interactions.

$$P = \frac{[\text{Drug}]_{\text{lipid phase}}}{[\text{Drug}]_{\text{aqueous phase}}}$$ **Equation 2**

Drug lipophilicity traditionally has been characterized by the determination of octanol/water partition coefficient ($K_{o/w}$) that is defined as the ratio of drug equilibrium concentrations in each phase formed by water and octanol, two immiscible solvents (34). However, in recent years, several flaws have been pointed in this membrane mimetic

system, because octanol is an isotropic solvent, contrary to the membrane phospholipidic bilayer, which has an anisotropic organization (3, 39).

Additionally, the octanol/water system does not take into account several crucial characteristics of biological membranes, which are constituted by phospholipids that have hydrophilic groups and can therefore establish electrostatic interactions (40). As so, the lack of biomembranes similarity, its oversimplification, not to mention the fact that octanol is an organic solvent relatively toxic, led to the development of more reliable and less pollutant alternative methods such as liposomes and micelles to characterize drug partition with biological membranes (41). In Figure 12 is possible to see the main differences between the determination of the partition coefficient in octanol/water systems and lipid/water systems.

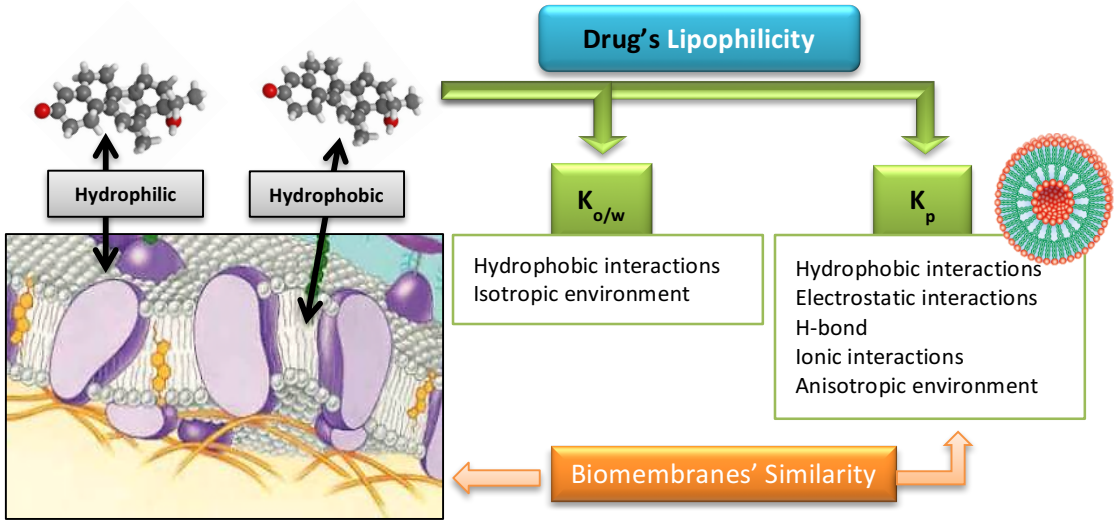


Figure 12: Schematic representation of the main differences between the determinations of partition coefficient in the octanol/water system and in the lipid/water systems as a function of drug lipophilicity.

Liposomes are increasingly being accepted to replace the octanol system once they are constituted by lipids that form a bilayer and can therefore mimic the chemical and structural characteristics of cell membranes, such as the anisotropic environment and ordered lipid organization (42). Because liposomes appear to simulate the different environments (polar surface and lipophilic core), they allow the study of the influence of ionic, H-bond and electrostatic interactions between the drug and the membrane (42) and, consequently, are a better model to describe the natural membrane behaviour. Hence, the determination of partition coefficient of the drug distribution between the aqueous and membrane phases using the liposome/water system (Kp) allows to obtain more realistic information and to characterize the active compounds interactions with phospholipids or other membrane components (36).

Due to the advantages presented, the K_p determination using liposomes/aqueous systems, was the selected method to evaluate the lipophilicity of the anthracyclines studied in the course of this work. The quantification analysis was performed by derivative spectroscopy that will be further described in the next subchapter.

2.1. Derivative spectroscopy

The partition coefficient (K_p) can be determined by UV-Vis spectrophotometry, because there is a difference in an absorbance parameter of the partitioning molecule (e.g., molar absorptivity, ε, and/ or wavelength of maximum absorbance, λ_{max}) when in aqueous solution and after its incorporation on the membrane (43-46). Batochromic deviations (λ_{max} deviations for higher wavelengths) indicate a decrease of polarity in the drug's surroundings and also an incorporation of investigated compounds into the hydrophobic part of lipid bilayer (47)), while hypsochromic deviations (λ_{max} deviations for lower wavelengths) are indicative of the presence of the drug in a more polar microenvironment (47)).

Derivative spectrophotometry was employed to the obtained data in ir order to eliminate the background interference of the medium caused by the light scattering of the vesicles and also to allow a better resolution of overlapping bands (45, 48, 49). Therefore, K_p can be calculated according to the following equation (45, 50):

$$D = D_w + \frac{(D_l - D_w)K_p[L]V_\varphi}{1 + K_p[L]V_\varphi} \qquad \textbf{Equation 3}$$

where D_T, D_w and D_m correspond to the total, aqueous and membrane absorbance of the compound, respectively; K_p is the partition coefficient (dimensionless); [L] the lipid concentration ($molL^{-1}$) and V_φ the lipid molar volume ($Lmol^{-1}$).

3. Membrane location

The study of membrane location allows a deeper understanding of the mode of action of drugs and can be assessed by fluorescence quenching of membrane bound extrinsic fluorophores (fluorescent probes), whose membrane location is known and well characterized. The deactivation of the probe fluorescence (quenching phenomenon) caused by the presence of a drug can, thus, be used to reveal the location of the drug in the membrane (44, 50-52).

3.1. Fluorescence probes

Fluorescence probes contain fluorophores groups that allow obtaining a great variety of information through analysis of their excitation and emission spectra, including their fluorescence quantum yield, the lifetime of the excited state and their polarization (14, 53). The information reported by a probe inserted in a membrane (natural or model membrane) can be used in the evaluation of membrane fluidity and also in the location of molecules in the membrane (54). Currently, there is a wide range of compounds employed as fluorescence probes in several studies. In this work, the fluorophores chosen for assessing the anthracyclines' membrane location were the TMA-DPH, (1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene) and DPH (1,6-diphenyl-1,3,5-hexatriene) (Figure 13). DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer (55). TMA-DPH, contains a DPH phenyl ring which locates within the hydrophobic acyl chains of the membrane phospholipids and a cationic group, that anchors the probe to the polar headgroups of the phospholipids (56, 57).

Accordingly, by using these amphipathic probes it is possible by one hand monitoring the interaction of the drug at the membrane interface (TMA-DPH) and, by other hand, to observe the effects of the drug on the lipid layer due to the parallel alignment of the probe within the hydrophobic chains of phospholipids (DPH) (58).

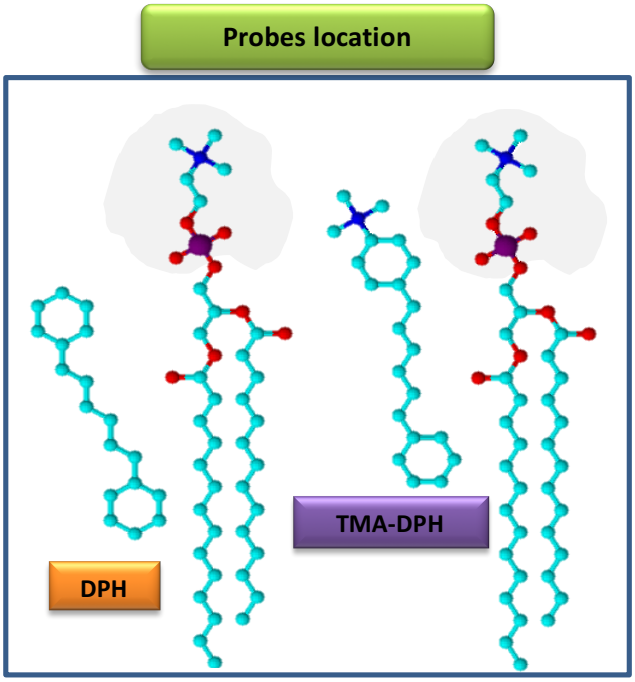


Figure 13: Schematic representation of the DPH and TMA-DPH membrane location.

3.2. Steady-state fluorescence quenching

The decrease in the emitted fluorescence, also known as fluorescence quenching, of a membrane bound fluorophore provides a measure of its accessibility to the drug (quencher) and can be related with the concentration of the quencher [*Q*], according to the Stern-Volmer equation (59):

$$\frac{I_0}{I} - 1 = K_{SV}[Q] \qquad \textbf{Equation 4}$$

where I_o and I are, respectively, the corrected fluorescence intensity of fluorophores in the absence and presence of drug and K_{SV} is the Stern-Volmer constant.

Plotting the values of corrected fluorescence intensities (I_o/I-1) as a function of drug concentration ([*Q*]) a linear relationship is obtained, where the slope is the Stern-Volmer constant (K_{SV}).

The fluorescence intensities are previously corrected to eliminate the inner filter effect, which occurs when the drug absorbs at the excitation wavelength of the fluorophore and decreases the effective exciting intensity, thereby decreasing the fluorescence emission intensity. Therefore, experimental fluorescence intensities should be corrected to eliminate this apparent quenching effect by (60):

$$I_{corr} = I \frac{A_Q}{A_F} \frac{1-10^{-A_F}}{1-10^{-A_Q}} \qquad \textbf{Equation 5}$$

where I_{corr} is the corrected fluorescence intensity, I the experimental fluorescence, A_Q and A_F, the absorbance of the sample in the absence and presence of the quencher.
Besides the correction for the inner filter effect, the Stern-Volmer equation should also be corrected to consider only the so-called effective concentration of the quencher ($[Q]_m$) (50, 61):

$$\frac{I_0}{I} - 1 = K_{SV}[Q]_m \qquad \textbf{Equation 6}$$

Indeed, since the extrinsic fluorophores used in quenching studies are inserted into the lipid matrix, the accessibility to these fluorophores is determined only by the fraction of drug molecules that partitions into the lipid phase. Therefore, from the total drug concentration used in the assays ($[Q]_T$) and the drugs' partition coefficient (K_p) between the aqueous and the lipid phases it is necessary to calculate the effective concentration of quencher ($[Q]_m$) that is able to reach the fluorophore and induce deactivation of its emitted fluorescence (50, 61):

$$[Q]_m = \frac{K_p[Q]_T}{1 + (K_P - 1)V_\varphi[L]} \qquad \textbf{Equation 7}$$

where $[L]$ is the lipid concentration (molL^{-1}) and V_φ is the lipid molar volume (Lmol^{-1}).

As previously mentioned, the fluorescence quenching studies are useful to predict the location of drugs in the membrane. When comparing several drugs that act as quenchers of the same fluorophore, the drug that leads to higher values of Stern-Volmer constant (K_{sv}) is the most effective in the fluorescence deactivation of the fluorophore and, thus, it is the one that is located closer to the fluorophore. When for the same drug, different fluorophores located at different membrane depths are used, it is possible to predict the drug location that will correspond to the location of the fluorophore that suffers higher fluorescence quenching by the drug.

3.3. Time-resolved fluorescence quenching

There are two kinds of mechanisms responsible for fluorescence quenching (59, 62): static quenching, where a non-fluorescent complex is formed between the fluorophore and the quencher; and dynamic or collisional quenching, where the quencher diffuses to the fluorophore during the lifetime of the excited state and upon contact, the fluorophore returns to the ground state, with emission of a photon. In general, quenching occurs without any permanent change in the molecules, i.e. without chemical reaction.

Time-resolved fluorescence measurements provide more information than the available from the steady-state, allowing to distinguish between static and dynamic quenching (59). Therefore, if the quenching process has a dynamic nature it will occur an equivalent decrease in fluorescence intensity and lifetime (59, 62):

$$\frac{I_0}{I} = \frac{\tau_0}{\tau}$$ **Equation 8**

where I_o, τ_o and I, τ are respectively, the corrected fluorescence intensity and lifetime of the fluorophore in the absence and presence of the quencher.

The decrease in lifetime occurs because quenching is an additional rate process that depopulates the excited state. Therefore, plotting the values of lifetime (τ_0/τ-1) as a function of drug concentration ($[Q_m]$) a linear relationship is obtained, where the slope is the dynamic constant (K_D):

$$\frac{\tau_0}{\tau} - 1 = K_D\,[\mathrm{Q_m}]$$ **Equation 9**

On the other hand, in the static quenching occurs the formation of a non-fluorescent complex between the fluorophore and the drug and, thus, the residual detectable fluorescence corresponds to the fraction of non-complexed fluorophores. The fraction of fluorophore molecules that are not complexed by the drug remains undisturbed and, consequently, the lifetime of the excited state remains also constant (59):

$$\frac{\tau_0}{\tau} = 1$$ **Equation 10**

4. Membrane biophysical properties

The membrane structure is very important not only for the characteristics of membrane permeability and interaction with exogenous molecules, but also for the action of endogenous molecules attached to the membrane (proteins, receptors, channels) and the molecular mechanisms involved in pharmacological effects exhibited by a variety of drugs [92, 93, 156]. Experimental work on membrane mimetic systems has demonstrated that their structural properties are strongly affected by membrane associated molecules. Many drugs are able to directly or indirectly influence cell membrane properties, such as interactions between proteins and phospholipids or the formation of complexes between ligand molecules and phospholipids or sterols that can lead to disruption of the membrane so that it becomes highly permeable (14). Moreover, some compounds affect the fluidity of membranes. Additionally, changes in membrane fluidity can affect receptor and enzyme activity and influence the ability of drugs to pass through the membrane, which in turn can affect their efficacy.

A single biophysical method is not sufficient for a detailed analysis of the complexity of membrane dynamics in the absence and presence of drug molecules. In this context, in the next topics are presented several techniques that, when combined, provide a detailed description of the membrane biophysical changes resultant from the interactions with drugs.

4.1. Steady-state anisotropy

The steady-state fluorescence anisotropy (r_{ss}) is based on determining the degree and extent of rotational diffusion of the fluorophore (probe) during the lifetime of the excited state. Small changes in the stiffness of the matrix surrounding the probe produce changes in the rotational movement of the probe and, as such, cause changes in the anisotropy (59). Therefore, the sample is excited with vertically polarized light and fluorescence intensities are measured with the emission polarizer oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation polarizer (59):

$$r_{ss} = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \qquad \textbf{Equation 11}$$

where G is an instrumental correction factor, given by the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (59):

$$G = \frac{I_{\perp}}{I_{\parallel}}$$ **Equation 12**

If the molecules of fluorophore are present in a highly ordered membrane, as seen in the gel or solid-crystalline state, their movement is highly restricted and it is induced a parallel orientation of the fluorophore molecules to the vertical excitation polarizer. As a result, the molecules of fluorophore emit polarized light because they remain immobile during the lifetime of the excited state. However, if the environment surrounding the fluorophore is the fluid state, the notorious free rotation of the fluorophore molecules pushes a random fluorophore orientation, resulting in a decrease in the emission of polarized light. The explanation for this decrease is based on the lack of alignment with the vertical excitation polarizer (Figure 14) (59).

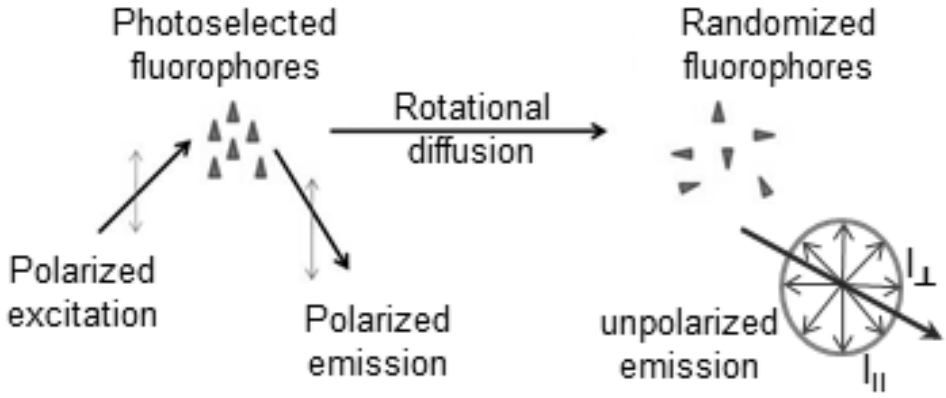


Figure 14: Effects of polarized excitation and rotational diffusion on the anisotropy of the emission. Adapted from (59).

4.2. X-ray diffraction

The basic structural element of biological membranes is the phospholipid bilayer with a lamellar structure. The membrane lamellar phase is a plain structure, whose interfaces are arranged horizontally and stacked regularly. The planes of the bilayers act as parallel mirrors and diffract the X-rays with phase differences between the incident waves (Figure 15) due to their spatial separations. The waves are scattered from lattice planes separated by a regular interplanar distance, *d*. If the scattered waves interfere constructively, they remain in phase and this only occurs if the path length of each incident wave is equal to an integer multiple (n) of the wavelength (λ). The path difference between two waves undergoing constructive interference is, thus, given by the Bragg's Law (63):

$$n\lambda = 2dsen(\theta)$$ **Equation 13**

The values of the angle of incidence, θ, depend on the *d* distances characteristics of lipid bilayers (63). The lamellar spacing (*s*) is correlated with the interplanar distance (*d*) according to the following equation (63):

$$s = \frac{n}{d}$$ **Equation 14**

In this context, it is possible to obtain information about the structure of membranes (Figure 15), both from the lamellar diffraction (bilayer thickness) and diffraction due to the packing of phospholipids (distance between the phospholipids head groups).

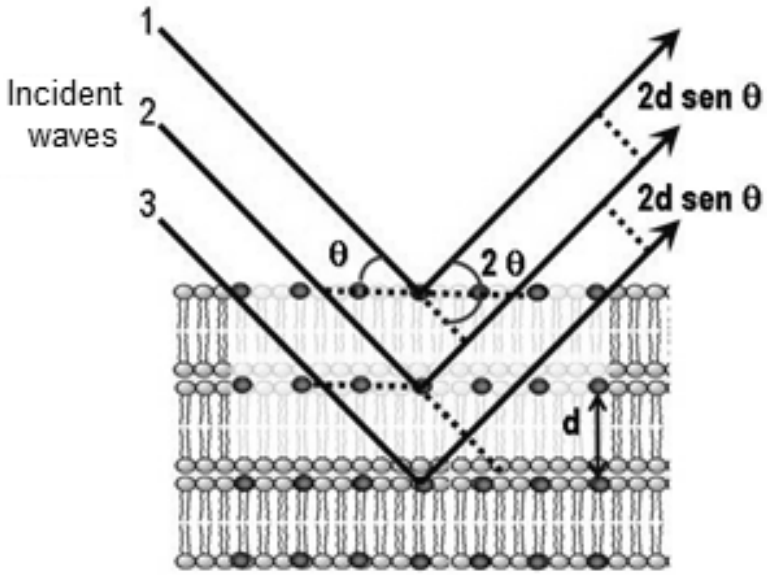


Figure 15: Schematic representation of the X-ray diffraction by phospholipid bilayers of thickness *d* in the direction perpendicular to its plane.

The long range distances (*d* = 10-100 Å) are determined by small-angle detection (small angle x-ray scattering or SAXS), since according to Bragg Law (Equation 16) for lower values of θ ($\theta < 5°$) higher values of *d* are obtained (64). Following the same reasoning for the determination of the short-range distances (*d* <10 Å) a wide-angle detection (wide angle x-ray scattering or WAXS) is required with values of θ of the order of 5 to 180 ° (64). In Figure 15 are presented the main features of the SAXS and WAXS experiments, as well as the main information that can be acquired. It can be also seen from Figure 16, that the lipid bilayers are MLVs contained in glass capillaries transparent to X-rays.

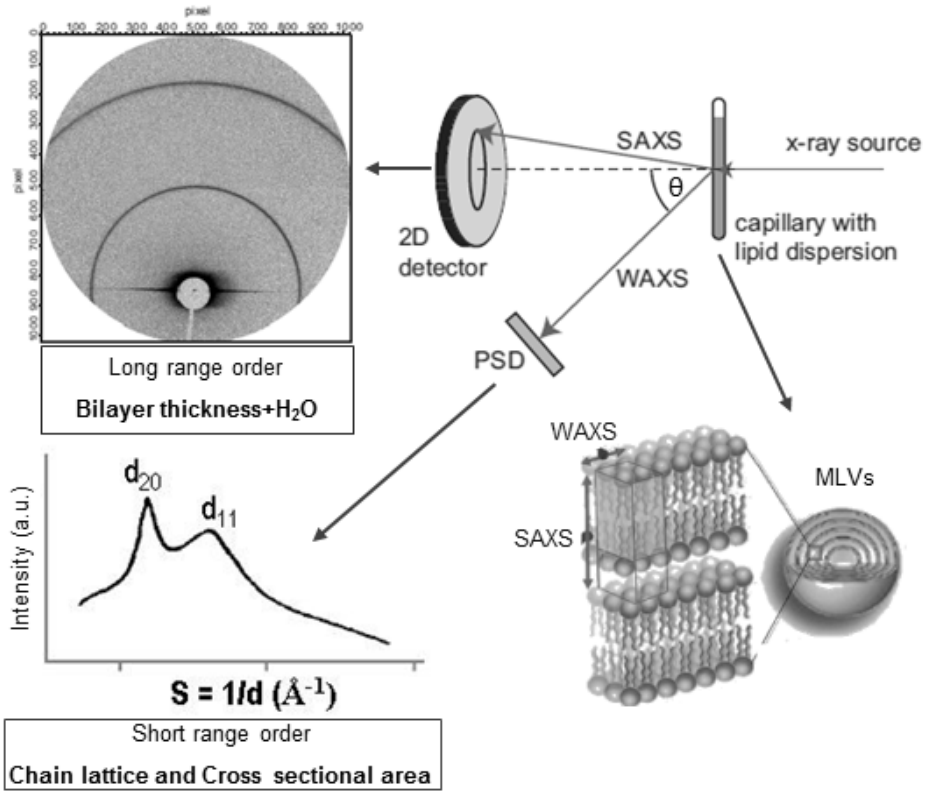


Figure 16: SAXS/WAXS experimental setup, scattering geometry and main information obtained from the x-ray diffraction methods. SAXS is recorded on a 2D detector, WAXS on a position-sensitive detector (PSD).

Synchrotron radiation from electron or positron storage rings offers several excellent features for performing X-ray diffraction studies, since high amounts of energy are required in order to obtain the highest resolution possible in SAXS and WAXS experiments.

4.2.1. Small angle x-ray scattering (SAXS)

As previously explained, the lipid molecules may undergo in a series of different phases depending on their structure, temperature, pressure and degree of hydration. With SAXS technique is possible to assign different lipid phases from the relation between the positions of the diffraction peaks (65): lamellar 1:1/1:2/1:3/1:4; hexagonal 1:1/1:√3/1:√4/1:√7, and cubic 1:1/1:√2/1:√3/1:√4/1:√5/1:√6/1:√8.

Moreover, it is even possible to identify interdigitated or partially interdigitated phases, once their distances (*d*) are smaller than the expected for a lipid bilayer.

The circular diffraction patterns that the 2 D detector provides can be converted to plots of Bragg peaks of intensity versus peak position (*s*). To obtain a more precise position for *s*, the diffraction peaks are fitted with Lorentzians, which also allows the determination of the

full width at half maximum (FWHM) of the peaks. From this parameter, it is also possible to determine the correlation length (ξ) through the following equation:

$$\xi = \frac{2\pi}{FWHM}$$ **Equation 15**

ξ provides indications about the correlation between the bilayers. If the correlation is high, it means that the frequency of appearance of *d* is high, i.e., a large number of bilayers present the same long-range distance, *d* (or thickness) determined by SAXS. The same parameter can be determined for the Bragg peaks in the studies of WAXS. In this case, ξ provides indications about the correlation between the phospholipid molecules. If the correlation is high, it means that the frequency of appearance of *d* is high, i.e., that a large number of phospholipids present the same short-range distance, *d* (packing distance) determined by WAXS (66).

4.2.2. Wide angle x-ray scattering (WAXS)

Through WAXS is possible to determine the type of molecular packing in the crystal lattice defined by the size of the unit cells of the network and, in the particular case of the membranes or membrane model systems, the packing of phospholipid molecules. In the fluid phase, L_{α}, membrane phospholipids are free to move and there is no real ordered network, which typically leads to very diffuse WAXS diffraction Bragg peaks (67).

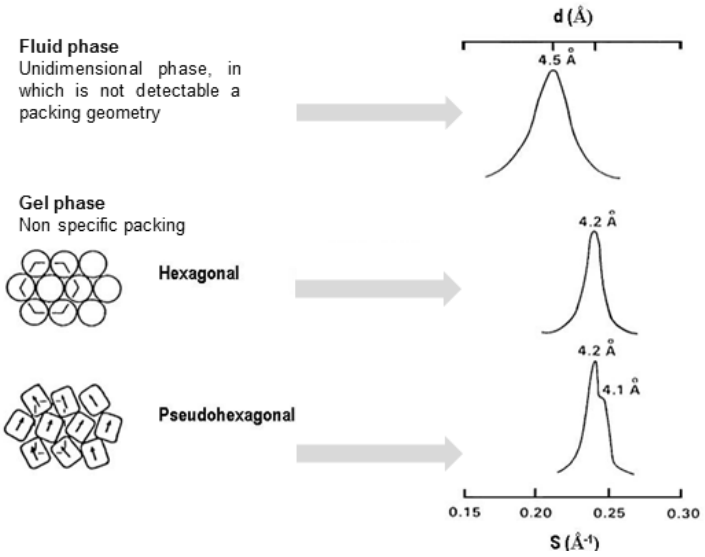


Figure 17: Typical examples of packing symmetry of hydrocarbon chains (left) and corresponding WAXS diffraction patterns (right). Adapted from (63).

In the gel phase, it is possible to obtain two types of WAXS diffractions. If the membrane is in the $P_{\beta'}$ phase or in the L_{β} phase, the packing is of the hexagonal type and translates into a single Bragg peak with a short-range distance $d \approx 4.2$ Å (63). If the membrane is in the $L_{\beta'}$ phase the packing is orthorrombic or pseudohexagonal as observed by the asymmetric Bragg peak (double) with short-range distances d_1 and $d_2 \approx 4.2$ Å ≈ 4.1 Å (63).
In Figure 17 are presented typical WAXS profiles according to the lipid phase and packing geometry. The interaction of anthracyclines with MLVs was studied during this work using both detection tecnhiques (SAXS and WAXS).

4.3. Langmuir isotherms

Monomolecular amphiphilic films show ordered phases similar to three-dimensional systems. The phases of an amphiphilic monolayer can be detected most conveniently in pressure area (π (mN/m)/area (Å^2/molecule) isotherms (Langmuir isotherms). Langmuir films produce surface-pressure area isotherms (Figure 18) with distinct lipid phases of varying compressibility: solid (S), liquid (L) and gas (G) (68). At a large surface area per molecule, monolayer behave like ideal gases, because there is no or little interactions between the lipids on the water surface. With compression, more lipids populate the surface area and, thus, the surface pressure increases. In this scenario, the lipids are in close contact but still have plenty of space to move around – the liquid phase (L). At least two types of liquid phase exist: a liquid-expanded (LE) and a liquid-condensed (LC) phase. In the LE phase, the monolayer becomes coherent, but the molecules still possess some degrees of freedom. The hydrocarbon chains in such a film are randomly oriented and still present gauche conformers. In the expanded phase, the area per molecule varies considerably with the surface pressure and no relation between the observed molecular area and the dimensions of the constituent molecules is apparent: the area per molecule is larger than the area associated with the cross-section of cylindrical alkyl chains. Under further compression, a second thermodynamic transition from the liquid to the condensed states can occur in the monolayer. This liquid expanded to liquid condensed (LE-LC) phase transition is characterised by a second constant pressure region in the isotherm which occurs at area per molecule comprised between 60 and 80 Å^2/molecule for phospholipid monolayers. In the LE-LC phase transition, condensed lipid domains appear in the expanded phase. As the molecular area is progressively reduced, the condensed phase, LC, is reached via a first order phase transition. Here, the amphiphiles exhibit a tilted phase

with a decreasing tilt angle (measured against the normal to the subphase). The film is relatively stiff but there is still some water present between the headgroups.
Further compression forces lipids to line up tightly, extend their acyl chains and form a solid phase and if the surface pressure is further increased the monolayer loses its integrity and collapses (X in Figure 18). Phase transitions in isotherms are often characterized by a plateaus (I) where the surface pressure during compression does not change.

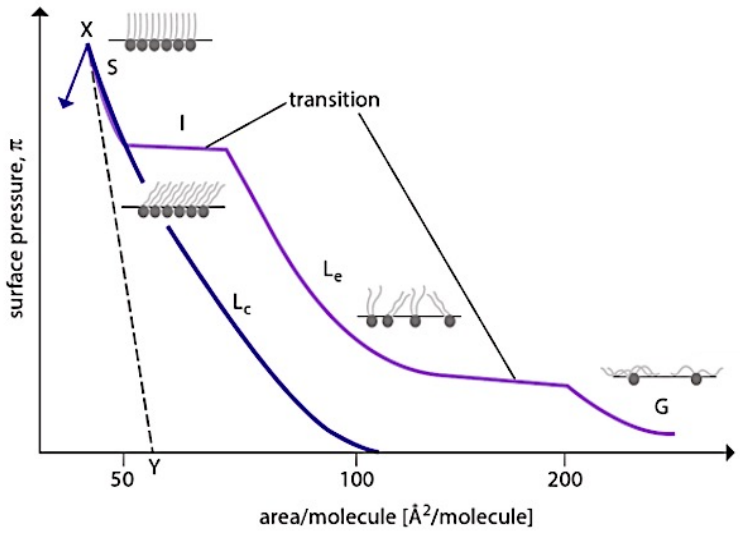


Figure 18: Langmuir isotherm with distinct lipid phases of varying compressibility: solid (S), liquid (L) and gas (G). Adapted from (33).

From π/A isotherms it is possible to calculate the minimum area per lipid molecule (A_o) and the elastic modulus (C_s^{-1}). The minimum area per lipid molecule corresponds to the molecular cross-section area and is by given the interception of the extrapolated lines from the LC phase (Y in Figure 17). The elastic modulus (C_s^{-1}) can be calculated from the π/A isotherms by the following equation (69, 70):

$$C_S^{-1} = -A(d\pi / dA) \qquad \textbf{Equation 16}$$

where A is the area per lipid molecule, and π is the surface pressure. C_S^{-1} relates to the proportionality between the increase in the surface pressure and that in the area strain (area decrease per unit area), thus a higher value of C_s^{-1} indicates a less compressible (71), i.e. more condensed, lipid monolayer.

4.3.1. Brewster Angle Microscopy (BAM)

BAM allows the morphological observation of the dynamic process of monolayer compression at the air-water interface (72). The main advantage of BAM is that the morphology of the monolayers at the air/water interface is visualized without the need of probes as it happens in the fluorescence microscopy (73). Thus, BAM is a powerful technique to visualize the two-dimensional organization of the monolayers, including the size and shape of the lipid domains (72). The lipid domains are attributed to the reflection caused by alkyl chains and the polar headgroups (74). In addition, BAM gives information about the fluidity of the monolayer in relation to the geometry of the domains observed at the air-water interface. Therefore, the contrast in the BAM images results from of the local differences in the monolayer refractivity index, caused by differences in the local molecular density or packing. In BAM pictures, the dark background corresponds to the fluid phase and the light gray areas to the condensed domains at the interface (75).

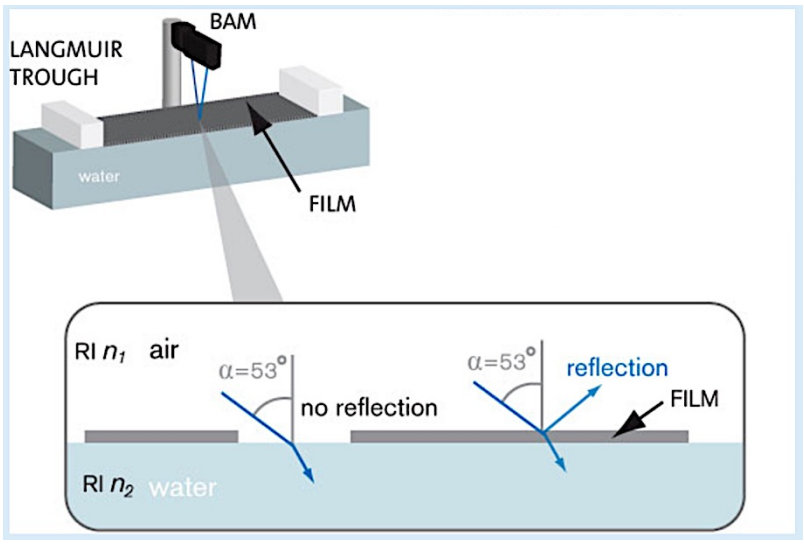


Figure 19: Principle of Brewster Angle Microscopy.

BAM is mounted in a trough and is composed by the optical components, namely a laser, the polarizers, lens and a charge-coupled device camera. It also possesses a video camera that allows to record the monolayer by both film and pictures. The BAM angle is determined using the Snell's law:

$$\tan \alpha = \frac{n_2}{n_1} \qquad \textbf{Equation 17}$$

where α is the Brewster angle in radians, n_1 the refractive index of air and n_2 the refractive index of water. Therefore, if a *p*-polarised light impinges at the air–water interface at the Brewster angle (53.1°), no light is reflected from the interface between the two media with

different refractive indices (Figure 19). Therefore, at the mentioned angle of incidence, the introduction of a Langmuir monolayer at the air/water interface modifies the Brewster angle conditions, and light is reflected. The BAM images obtained are commonly digitized and filtered in order to reduce diffraction fringes caused by the coherent nature of the laser beam and, finally, processed to improve their quality.

4.3.2. Polarization-Modulation Infrared reflection-absorbance spectroscopy (PM-IRRAS)

Langmuir isotherms provide important information about the phases formed by amphiphiles; however, such data do not provide a complete molecular level description of monolayer films. To acquire structural information such as chain conformation, hydrogen bonding and ionic interactions in amphiphiles, spectroscopic techniques are required. The most powerful of which is a variant of infrared (IR) spectroscopy known as infrared reflection–absorption spectroscopy (IRRAS). A major difficulty addressed is the strong signal that arises from the liquid and vapor phases of water, that mask the vibrations of important vibrational groups, as the amide, especially important in the study of proteins and drugs with this chemical group (76). In this regard, the PM-IRRAS (polarization modulation infrared reflection absorption spectroscopy) was developed by Baldez *et al.* (76). PM-IRRAS is a more sensitive technique that allows to minimize the ignal of interferers as the water and carbon (77).

PM-IRRAS is associated with a trough, which must have an adequate size (≥ 6 cm) in order to minimize the meniscus interferences. The PM-IRRAS uses a polarization modulation technique (76). This technique is based on the observation that when incident mid-IR radiation is applied onto aqueous monolayers films, a small fraction (6%) of the light is reflected from the molecular constituents of the surface (78). The data is generally presented as plots of reflectance–absorbance versus wavenumber. The reflectance absorbance intensity is determined by (78, 79):

$$RA = -\log\frac{R}{R_0}$$ **Equation 18**

where R and R_0 are, respectively, the reflectivity values of the monolayer-covered and pure buffer solution surfaces.

The vibration modes from the main constituent groups of the phospholipids as well as the structural information that can be taken from them are presented in Table 3. The symmetric

and asymmetric wavenumbers of the methylene stretching vibrations [$\nu_s(CH_2)$ and $\nu_{as}(CH_2)$], around 2850 and 2920 cm^{-1}, respectively, are used to characterize the state of conformational order in the lipid monolayer. The wavenumber values can be used to monitor the average *trans/gauche* isomerisation in the systems. Shifts to higher wavenumbers correspond to an increase in number of *gauche* conformers. Furthermore, the bandwidths of the CH_2 stretching bands give dynamic information about the system; the bandwidth increase implies an increase in fluidity. The CH_2 scissoring [$\nu_{sc}(CH_2)$] vibrations give information about the packing geometry of the phospholipids; a single vibration around 1468 cm^{-1} is evidence of an hexagonal packing but if a spectral doublet appears at 1462/1674 cm^{-1} the chains are conformationally ordered and packed in an orthorhombic perpendicular geometry. The stretching ν(C=O) band localized between 1710 and 1740 cm^{-1} is used to investigate the interfacial region of phospholipids. This band is sensitive to hydrogen bonding and provides information about the carbonyl groups' accessibility to water and its shift to higher wavenumbers indicates C=O group dehydration. Also, the asymmetric vibration of the phosphate ν_{as} (PO^{2-}) localized at 1210-1250 cm^{-1} is sensitive to ion binding and hydration.

Table 3: IR modes of vibration used for IRRAS analysis.

Information	*Vibration mode*	*Wavenumber (cm-1)*	*Comments*
Conformational order	CH_2 sym. stretch	2849-2854	Deviation to lower wavenumbers implies changes in the conformation from gauche to trans and, thus, a more ordered membrane.
	CH_2 asym. stretch	2916-2924	
Packing	CH_2 scissoring	1462; 1474; 1468	Orthorrombic. Hexagonal.
Binding and hydration	PO^{2-} sym. stretch	1090	Deviation to lower wavenumbers indicates increase in the number of hydrogen bonds.
	PO^{2-} asym. stretch	1220-1250	
Hydration and hydrogen bonding	C=O (esther)	1710-1740	Deviation to lower wavenumbers points to the formation of new or stronger hydrogen bonds.

Chapter V

Progress beyond the state-of-art

In this chapter are presented the papers that this work has originated.

1. A biophysical approach to daunorubicin interaction with model membranes: relevance for the drug's biological activity

2. Influence of doxorubicin on model cell membrane properties: insights from *in vitro* and *in silico* studies

3. A synchrotron X-ray assessment regarding the nature of anthracyclines–lipid interactions

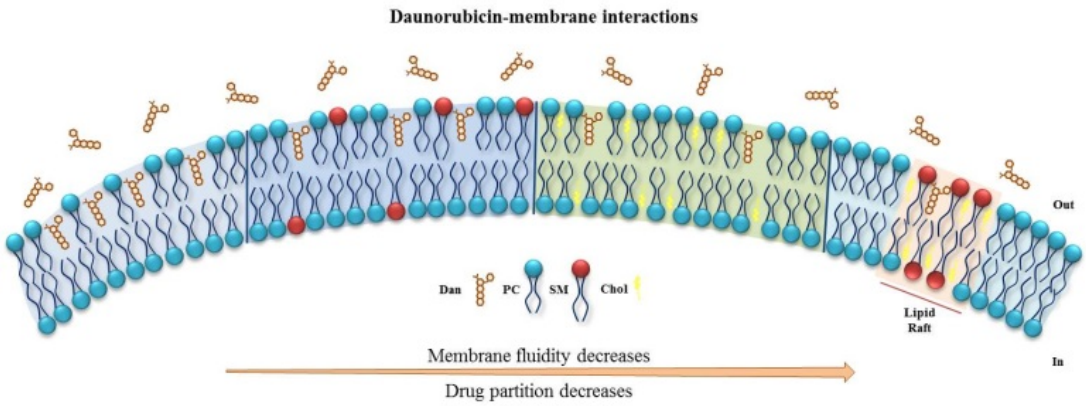
4. Daunorubicin and doxorubicin molecular interplay with 2D membrane models

5. The daunorubicin interplay with mimetic model membranes of cancer cells: a biophysical interpretation

6. Doxorubicin molecular interactions with cancer membrane models: *in vitro* and dynamic simulation studies

7. Synchrotron SAXS and WAXS investigation of the interactions of anthracyclines with lipid membranes

1. A biophysical approach to daunorubicin interaction with model membranes: relevance for the drug's biological activity



HIGHLIGTHS

- Daunorubicin's membrane concentration and its effect in membrane fluidity are highly dependent on the lipid composition.
- The anticancer drug partition is reduced in the presence of sphingomyelin and cholesterol, that together decrease the overall membrane fluidity.
- Interplay of daunorubicin is mainly driven by electrostatic and hydrophobic interactions.
- A decrease of membrane fluidity is more pronounced in the acyl regions of the bilayer, where the drug has a preferential location.
- Daunorubicin-membrane interaction can be correlated with the drug's bioaccumulation and action.

A biophysical approach to daunorubicin interaction with model membranes: relevance for the drug's biological activity

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Daunorubicin is extensively used in chemotherapy for diverse types of cancer. Over the years, evidence has suggested that the mechanisms which daunorubicin cause cytotoxic effects are also associated with interactions at the membrane level. The overlooked perspective of biological membranes as passive platforms evolved to a rather dynamic and complex concept, where the fate of drugs can be defined. The present work aimed, therefore, to study the interplay between daunorubicin and mimetic membrane models composed of different ratios of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol). In order to accomplish that, several biophysical parameters were assessed using liposomes as mimetic model membranes. Thereby, the ability of daunorubicin to partition into lipid bilayers, its apparent location within the membrane and its effect in membrane fluidity were investigated.

The results showed that daunorubicin has greater affinity for lipid bilayers composed of DMPC, followed by DMPC:SM, DMPC:Chol and lastly by DMPC:SM:Chol. The addition of SM or Chol into DMPC membranes increases the complexity of the model membrane but also decreases its fluidity, which, in turn, reduces the amount of anticancer drug that can partition into these mimetic models. Fluorescence quenching studies suggest a broad distribution of the drug across the bilayer thickness, with a preferential location in the phospholipid tails. The gathered data supports that daunorubicin permeates all types of membranes in different degrees, interacts with phospholipids through electrostatic and hydrophobic bonds and causes alterations in the biophysical properties of the bilayers, namely in membrane fluidity. A more pronounced effect can be observed in the acyl region of the phospholipids, where the anticancer compound causes a decrease in membrane fluidity. Ultimately, such outcomes can be correlated with daunorubicin's biological action, where membrane structure and lipid composition have an important role. In fact, the results indicate that the intercalation of daunorubicin between the phospholipids can also take place in rigid domains, such as rafts that are known to be involved in different receptor processes important for cellular function.

drug-membrane interactions | daunorubicin | partition | location | fluidity

Daunorubicin, a compound isolated in Italy in 1963 from *Streptomyces peucetius* cultures [1] was the first anthracycline antibiotic to be used in cancer treatment. Despite its extensive clinical application for the treatment of acute lymphoblastic or myeloblastic leukemias [2], a major problem with daunorubicin administration is the dose-dependent toxicity in healthy tissues, especially cardiotoxicity [2]. Daunorubicin continues to be widely studied because its mechanisms of action are still a matter of substantial controversy [2]. Nevertheless, it is thought that daunorubicin exhibits cytotoxic activity mainly through intercalation with DNA, alteration of topoisomerase II which, in turn, inhibits DNA and RNA synthesis and leads to apoptosis, disturbance of membrane fluidity and ion transport, and production of oxygen radicals [3].

In addition to intracellular targets, strong evidence suggests that the cell membrane can itself be a target for this type of agents [4]. In fact, in 1982 Tritton *et al*. demonstrated that anthracyclines, without entering the cell, could exert their cytotoxic effect only through their interplay with the membrane [5]. Later on, Escriba *et al*. showed that daunorubicin reduced the formation of the nonlamellar hexagonal (H_{II}) phase of liposomes composed by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and stabilized the bilayer structure just by interacting with membrane phospholipids [6]. The repercussion of such interaction is an alteration of various cellular events involved in signal transduction, in which these lipid phases are involved [6]. The gathered data highlighted the role of the cell membrane and revealed that membrane lipids hold a fundamental function in signal transduction that can be influenced by xenobiotics, such as anthracyclines.

The transport of daunorubicin across biological membranes, an important factor that can influence the drug's pharmacokinetics properties, is determined by the balance between lipophilicity and hydrophilicity and by its charge state [7]. The determination of the drug's partition coefficient, which gives information regarding the lipophilicity of a compound, can help to infer about the process of passive diffusion. However, it also becomes relevant to take into account the drug's degree of ionization, since it can affect the ability of the compound to move through the bilayer. Several studies describe the transport of daunorubicin through cell membranes as a process of passive diffusion [8, 9]. However, such explanation only takes into consideration the non-ionized form of the drug. Since most of the daunorubicin molecules are charged at physiological pH, it seems unlikely that most of them cross the cell by simple diffusion. In this context, Regev R. et al. studied the transport of daunorubicin across the membrane and demonstrated that it occurs by a passive "flip-flop" mechanism, rather than by diffusion down a continuous concentration gradient [10].

Although the biological action of daunorubicin has been broadly described, the biophysical mechanisms dependent on the interactions with membranes are far from being completely understood. In 1996, Gallois *et al*. demonstrated that the incorporation of daunorubicin molecules within the bilayers was independent of their charge, but the amount of neutral and positively charged drug forms embedded were dependent on the membrane

electrostatic parameters [11]. In 1998, the same authors investigated the interplay of daunorubicin with large unilamellar vesicles (LUVs) composed by phosphatidylcholine, phosphatidic acid and cholesterol, and showed that, due to its lipophilicity and chemical nature, daunorubicin binds to the membrane through both electrostatic and hydrophobic interactions [12]. Later on, Escriba *et al*. described that different lipid content can determine how daunorubicin binds to membranes. While the presence of phosphatidylserine greatly increased both the drug's affinity and stoichiometry of binding to model lipid membranes, the presence of cholesterol caused a reverse effect [13]. A strong affinity of daunorubicin to cardiolipin, a lipid abundant in heart tissue has also been described [14]. As a consequence of electrostatic interactions, a complex is formed that is thought to be involved in the cardiotoxicity of this drug [14]. Finally, it was reported that daunorubicin triggered cell death is mediated by a signaling pathway that starts with an early production of sphingomyelin-derived ceramide [15], which emphasizes the importance of the cell membrane's lipid content for this anticancer drug's activity. Recent studies have also shown that daunorubicin induces apoptosis via signaling through plasma membrane lipid rafts, where the death receptor pathway is involved [16, 17]. The process is initiated by the activation of a sphingomyelinase, which in turn causes the hydrolysis of sphingomyelin (SM) and generates ceramide. Later on, ceramide is responsible for triggering different signaling pathways among which a stress-activated protein kinase cascade plays a crucial role.

The different structural, biophysical and chemical aspects of the membrane can define whether and how the cell will react to the drug and if the drug will cross this boundary. On the other hand, the drug's properties can also modulate its penetration, conformation and/or location within membranes [26]. Simultaneously, drugs can initiate membrane adjustments that can vary from changes in lipid conformation and surface charge to alteration in the fluidity and, consequently, in cell function [7]. For these reasons, drug-membrane interactions become important as to understand the mechanisms of multi-drug resistance or the development of undesirable side effects [13]. Such knowledge is crucial, especially for anthracyclines, since these drawbacks are the reason why their therapeutic index need to be improved. Additionally, due to the huge variety in membrane's composition and structure, those interactions can significantly affect the drug's activity and toxicity [26]. For that reason, the goal of the current work is to elucidate whether and how the features of daunorubicin can affect the biophysical characteristics of biological membranes and what is the importance of lipid composition and membrane structure in the drug's pharmacokinetic and pharmacodynamic properties.

Since biological membranes are too complex to accurately assess biophysical parameters, liposomes were used as membrane model systems. The main lipid components of biological membranes are glycerophospholipids, sphingolipids and sterols and, for that reason, were chosen to develop the model systems. The mimetic models were developed in order to simulate several biological environments that daunorubicin can encounter. Accordingly, the purpose was to study the role of different types of membrane organization and fluidity in daunorubicin interaction, using LUVs constituted of different molar ratios of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), SM and cholesterol (Chol). The lipids choice is explained as follows: i) phosphatidylcholines are among the most abundant phospholipids of natural plasma membranes [27] and were, therefore, included in the mimetic systems as the dominant constituent; ii) SM, the major sphingolipid found in membranes, plays several roles in biological membranes and is involved in many cellular functions [28], iii) sterols are the main non-polar lipids of cell membranes, being cholesterol the most abundant and one of the most important regulators of lipid organization [29] and thus it was added in order to mimic cellular targets enriched in cholesterol domains. The presence of cholesterol together with sphingomyelin, known to control membrane fluidity and permeability, is important for the preservation of its organization and, consequently, contributes to the cell membrane's physiology and function [30]. In addition, cholesterol and sphingomyelin form liquid ordered domains in a more fluid matrix, the so called lipids rafts, which are fundamental for membrane organization and where several proteins and other biomolecules are embedded [31]. Furthermore, in a pharmacological context, it is crucial to know if a drug partitions into specific lipid domains and how this partition can have implications in specific functional effects.

Materials and Methods

Reagents. Daunorubicin (DAN) was obtained from Biovision (Milpitas, California). The lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). N-(2-Hydroxyethyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). All reagents were used without further purification. Drug solutions were prepared with HEPES buffer (10 mM, pH 7.4). The buffer was prepared with double deionized water (conductivity inferior to 0.1 μScm−1) and ionic strength was adjusted with NaCl (I=0.1 M).

Liposomes Preparation. Liposomes were prepared according to an adapted classic thin film hydration method [32]. The molar ratio mixture of the lipids (DMPC, DMPC:SM [8:2], DMPC:Chol [8:2] and DMPC:SM:Chol [7:1.5:1.5]) was dissolved in chloroform/methanol (3:2 v/v) and the organic solvents were evaporated under a stream of nitrogen in a rotative evaporator (Buchi R-200, equipped with a thermostated bath Buchi R-490, Switzerland). The resultant dried lipid film was dispersed in HEPES (pH 7.4) and the mixture was vortexed to form multilamellar vesicles (MLVs). To obtain large unilamellar vesicles (LUVs) the MLVs were extruded ten times (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) through policarbonate filters with a diameter pore of 100 nm (Nucleopore) at 60 °C (temperature above the main phase transition temperature of the lipids).

For fluorescence studies, the fluorescence probe (DPH or TMA-DPH) was co-dissolved with the lipid in the

organic solvents mixture to give a probe/lipid molar ratio of 1:300 in order to prevent changes in the membrane's structure. The labeled LUVs were similarly obtained by evaporation of the organic solvents, followed by hydration of the dried lipid film and extrusion of the MLVs.

Size and Zeta Potential characterization by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS). Particle size and zeta potential were assessed using a Brookhaven™ BI-MAS and Zeta-Pals (Brookhaven Instruments, Holtsville, NY, USA). Both size and zeta potential distribution of the extruded liposomes, with and without drug, were determined at pH 7.4 (HEPES buffer) at 37±0.1 °C. Lipid concentration was kept constant at 500 μM and daunorubicin concentrations ranged from 0 to 75 μM. The size and zeta potential values obtained were calculated from at least three independent assays.

Determination of Partition Coefficients by derivative UV-Vis spectrophotometry. Partition coefficient (Kp) of daunorubicin in the different model membranes (LUVs) was assessed by derivative UV-Vis spectrophotometry. A set of samples was prepared by the addition of anticancer drug HEPES buffered solutions with a fixed drug concentration (40 μM) and increasing concentrations of lipid solution (in the range of 0–1500 μM). The corresponding references solutions were prepared identically but without daunorubicin. All samples and references were incubated during 30 min at 37 °C. The absorption spectra of all solutions were recorded at 37 °C using a multidetection microplate reader (Synergy™ HT, BioTek Instruments Inc, Winooski, VT, USA). The mathematical treatment of the results obtained was performed using a developed routine, the Kp Calculator [33], where each reference spectrum is subtracted from the correspondent sample spectrum to eliminate the scattering from the vesicles and its second and third derivative spectra are determined to enhance the ability to detect minor spectral features and to improve the band resolution. The Kp values are then calculated using a nonlinear regression method that fits equation 1 to the experimental data (D_T vs [L]):

$$D_T = D_W + \frac{(D_m - Dw)Kp[L]V_m}{1 + Kp[L]V_m} \quad (1)$$

where, DT is the second or third derivative intensity ($D=d^nAbs/d\lambda^n$) obtained from the absorbance values of the total amount of daunorubicin, the daunorubicin distributed on the lipid membrane phase (D_m) and the daunorubicin distributed in the aqueous phase (D_w). [L] represents the lipid concentration (in M) and V_m is the lipid molar volume. The V_m for the mimetic systems studied (DMPC, DMPC:SM, DMPC:Chol and DMPC:SM:Chol) were 0.663, 0.682, 0.596 and 0.633 L mol−1, respectively. The obtained Vm values were calculated using the specific volume of each lipid and taking into account the different lipid molar ratio used for each model membrane. The Kp values obtained are adimentional and presented as the mean ± standard deviation calculated from at least three independent assays.

Drug Location Studies Performed by Fluorescence Quenching. Daunorubicin location within the model systems was assessed by fluorescence studies that included steady-state and time-resolved measurements, according to an already described method [34]. These experiments were conducted by incubating the anticancer-drug with DPH and TMA-DPH labeled liposomes. The samples containing a fixed concentration of lipid (500 μM) and increasing concentrations of daunorubicin (0–75 μM), were incubated at 37 °C during 30 min in the dark. The fluorescence measurements were carried out at 37 °C, at excitation and emission wavelengths defined at 357 nm and 429 nm, and 359 nm and 429 nm, for DPH and TMA-DPH, respectively. Fluorescence steady-state measurements were performed in a Jasco FP-6500 spectrofluorometer (Jasco, Great Dunmow, UK) equipped with a constant temperature cell holder and all data was recorded in a 1 cm path length cuvette. The fluorescence intensity values obtained were corrected for inner filter effects (quencher absorbance) at the excitation wavelength [35]. The fluorescence time-resolved measurements were done using a Fluorolog Tau-3 Lifetime system. The modulation frequencies were acquired between 10 and 200 MHz, with an integration time of 10 s. The fluorescence emission was detected with a 90° scattering geometry. All measurements were made using Ludox as a reference standard (τ=0.00 ns).

Membrane Fluidity Studies Performed by Fluorescence Anisotropy. The influence of daunorubicin on the membrane's fluidity, in the different model membranes developed, was assessed by fluorescence anisotropy studies. The samples were prepared with a fixed concentration of lipid (500 μM), labeled with DPH or TMA-DPH, and increasing concentrations of daunorubicin (0, 40 and 75 μM), and incubated for 30 min at 37 °C in the dark. The steady-state fluorescence anisotropy measurements (r_s) were recorded between 10 and 60 °C with intervals of 2 °C. The experiments were performed in Jasco FP-6500 spectrofluorometer used for the location studies with polarizers inserted (excitation/emission wavelengths were set as described for each fluorescent probe) and equipped with a Peltier temperature controller. The results obtained are presented as the mean ± standard deviation calculated from at least three independent assays.

Statistical analysis. Statistical analysis was performed using IBM® SPSS® Statistics software (v.20.0.0.0, IBM, Armonk, NY, USA). The measurements were repeated at least three times and data was expressed as mean ± standard deviation (SD). Data was statistically analyzed through the one-way analysis of variance (ANOVA) method and differences between groups were compared by Bonferroni and Tukey post-hoc tests in which a p value lower than 0.05 ($p<0.05$) was considered statistically significant.

Results and discussion

Size and Zeta potential characterization

The different mimetic systems were analyzed regarding their size and overall charge in the absence and presence of daunorubicin and the results obtained are depicted in Table 1 for all model membranes studied. The size measurements demonstrate that the different model systems present an average diameter between 100-110

Table 1. Characterization of liposomes size diameter and zeta potential with increasing daunorubicin concentration. Studies conducted at pH 7.4 and 37 °C. [a]

MODEL	SIZE (nm)				ZETA (mV)			
	0 μM	5μM	40 μM	75 μM	0 μM	5μM	40 μM	75 μM
DMPC	113 ± 2	114 ± 2	113 ± 3	115 ± 1	-14 ± 2	3.8* ± 0.9	15* ± 1	20* ± 1
DMPC:SM	100 ± 5	104 ± 6	117* ± 5	120* ± 6	-18 ± 1	-6.5* ± 0.8	17* ± 2	19* ± 1
DMPC:Chol	102 ± 3	118* ± 2	122.9* ± 0.3	120* ± 4	-8.0 ± 0.7	-4* ± 2	7.1* ± 0.7	12* ± 1
DMPC:SM:Chol	107 ± 4	117* ± 5	119* ± 5	122* ± 5	-16 ± 1	-7* ± 1	8* ± 1	11* ± 1

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the corresponding sample without daunorubicin.

nm independently of their composition, a size intended to mimic the curvature of biological membranes [7].
However, it can be noted that daunorubicin influences the liposome size in a concentration-dependent way, indicating that the compound interacts with and probably permeates the different types of membranes. The anticancer drug has a bigger effect in the size of models containing Chol, probably due to the formation of a hydrogen bond between the protonated NH^{3+} group of daunorubicin and the OH group of Chol.
Regarding zeta potential values, the model membranes present an overall negative surface charge that suffers a significant alteration in the presence of the anticancer drug as can be seen in Table 1. This outcome can be explained by the fact that daunorubicin molecules are positively charged at physiological pH, thereby, interacting with the phospholipid by electrostatic interactions.
Overall, the results points towards an interplay of daunorubicin with model membranes driven through both electrostatic interactions and hydrogen bonds.

Daunorubicin Partition Coefficients

Drug-membrane interaction is strongly influenced by drug lipophilicity, which can affect the drug's pharmacodynamics and pharmacokinetics [36]. In this context, the assessment of the partition coefficient (Kp) can be crucial to understand and explain its pharmacological activity [37]. The daunorubicin partition coefficient in the several model membranes was determined by derivative UV-Vis spectrophotometry [32, 38, 39]. This technique, based on the fact that the drug´s spectral characteristics (λ_{max}) change when it permeates from the aqueous to the lipid medium, allows the quantification of the drug distribution in each phase. Furthermore, it also provides a better resolution of the overlapped bands and eliminates the lipid light scattering interference by the use of the derivative method.
In Fig. 1 (A) from supplementary material is represented, as an example, the third-derivative absorption spectra of daunorubicin with increasing lipid concentration, calculated to improve the resolution of the spectra and to eliminate the interference caused by the vesicles scattering. From these spectra it is possible to observe a slight shift in the λ_{max}, which indicates that the drug partitions from the aqueous to the lipid phase of the liposomes. The best fit of equation 1 to the third-derivative data was achieved at 546 nm and is shown in Fig. 1 (B) from supplementary material.

The experimental daunorubicin partition coefficients obtained for each mimetic system (expressed as Kp and logD) along with the theoretical octanol/water partition coefficient (expressed as logP), predicted using Marvin sketch calculator software (ChemAxon), are depicted in Table 2.
The gathered experimental results (Table 2) reveal that daunorubicin presents a significant partition into the several mimetic models. When comparing the experimental results (experimental logP) and the values predicted for the octanol/water system (theoretical logP) it is possible to observe a significant variance between them. Such inconsistency is related to the differences between these techniques and can be explained by the fact that the octanol-water system does not take into account several crucial characteristics of biological membranes. Indeed, membranes are constituted by amphiphilic phospholipids that can, establish electrostatic and hydrophobic interactions. Furthermore, the compound's partition is highly dependent on the lipid composition of the mimetic models studied, emphasizing the fact that using liposomes it is possible to obtain more realistic information about daunorubicin lipophilicity and, therefore it's *in vivo* membrane partition.
On the other hand, the pKa values (9.17) calculated using Marvin sketch calculator software predict that nearly 80% of daunorubicin molecules are in the cationic form at pH 7.4, which means that the drug-membrane interaction should be mainly driven by electrostatic interaction with the polar headgroups of the phospholipids. For that reason, the theoretical logP value is much smaller than the experimental logP because it only reflects the hydrophobic interactions of the small amount of molecules that are in the neutral form at physiological pH. Hence, daunorubicin interplays with membranes through: electrostatic forces of the amino sugar moiety (positively charged) and the lipid phosphate groups (negatively charged); and hydrophobic interactions between the dihydroanthraquinone residue and the lipid fatty acid chains.
Comparing the results acquired for the different mimetic systems it becomes clear that the drug concentration between the membrane and aqueous medium is different from model to model, depending on lipid composition and biophysical characteristics, particularly membrane fluidity (see section 3.4). Therefore, daunorubicin presents higher partition for the model composed by DMPC, followed by DMPC:SM, DMPC:Chol and lastly by DMPC:SM:Chol. The addition of SM or Chol into DMPC membranes increases the complexity of the

system but also decreases its fluidity, which, in turn, reduces the amount of anticancer drug that can partition into these mimetic models. Ultimately, the presence of SM together with Chol confers the membrane an increased organization and lipid packing (see section 3.4) and, consequently, hinders daunorubicin partition into the lipid membrane. Nevertheless, a small partition can be observed for this model, probably due to the drug's planar structure that allows it to intercalate between the phospholipids of the bilayer, just as it does with the DNA strands, even in more rigid membranes where the lipid-lipid interactions are stronger.

Table 2. Experimental (Exp.) and theoretical (Theo.) partition Coefficients (expressed as Kp and logP) of daunorubicin for the different model membranes at pH 7.4 and 37 °C. [a]

MODEL	Kp	Exp. logP	Theo. logP
DMPC	3363 ± 41*	3.53 ± 0.01*	0.91
DMPC:SM	2983 ± 63*	3.47 ± 0.01*	
DMPC:Chol	1413 ± 82*	3.15 ± 0.04*	
DMPC:SM:Chol	869 ± 57*	2.94 ± 0.03*	

[a] All values represent the mean ± standard deviation (n=3).
*$p < 0.05$ statistically different from the other models.

The drug's membrane concentration (Kp) values demonstrated that daunorubicin permeates the several models in different degrees. Nevertheless, its partition appears to be mainly driven by electrostatic (that can be measured using liposome/water system, contrary to the octanol/water method) and hydrophobic interactions in all models. Such outcomes are in agreement with previous studies [40-43] and evidence the fact that the membrane partition of specific molecules is dependent on the lipid composition and can help to explain its biodistribution. Furthermore, the transport of daunorubicin across membranes probably occurs by an initial electrostatic binding to phospholipids of the mimetic systems, followed by a "flip-flop" mechanism within the bilayer, as demonstrated by Regev *et al.* (2005) [10].

Drug location studies using different fluorescent probes

The daunorubicin location within the bilayer was also evaluated by fluorescence quenching of two probes [44]. The quenching efficiency of daunorubicin in the several models was determined by adding increasing drug concentration to liposomes labelled with DPH and TMA-DPH. Such knowledge is only possible to obtain due to the fact that the fluorophore position in the membrane of each fluorescent probe (TMA-DPH and DHP) is well defined and documented. While DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer [45], TMA-DPH is reported to be anchored in the polar head groups region of phospholipids due to its charged group [46].

The drug's efficiency to quench the fluorophore is associated with its proximity to the probe. In this sense, because the fluorophore is inserted in the membrane, only the drug that partitions into the lipid phase will be able to act as a quencher. In order to determine the daunorubicin quenching efficiency it is important to calculate its membrane concentration ($[Q]_m$) that is given by equation 2:

$$[Q]_m = \frac{K_p[Q]_T}{K_p\alpha_m + (1 - \alpha_m)} \quad (2)$$

where α_m is the volume fraction of membrane phase ($\alpha_m = V_m/V_T$; V_m and V_T represent the volumes of the membrane and water phase, respectively).

Daunorubicin molecules have effectively been able to quench all probes in all mimetic membranes. The wavelength corresponding to the absorption band of the probes did not change with daunorubicin presence and no additional band was observed at a longer wavelength. As such, the quenching of fluorescence was analyzed by the modified Stern-Volmer equation (3):

$$\frac{I_0}{I} = K_{SV}[Q]_m + 1 = \frac{\tau_0}{\tau} \quad (3)$$

where I and I_0 are the steady state fluorescence intensities with and without the quencher (daunorubicin), respectively; K_{SV} is the Stern-Volmer constant; $[Q]_m$ is the daunorubicin membrane concentration; and τ_0 and τ are the fluorophore lifetime in the absence and presence of daunorubicin, respectively. The molecular contact between the probe (fluorophore) and the drug (quencher) can be due to different molecular interactions and can be observed by the decrease in fluorescence intensity (fluorescence quenching) as represented in Fig. 2 from supplementary material. The quenching process can result from collisional encounters between the fluorophore and quencher, and is therefore called collisional or dynamic quenching, or it can be due to the formation of a complex, which is called static quenching [44]. These two types of quenching can be distinguished by lifetime measurements. This happens because while complexed fluorophores are nonfluorescent, and the observed fluorescence is just from the uncomplexed fluorophores, the uncomplexed fraction is unperturbed, and hence its lifetime is about the same as τ_0. In this context, for static quenching $\tau_0/\tau=1$ while for dynamic quenching $I_0/I=\tau_0/\tau$ [44]. Accordingly, besides the determination of the fluorescence emission intensity, fluorescence lifetimes measurements were also performed.

In many cases, the fluorescent probe can be quenched both by collisions and by formation of a complex with the same quencher. Such events result in an upward curvature of the Stern-Volmer plots, a positive deviation that can be seen in Fig. 2 from supplementary material. Accordingly, the dynamic portion (K_D) of the observed quenching is determined by lifetime measurements, through the following equation (equation 4) [44]:

$$\frac{\tau_0}{\tau} = K_D[Q]_m + 1 \quad (4)$$

After knowing the dynamic component, the static contribution may be calculated by linearization of the following equation (equation 5) [44]:

$$\frac{I_0}{I} \times \frac{1}{K_D[Q] + 1} = K_S[Q]_m + 1 \quad (5)$$

This modified form of the Stern-Volmer equation is second order in $[Q]_m$, which accounts for the upward curvature observed when both static and dynamic quenching occur for the same fluorophore.

In Fig. 2 from supplementary material, the Stern–Volmer plots of $I_0/I-1$ and $\tau_0/\tau-1$ as a function of daunorubicin membrane concentration are shown. From its observation, it is possible to conclude the existence of both collisional and static quenching processes, which means that daunorubicin must diffuse to the fluorophore during the lifetime of the excited state but it also might form a non-fluorescent complex with the probe.

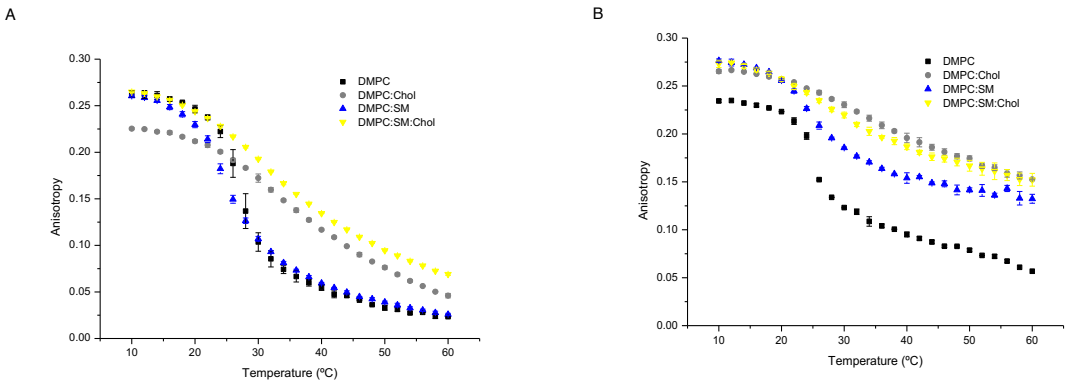


Fig. 1. Steady-state anisotropy of DPH (A) and TMA-DPH (B) as a function of temperature in each mimetic model, at pH 7.4. Results present the mean of at least three independent assays.

Table 3: Fluorescence quenching parameters (K_D, dynamic constant; K_S, static constant; K_{SV}, Stern–Volmer constant) calculated from the quenching of TMA-DPH and DPH by daunorubicin in the different mimetic models at pH 7.4 and 37°C. [a]

Model	Probe	K_D (M^{-1})	K_S (M^{-1})	K_{SV} (M^{-1})
DMPC	DPH	17* ± 1	81* ± 2	98* ± 3
	TMA-DPH	34* ± 2	12* ± 1	45* ± 1
DMPC:SM	DPH	41* ± 2	38* ± 4	80* ± 1
	TMA-DPH	28* ± 2	13* ± 2	42* ± 2
DMPC:Chol	DPH	8.1 ± 0.1	11* ± 1	18.8* ± 0.5
	TMA-DPH	7 ± 1	~0	7* ± 1
DMPC:SM:Chol	DPH	31* ± 1	51* ± 1	82* ± 2
	TMA-DPH	20.2* ± 0.3	29.0* ± 0.2	49* ± 1

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other probe, for each biophysical parameter.

According to the previous description of the quenching behavior, the quenching parameters values, namely the dynamic constant (K_D), the static constant (K_S) and the Stern–Volmer constant (K_{SV}), obtained from this technique are shown in Table 3.

From the analysis of Table 3, it can be seen that the quenching process results from dynamic and static interactions, except for the model composed by DMPC:Chol where it results only from dynamic interactions. The observation of the obtained data leads to the conclusion that all probes were quenched by daunorubicin but with different quenching efficiency depending on the type of mimetic model used. Daunorubicin induced a reduction of the light emitted by both probes DPH and TMA-DPH incorporated into liposomes in a concentration dependent manner, indicating a broad distribution of the drug across the bilayer thickness.

Nevertheless, the decrease of the probe's fluorescence was more pronounced for DPH than for TMA-DPH in all the model systems, which translates into higher values of K_{sv}. This fact suggests that the drug is buried inside the lipid bilayer but also near the phospholipid head. Once more, it appears that two types of interactions coexist and are responsible for the distribution of daunorubicin at the membrane level: electrostatic interactions between the positive group of the anticancer drug and the negative pole of the phospholipid headgroup and hydrophobic interactions between the drug's dihydroanthraquinone residue and the lipid fatty acid chains. Furthermore, the membrane location of daunorubicin can be explained by the fact that being an amphiphilic molecule permeates the lipid bilayer (quenches DPH and TMA-DPH), which is in turn related with the partition coefficients determined for this compound in the different model systems.

Effect of Daunorubicin in membrane fluidity

Alteration of membrane fluidity can severely affect the cell's functional properties and also activate apoptotic pathways which ultimately result in cell death. Since anticancer drugs interact with membranes in order to reach their intracellular target, it is crucial to understand how these compounds can induce perturbation in the physicochemical state of the phospholipid bilayer. Membrane fluidity studies were performed by steady-state anisotropy [49], that reports the microenvironment around the fluorescent probe once it conditions the degree of rotational motion of the probe [50].

The effect of temperature on the DPH and TMA-DPH fluorescence anisotropy of the different model membranes without daunorubicin is presented in Fig. 1. For all model systems developed, the anisotropy values decrease with increasing temperature in greater extent in the acyl chain region (given by the probe DPH) than in the phospholipid head region (given by the TMA-DPH probe). It is well documented that the

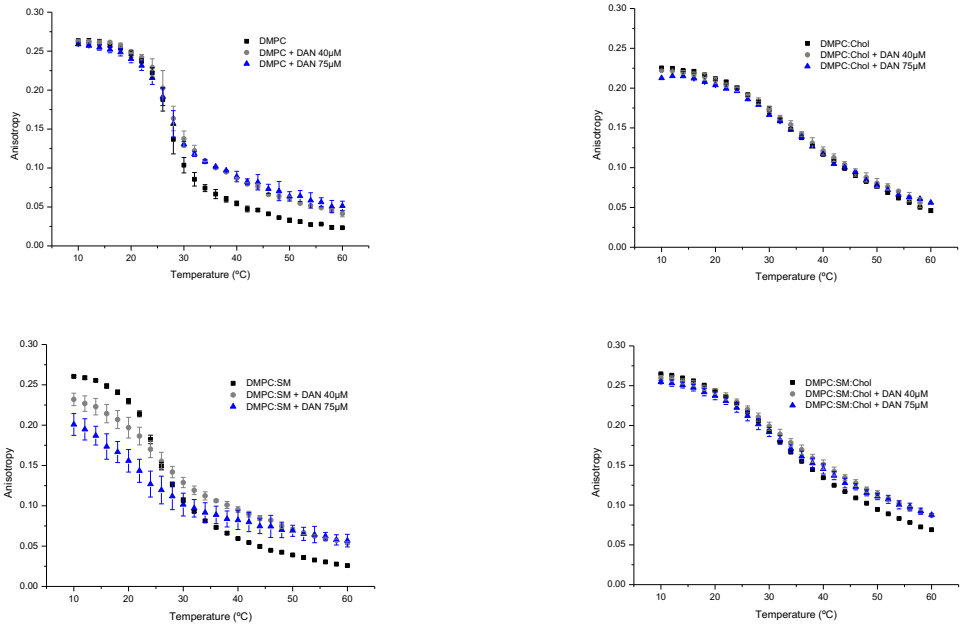


Fig. 2. Steady-state anisotropy of DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of DAN 40 μM (●) and 75 μM (▲), at pH 7.4. Results present the mean of at least three independent assays.

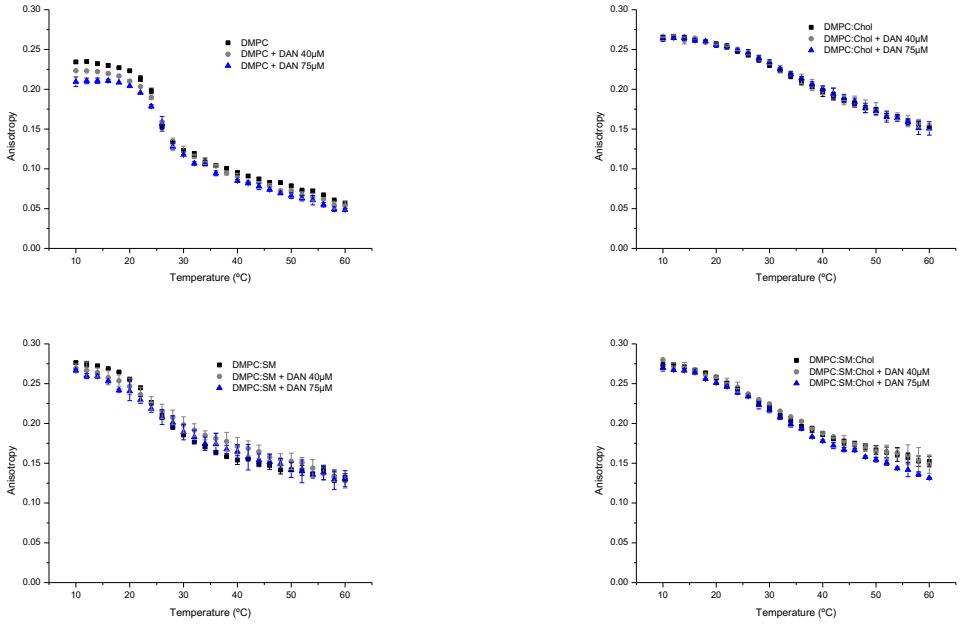


Fig. 3. Steady-state anisotropy of TMA-DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of DAN 40 μM (●) and 75 μM (▲), at pH 7.4. Results present the mean of at least three independent assays.

membrane is characterized by a fluidity gradient from the aqueous interface to the bilayer interior, where the acyl chain end presents an increased disorder [51].

Furthermore, from the graphical analysis, one can observe that while the main phase transition increases the cooperativity of the process decreases with increasing membrane lipid composition and complexity, for both probes. In fact, the simplest model composed of only DMPC, presents much lower anisotropy values for both probes than the other systems. Evidence suggest that the presence of SM and Chol in membranes promotes the formation and maintenance of specific domains in the liquid-ordered phase, more condensed and organized, which results in higher anisotropy values [52].

The influence of daunorubicin on the DPH and TMA-DPH fluorescence anisotropy as a function of temperature for each model system studied is shown in

Fig. 2 and Fig. 3, respectively. Overall the results demonstrate that the anticancer drug influences the bilayer fluidity in such a way that depends on the mimetic model membrane studied and the location of probe used (i.e., the type of microenvironment). Therefore, while a decrease in membrane fluidity takes place in the acyl region, almost no significant change in membrane fluidity near the phospholipids head groups is observed. This can be explained by the fact that, although daunorubicin is positively charged at pH 7.4 and it can establish electrostatic interactions between the phospholipids headgroups, its backbone is deeply buried in the membrane. For this reason, the major alterations in membrane fluidity are along the acyl chains as revealed by the DPH results.

On the other hand, daunorubicin membrane incorporation due to the hydrophobic interactions is easier when the phospholipid bilayer is less packed and the interplay between the acyl chains of the lipid molecules are not so strong, as in the case of DMPC. As consequence, a more pronounced effect in DMPC anisotropy can be observed in the presence of daunorubicin, contrary to the rest of the other membranes that contain SM and Chol. It can also be verified that Chol presence impairs the compound's permeation in the membrane and therefore, small changes in fluidity can be observed, which ultimately are in agreement with drug partition studies. The outcomes allow one to infer that the anticancer drug leads to an ordering effect of the bilayer, which is manifested by the decrease of fluidity, except for membranes containing Chol.

Daunorubicin is a molecule that possesses an aromatic ring wherein its planar structure bears resemblance to the structure of cholesterol. Evidence suggests that there is a competition between Chol and drugs presenting an identical planar structure [53].

The membrane fluidity studies clearly demonstrate such event and a bigger influence of daunorubicin along the membrane is achieved in the systems composed by DMPC and DMPC:SM. In this sense, daunorubicin interaction with those models is facilitated probably because there is no competition between the drug and Chol for the place within the bilayer. Furthermore, it is possible that the interactions between daunorubicin and Chol involve the formation of hydrogen bonds between the protonated NH3+ group of daunorubicin and the OH group of Chol, as already mentioned. In fact, size measurements shown that the size of liposomes containing Chol increased in the presence of daunorubicin, which is probably associated with daunorubicin adsorption in the surface. For that reason, only small changes in the membrane fluidity near the phospholipids heads can be observed. These findings are, once again, consistent with the previous results of partition and location, where it has been observed that the anticancer drug establishes electrostatic interactions with the negative phosphate group, hydrophobic interactions with the hydrocarbon chains of the phospholipids and can also form H bonds with Chol. On the other hand, the presence of Chol in bilayers leads to a decrease in the overall effects caused by daunorubicin in membrane fluidity. This fact is especially pronounced in the model containing SM, a more rigid and compact model due to its lipid composition, where the anticancer drug cannot permeate in a higher degree comparatively to the other mimetic systems.

Albeit in a smaller degree when compared with simpler model membranes, daunorubicin partitions in bilayers that contain SM and Chol, which can be correlated with its biological action. Lipid rafts are small, heterogeneous, highly dynamic platforms for signaling molecules that modulate various cellular functions, including cell survival [22]. These particular structures within the membrane are enriched in sterol- and sphingolipids [23] and play important roles in the activation of signaling pathways induced by several anticancer drugs [24]. In fact, evidence reveals that such regions are implicated in drug induced apoptosis [25]. It has been shown that different anticancer drugs can produce changes in membrane rafts lipid content, which compromises their structure and integrity and, consequently, impairs cell growth and triggers cellular death [24]. It was also demonstrated that some cancer cell lines contain more lipid rafts than their normal counterparts and, when depleted of cholesterol were more sensitive to induced cell death [22]. Furthermore, resistance to chemotherapy exhibited by cancer cells is a major clinical problem that leads to therapy failure [18]. This phenomenon has been associated with the presence of molecular "pumps" in tumor cell membranes, such as p-glycoprotein (Pgp), which actively transport the anticancer drugs to the exterior of the cell [19]. Pgp appears to be associated with specific membrane microdomains (lipid rafts), where its activity and function can be regulated by changes in the membrane lipid environment [20, 21]. It has also been evidenced by several structure–activity studies that hydrophobicity and the presence of planar aromatic regions, which occurs in the case of daunorubicin, favor the drug-Pgp interaction [54]. Besides, it was also reported that compounds able to increase membrane fluidity cause an inhibition of Pgp function [55]. Nevertheless, our results showed that daunorubicin is not capable of producing significant alteration in membrane fluidity of models that include Chol. Since it has also been demonstrated that Pgp seems to be associated with the referred microdomains and that the drug's binding sites are via the lipid membrane [20], it is possible to correlate the gathered results with the resistance problem associated with daunorubicin administration.

The present work also highlights the importance and contribution of biological membranes for anticancer drug's pharmacological activity, since it is where several events associated with the compounds cytotoxicity take place, such as apoptosis activation through signaling platforms (lipid rafts).

Conclusions

The interplay between daunorubicin and membranes composed of different lipid contents is expressed as modifications in the overall membrane biophysical properties. In fact, the anticancer drug showed a high partition with membranes, which seems to be greatly driven by electrostatic interactions. Furthermore, the use of two fluorescent probes with different location within the membrane allowed one to confirm that daunorubicin is placed within phospholipids and is positioned between the polar region (where it establishes electrostatic interactions with the negative phosphate group) and the membrane core (where it establishes hydrophobic interactions with the acyl chain tails). As a consequence of its location, the compound induces a decrease in membrane fluidity in the hydrophobic tails of the

phospholipids, in bilayers where cholesterol is not present.

These interactions are intrinsically dependent on the lipid composition and the membrane physical state and structure, which can favor or restrain the drug's mechanism of action and/or its side effects. Moreover, such characteristics are a key factor that influences the anticancer drug partition, its location within the membrane and, ultimately, membrane fluidity. Indeed, the differences in drug incorporation within cell membranes and the alteration in membrane fluidity caused by daunorubicin reveal that the plasma membrane is a crucial place for the anticancer drug's bioaccumulation and action, but also for its cytotoxicity and chemoresistance problem.

The combination of daunorubicin-membrane interplay assessed in this work with previous studies leads to the idea that, when designing and developing new drugs, the different membrane characteristics should be taken into account. In fact, the results demonstrated that the different properties of cell membrane can modulate daunorubicin behavior. For that reason, membrane-lipid therapy, that considers the plasma membranes as targets for drugs, emerges as a promising way of treating cancer. This approach might help the future development of drugs with increased specificity and, therefore, higher efficiency against cancer.

ACKNOWLEDGMENTS Ana Catarina Alves and Cláudia Nunes thank FCT (Lisbon) for the fellowships (SFRH/BD/82443/2011 and SFRH/BPD/81963/2011), respectively. Additionally, this work was financial support from FCT/MEC through National Funds and co-financed by FEDER, under the partnership agreement PT2020-UID/MULTI/04378/2013-POCI/01/0145/FERDER/007728.

[1] A. Grein, C. Spalla, Canevazz.G and A. Dimarco, Descrizione E Classificazione Di Un Attinomicete (Streptomyces Peucetius Sp. Nova) Produttore Di Una Sostanza Ad Attivita Antitumorale - La Daunomicina, Giornale Di Microbiologia, 11 (1963) 109-&.

[2] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo and L. Gianni, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity, Pharmacological reviews, 56 (2004) 185-229.

[3] D.A. Gewirtz, A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin, Biochemical pharmacology, 57 (1999) 727-741.

[4] P.V. Escriba, Membrane-lipid therapy: a new approach in molecular medicine, Trends in Molecular Medicine, 12 (2006) 34-43.

[5] T.R. Tritton and G. Yee, The Anticancer Agent Adriamycin Can Be Actively Cyto-Toxic without Entering Cells, Science, 217 (1982) 248-250.

[6] P.V. Escriba, M. Sastre and J.A. Garciasevilla, Disruption of Cellular Signaling Pathways by Daunomycin through Destabilization of Nonlamellar Membrane Structures, Proceedings of the National Academy of Sciences of the United States of America, 92 (1995) 7595-7599.

[7] M. Lucio, J.L.F.C. Lima and S. Reis, Drug-Membrane Interactions: Significance for Medicinal Chemistry, Current medicinal chemistry, 17 (2010) 1795-1809.

[8] J.M. Siegfried, T.G. Burke and T.R. Tritton, Cellular transport of anthracyclines by passive diffusion. Implications for drug resistance, Biochemical pharmacology, 34 (1985) 593-598.

[9] M.C. Willingham, M.M. Cornwell, C.O. Cardarelli, M.M. Gottesman and I. Pastan, Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs, Cancer research, 46 (1986) 5941-5946.

[10] R. Regev, D. Yeheskely-Hayon, H. Katzir and G.D. Eytan, Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism, Biochemical pharmacology, 70 (2005) 161-169.

[11] L. Gallois, M. Fiallo, A. Laigle, W. Priebe and A. GarnierSuillerot, The overall partitioning of anthracyclines into phosphatidyl-containing model membranes depend neither on the drug charge nor on the presence of anionic phospholipids., Abstracts of Papers of the American Chemical Society, 211 (1996) 51-Carb.

[12] L. Gallois, M. Fiallo and A. Garnier-Suillerot, Comparison of the interaction of doxorubicin, daunorubicin, idarubicin and idarubicinol with large unilamellar vesicles - Circular dichroism study, Biochimica Et Biophysica Acta-Biomembranes, 1370 (1998) 31-40.

[13] P.V. Escriba, A.V. Ferrermontiel, J.A. Ferragut and J.M. Gonzalezros, Role of Membrane-Lipids in the Interaction of Daunomycin with Plasma-Membranes from Tumor-Cells – Implications in Drug-Resistance Phenomena, Biochemistry, 29 (1990) 7275-7282.

[14] H.S. Schwartz and P.M. Kanter, Chemical interactions of cardiolipin with daunorubicin and other intercalating agents, European Journal of Cancer (1965), 15 (1979) 923-928.

[15] J.P. Jaffrezou, T. Levade, A. Bettaieb, N. Andrieu, C. Bezombes, N. Maestre, S. Vermeersch, A. Rousse and G. Laurent, Daunorubicin-induced apoptosis: Triggering of ceramide generation through sphingomyelin hydrolysis, Embo Journal, 15 (1996) 2417-2424.

[16] S. Grazide, N. Maestre, R.J. Veldman, C. Bezombes, S. Maddens, T. Levade, G. Laurent and J.P. Jaffrezou, Ara-C- and daunorubicin-induced recruitment of Lyn in sphingomyelinase-enriched membrane rafts, FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 16 (2002) 1685-1687.

[17] M.-T. Dimanche-Boitrel, O. Meurette, A. Rebillard and S. Lacour, Role of early plasma membrane events in chemotherapy-induced cell death, Drug Resistance Updates, 8 (2005) 5-14.

[18] F.E. Koehn, Natural Products and Cancer Drug Discovery, Springer, 2013.

[19] Cancer multidrug resistance, Nat Biotech, (2000).

[20] S. Modok, C. Heyward and R. Callaghan, P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment, Journal of lipid research, 45 (2004) 1910-1918.

[21] S.M. dos Santos, C.C. Weber, C. Franke, W.E. Muller and G.P. Eckert, Cholesterol: Coupling between membrane microenvironment and ABC transporter activity (vol 354, pg 216, 2007), Biochemical and Biophysical Research Communications, 408 (2011) 193-193.

[22] Y.C. Li, M.J. Park, S.K. Ye, C.W. Kim and Y.N. Kim, Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents, American Journal of Pathology, 168 (2006) 1107-1118.

[23] S.K. Patra, Dissecting lipid raft facilitated cell signaling pathways in cancer, Biochimica Et Biophysica Acta-Reviews on Cancer, 1785 (2008) 182-206.

[24] K.S. George and S. Wu, Lipid raft: A floating island of death or survival, Toxicology and applied pharmacology, 259 (2012) 311-319.

[25] C. Bezombes, G. Laurent and J.P. Jaffrezou, Implication of raft microdomains in drug induced apoptosis, Current medicinal chemistry. Anti-cancer agents, 3 (2003) 263-270.

[26] J.K. Seydel and M. Wiese, Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling, Wiley-VCH Verlag GmbH & Co, 2002.

[27] P.V. Escriba, J.M. Gonzalez-Ros, F.M. Goni, P.K. Kinnunen, L. Vigh, L. Sanchez-Magraner, A.M. Fernandez, X. Busquets, I. Horvath and G. Barcelo-Coblijn, Membranes: a meeting point for lipids, proteins and therapies, Journal of cellular and molecular medicine, 12 (2008) 829-875.

[28] J.P. Slotte, Biological functions of sphingomyelins, Progress in Lipid Research, 52 (2013) 424-437.
[29] F.R. Maxfield and I. Tabas, Role of cholesterol and lipid organization in disease, Nature, 438 (2005) 612-621.
[30] P. Wydro, Sphingomyelin/phosphatidylcholine/cholesterol monolayers - analysis of the interactions in model membranes and Brewster Angle Microscopy experiments, Colloids and Surfaces B-Biointerfaces, 93 (2012) 174-179.
[31] D. Lingwood and K. Simons, Lipid Rafts As a Membrane-Organizing Principle, Science, 327 (2010) 46-50.
[32] C. Nunes, D. Lopes, M. Pinheiro, C. Pereira-Leite and S. Reis, In vitro assessment of NSAIDs-membrane interactions: significance for pharmacological actions, Pharmaceutical research, 30 (2013) 2097-2107.
[33] L.M. Magalhaes, C. Nunes, M. Lucio, M.A. Segundo, S. Reis and J.L. Lima, High-throughput microplate assay for the determination of drug partition coefficients, Nature protocols, 5 (2010) 1823-1830.
[34] J. Brittes, M. Lucio, C. Nunes, J.L. Lima and S. Reis, Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects, Chemistry and physics of lipids, 163 (2010) 747-754.
[35] A. Coutinho and M. Prieto, Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrylamide: A laboratory experiment for undergraduate students, Journal of Chemical Education, 70 (1993) 425.
[36] J. Marcelino, J.L. Lima, S. Reis and C. Matos, Assessing the effects of surfactants on the physical properties of liposome membranes, Chemistry and physics of lipids, 146 (2007) 94-103.
[37] M.N. Martinez and G.L. Amidon, A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals, Journal of clinical pharmacology, 42 (2002) 620-643.
[38] C. Pereira-Leite, C. Nunes, J.L. Lima, S. Reis and M. Lucio, Interaction of celecoxib with membranes: the role of membrane biophysics on its therapeutic and toxic effects, The journal of physical chemistry. B, 116 (2012) 13608-13617.
[39] C. Nunes, G. Brezesinski, D. Lopes, J.L. Lima, S. Reis and M. Lucio, Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality, The journal of physical chemistry. B, 115 (2011) 12615-12623.
[40] L. Gallois, M. Fiallo, A. Laigle, W. Priebe and A. Garnier-Suillerot, The overall partitioning of anthracyclines into phosphatidyl-containing model membranes depends neither on the drug charge nor the presence of anionic phospholipids, European journal of biochemistry / FEBS, 241 (1996) 879-887.
[41] L. Dupou-Cezanne, A.M. Sautereau and J.F. Tocanne, Localization of adriamycin in model and natural membranes. Influence of lipid molecular packing, European journal of biochemistry / FEBS, 181 (1989) 695-702.
[42] L. Calzolai, E. Gaggelli, A. Maccotta and G. Valensin, Interaction of Daunomycin with Dipalmitoylphosphatidylcholine Model Membranes. A 1H NMR Study, Journal of magnetic resonance. Series B, 112 (1996) 228-235.
[43] S. Banuelos, J.L. Arrondo, J.M. Canaves, J.A. Ferragut and A. Muga, The interaction of daunomycin with model membranes. Effect of the lipid physical state and the lipid composition, European journal of biochemistry / FEBS, 213 (1993) 1269-1275.
[44] J.R. Lakowicz, Quenching of Fluorescence, in: Principles of Fluorescence Spectroscopy, Springer, 2006, pp. 278-327.
[45] R.D. Kaiser and E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth, Biochemistry, 37 (1998) 8180-8190.
[46] D. Illinger, G. Duportail, Y. Mely, N. Poirel-Morales, D. Gerard and J.G. Kuhry, A comparison of the fluorescence properties of TMA-DPH as a probe for plasma membrane and for endocytic membrane, Biochimica et biophysica acta, 1239 (1995) 58-66.
[47] M.C. Erickson, Chemistry and Function of Phospholipids, in: C.C. Akoh, D.B. Min (Eds.) Food Lipids: Chemistry, Nutrition, and Biotechnology, Taylor & Francis Group, LLC, 2008, pp. 44.
[48] G. Karp, The Structure of Function of the Plasma Membrane, in: Cell and Molecular Biology: Concepts and Experiments, John Wiley & Sons, Inc., 2010, pp. 133-134.
[49] J.R. Lakowicz, Fluorescence Anisotropy, in: Principles of Fluorescence Spectroscopy, Springer, 2006, pp. 366-375.
[50] M. Lucio, H. Ferreira, J.L. Lima and S. Reis, Interactions between oxicams and membrane bilayers: an explanation for their different COX selectivity, Medicinal chemistry (Shariqah (United Arab Emirates)), 2 (2006) 447-456.
[51] J.M. Collins, R.N. Dominey and W.M. Grogan, Shape of the fluidity gradient in the plasma membrane of living HeLa cells, Journal of lipid research, 31 (1990) 261-270.
[52] Y. Barenholz, Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications, Sub-cellular biochemistry, 37 (2004) 167-215.
[53] C. Tessier, P. Nuss, G. Staneva and C. Wolf, Modification of membrane heterogeneity by antipsychotic drugs: an X-ray diffraction comparative study, J Colloid Interface Sci, 320 (2008) 469-475.
[54] A. Seelig and E. Landwojtowicz, Structure-activity relationship of P-glycoprotein substrates and modifiers, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 12 (2000) 31-40.
[55] S. Orlowski, S. Martin and A. Escargueil, P-glycoprotein and 'lipid rafts': some ambiguous mutual relationships (floating on them, building them or meeting them by chance?), Cellular and Molecular Life Sciences, 63 (2006) 1038-1059.

Supplementary material

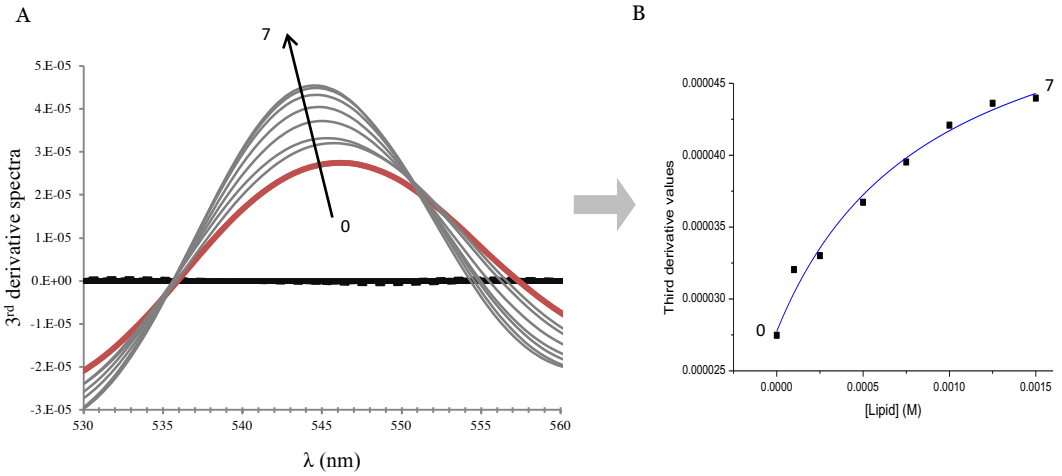


Fig. 1. Third-derivative absorption spectra (A) of daunorubicin (40μM) (red line, 0) alone, incubated in DMPC:ES model membrane at 37 °C with increasing lipid concentration (7, represents the maximum lipid concentration) and the model membrane without drug (black dashed lines). Plot (B) represents the best fitting curve to experimental third-derivative spectrophotometric data (DT vs. [L]) using a nonlinear regression method at a wavelength of 546 nm.

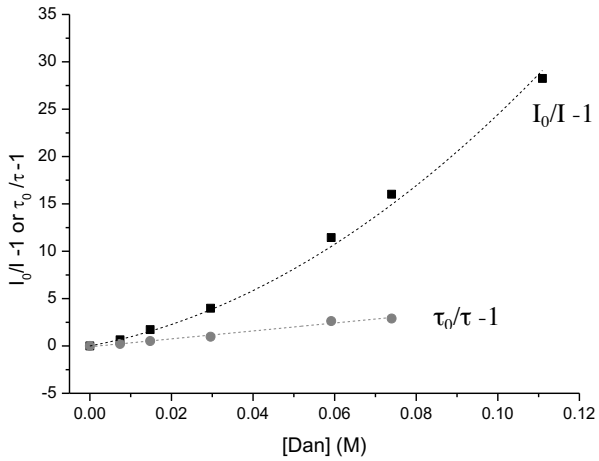
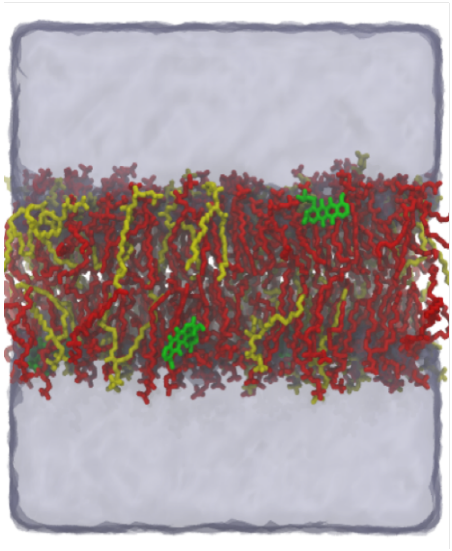


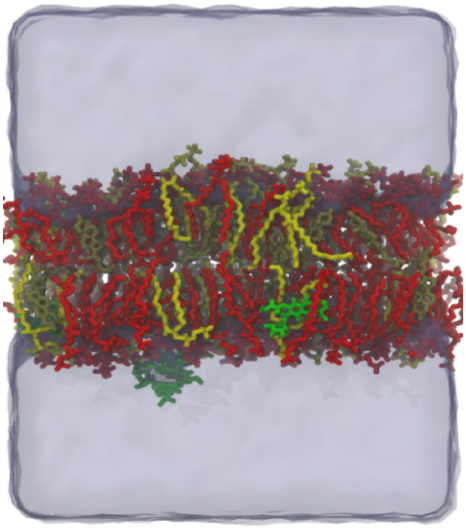
Fig. 2. Stern–Volmer plots of the probe DPH in the DMPC:SM model at pH 7.4 and 37 °C with increasing daunorubicin concentrations: square symbols (■) represent the Stern–Volmer plot obtained by steady-state fluorescence measurements ($I_0/I-1$) and circle symbols (●) represent the Stern–Volmer plot obtained by lifetime fluorescence measurements ($\tau_0/\tau-1$).

2. Influence of doxorubicin on model cell membrane properties: insights from *in vitro* and *in silico* studies

DMPC:SM
8:2

DMPC:SM:Chol
7:1.5:1.5



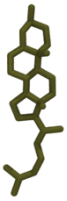




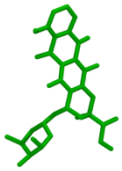
DMPC



SM



Chol



Dox

Influence of doxorubicin on model cell membrane properties: insights from *in vitro* and *in silico* studies

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Submitted to Scientific Reports

Despite doxorubicin being one of the most commonly used chemotherapy agents there still remain significant holes in our knowledge regarding its delivery efficacy and an observed resistance mechanism to its efficacy that is postulated to involve the cell membrane. One possible mechanism is the efflux by the protein P-gp, which is found predominantly in cholesterol enriched domains. Thereby, a hypothesis for the vulnerability of doxorubicin to efflux through P-gp is its enhanced affinity for the ordered cholesterol rich regions of the plasma membrane. Thus, we have studied doxorubicin´s interaction with model membranes in a cholesterol rich, ordered environment and in liquid-disordered cholesterol poor environment of the rest of the plasma membrane. We have combined three separate experimental protocols: UV-Vis spectrophotometry, fluorescence quenching and steady-state anisotropy and computational molecular dynamics modeling. Together our results show that the presence of cholesterol induces a change in membrane structure and doesn´t impair doxorubicin's membrane partition, but reduces the drug's influence on membrane fluidity without directly interacting with it. It is thus possible that the resistance mechanism that lowers the efficacy of doxorubicin, results from an increased density in membrane regions where the efflux proteins are present. This work can be seen as a successful approach combining experimental and computational studies of membrane based systems to unveil the behavior of drugs and candidate drug molecules.

SAXS | WAXS | anthracyclines | drug-membrane interactions

Doxorubicin (as shown in Fig. 1) is one of the most widely prescribed anticancer drugs[1]. The most prevalent opinion is that the anticancer activity of the drug is mainly due to direct interaction with nucleic acids, leading to DNA damage and inhibition of DNA synthesis. This is, however, still the subject of considerable debate[1]; there is also strong evidence suggesting interaction with cell membranes plays a role in its activity[2]. Clearly, even if the dominant mechanism is interaction with the nucleic acid, to reach this target doxorubicin must pass through a variety of other organelles to reach the DNA[3]. First, the drug must pass the selective barrier to entry into the cell that is the cell membrane, then the plasma and, finally, the nuclear membrane; interaction with lipid membranes is an unavoidable step in doxorubicin activity, whatever the exact mechanism of action proves to be.

Drug-membrane interactions produce alterations in the physical properties of the cell membrane, as a result of the behaviour of the drug within the membrane; how the drug orients in the membrane, which components of the lipid membrane the drug interacts with and what interactions within the membrane the drug disrupts, will all alter the physical properties of the membrane[4]. This can, in turn, lead to changes in cell signaling and the function of membrane proteins, e.g. transporters and ion channels[5].

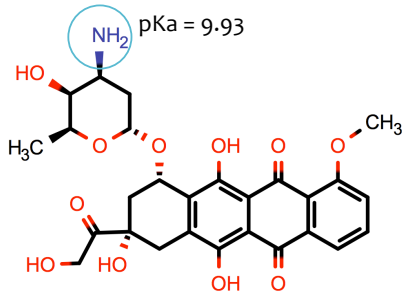


Fig. 1. Molecular structure and pKa value of doxorubicin.

It was, for example, found that Daunorubicin (a doxorubicin analog) causes alteration of both G-proteins and protein kinase C-associated signaling pathways, through destabilizing the nonlamellar membrane structures that are involved in their location and activity[6,7]. As these proteins participate in fundamental functions of the cell, including proliferation and differentiation, their modulation could be responsible for the antitumor activity of some drugs[6].

Within the membrane, the lipids are heterogeneously distributed into dynamic domains. Domains enriched in cholesterol and sphingolipids are thought to form a highly ordered, liquid-ordered phase (l_o), *rafts* that metaphorically float in a liquid-disordered phase (l_d) matrix[8]. The function of several membrane proteins is highly dependent on their association with these domains[9,10]. Among the proteins thought to be associated with lipid rafts[11] is P-glycoprotein (P-gp)[12] that effluxes a variety of hydrophobic, neutral, and positively charged drugs from the cell; P-gp is a component of the normal cellular defense system against xenobiotics[13]. The P-gp protein has been shown to be highly sensitive to membrane fluidity, thus, any changes in membrane fluidity will alter the ability of P-gp to efflux drug molecules[11].

Doxorubicin is among the substrates effluxed by P-gp; thus, in this case, this protein plays a key role in drug resistance[14]. What is not so well understood, however, is: 1) how doxorubicin partitions between the l_o rafts and the l_d matrix and 2) how doxorubicin alters the physical properties of the lipid rafts. These questions are highly relevant to doxorubicin as a drug because how

doxorubicin partitions between the two phases of the plasma membrane will determine its susceptibility to P-gp; if more of it is in the rafts where P-gp is located then it will be effluxed with greater efficiency and how the presence of doxorubicin affects the structure of the l_o raft can not only effect P-gp function, but could result in the modulation of the physicochemical properties of the membrane. This could, in turn, initiate different processes implicated in the anticancer cytotoxic effects. Several anticancer drugs, in fact, induce modifications in the content of lipid rafts, leading to growth inhibition and activation of tumor cells apoptosis[9].

In light of all of this, we set out to apply a combined protocol of *in vitro* experimental analysis techniques, including UV-Vis spectrophotometry and fluorescence quenching and steady state anisotropy, both using membrane bound fluorescent probes and *in silico* computational modeling, to study the interaction of doxorubicin with the plasma membrane. Properties studied include, effect of doxorubicin on membrane fluidity, distribution of doxorubicin between the l_o and l_d phases and, through computational molecular dynamics modeling, the microscopic details of the membrane-doxorubicin interactions. Altogether we show that cholesterol, present in the l_o rafts but is not so prevalent in the l_d matrix alters the behavior of doxorubicin in the membrane without directly interacting with it, in a way that promotes increased location of doxorubicin to the l_o rafts, possibly increasing uptake by the P-gp efflux protein. We propose that this could possibly be a mechanism that contributes to the doxorubicin chemoresistance problem.

Results and Discussion

Doxorubicin Partition Coefficients

The partitioning of drugs into biological membranes plays a significant role in their uptake, transport, bioavailability and distribution[15]. In fact, doxorubicin needs to cross the cell membrane to reach its intracellular targets, such as the DNA, and the interaction with lipids can strongly influences the drug's pharmacokinetic and pharmacodynamic properties[15]. The partition coefficient (Kp), is an important property to determine, as it provides information concerning the distribution of drugs between aqueous and lipid phases, of direct relevance to the pharmacological activity of the drug. The doxorubicin partition coefficient in several model membranes was determined through derivative UV-Vis spectrophotometry. This technique, based on the fact that the spectral characteristics (λ_{max}) of the drug change when it permeates from the aqueous to the lipid medium, allows for the quantification of drug distribution between each phase. Furthermore, it also provides a better resolution of the overlapped bands and eliminates the lipid light scattering interference through the use of the derivative method.

Fig. 2 (A) shows, as an example, the third-derivative absorption spectra of doxorubicin with different concentrations of liposomes composed of DMPC:SM [8:2] at pH 7.4 and at 37°C. The use of the third-derivative allowed for the elimination of the residual background signal caused by scattering from the liposomes. The best fit of equation 1 to the third-derivative data was achieved at 544 nm and is shown in Fig. 2 (B).

The experimental values of doxorubicin partition coefficient with DMPC:SM and DMPC:SM:Chol (expressed as Kp and logD) are depicted in Table 1. Traditionally, the partition coefficient is assessed in octanol/water systems. The octanol/water logD of doxorubicin at pH 7.4, obtained using Marvin sketch calculator software (ChemAxon), was 0.02. Thereby, a lack of correlation between the doxorubicin partition coefficients obtained in liposome/buffer and octanol/water systems can be observed. Such difference is due to the fact that the biphasic octanol/water system can only account for hydrophobic interactions. Liposomes, on the other hand, provide an anisotropic environment that can better mimic membranes, which are composed by amphiphilic phospholipids and can, therefore, establish electrostatic and hydrophobic interactions.

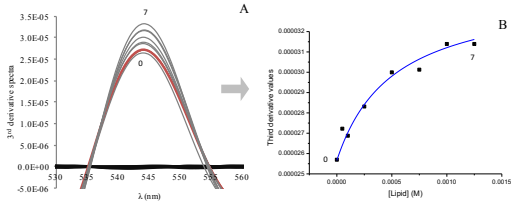


Fig. 2. Third-derivative absorption spectra (A) of doxorubicin (40µM) (red line, 0) alone, incubated in DMPC:SM model membrane at 37°C with increasing lipid concentration (7 represents the maximum lipid concentration) and the model membrane without drug (black lines). (B) represents the best fitting curve to experimental third-derivative spectrophotometric data (DT vs. [L]) using a nonlinear regression method at a wavelength of 544 nm.

In fact, the pK_a value (9.93), calculated using Marvin sketch calculator software, predict that nearly 80% of doxorubicin molecules are in the cationic form at pH 7.4, providing evidence that not only hydrophobic intermolecular forces drive the doxorubicin's partition. In fact, the octanol/water partition coefficient value is much smaller than that calculated by the liposome/buffer system, since it only reflects the hydrophobic interactions of a small amount of molecules that are in the neutral form at pH 7.4. Hence, the use of a liposomes/buffer system allows one to obtain more realistic information regarding doxorubicin lipophilicity and, therefore, it's *in vivo* membrane partition.

Furthermore, the gathered experimental results reveal that doxorubicin exhibits a similar membrane partitioning with both DMPC:SM and DMPC:SM:Chol liposomes. This suggests that the positively charged doxorubicin interacts with the phospholipid polar head groups of both models and that the presence of Chol does not impair such partition.

Table 1: Partition Coefficients (expressed as Kp and logD) of doxorubicin in DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] at pH 7.4 and 37°C.[a]

MODEL	Kp	Experimental logD
DMPC:SM	8195 ± 406	3.91 ± 0.02
DMPC:SM:Chol	8707 ± 523	3.94 ± 0.03

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other model.

Drug location studies using different fluorescent probes

The location of doxorubicin in the membrane was tracked by fluorescence quenching of membrane bound probes (TMA-DPH and DPH), since this technique provides a measure of the accessibility of the drug to each probe[16]. Such knowledge is only possible to obtain due to the fact that the fluorophore position in the membrane is well defined and documented. Therefore, while DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer[17], TMA-DPH is reported to be anchored in the polar head groups region of phospholipids due to its charged group[18]. According to this, in the current study the steady-state fluorescence intensities and lifetimes were measured in liposomes labeled with TMA-DPH and DPH probes. The theory and equations behind this technique are presented in the *Supporting Information*.

In Fig. 3, the Stern–Volmer plots of I_0/I-1 and τ_0/τ-1 as a function of doxorubicin membrane concentration are shown, for the DMPC:SM [8:2] model.

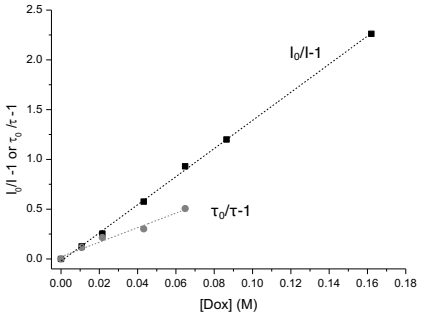


Fig. 3. Stern–Volmer plots of the probe DPH in DMPC:SM [8:2] model at pH 7.4 and 37°C with increasing doxorubicin concentrations: square symbols (■) represent the Stern–Volmer plot obtained by steady-state fluorescence measurements ($I_0/I-1$) and circle symbols (●) represent the Stern–Volmer plot obtained by lifetime fluorescence measurements ($\tau_0/\tau-1$).

According to the description of the quenching behavior (*Supporting Information*), the quenching parameter values, namely the dynamic constant (K_D), the static constant (K_S) and the Stern–Volmer constant (K_{SV}), obtained from this technique are depicted in Table 2.

From the analysis of Table 2, it is possible to conclude that the quenching process, in all mimetic models systems studied and probes used, results from both dynamic and static interactions. This behavior indicates that doxorubicin must diffuse into the fluorophore during the lifetime of the excited state, however, it can also form a non-fluorescent complex with the probe. According to the results, doxorubicin was able to quench both probes, DPH and TMA-DPH. Nevertheless, the K_{SV} values demonstrate that the decrease of the DPH fluorescence was more pronounced than for TMA-DPH in the membrane model composed of DMPC:SM [8:2]. This suggests that doxorubicin interacts with the lipid head groups of the phospholipids, however, the drug's dihydroanthraquinone residue may also interact with the lipid fatty acid chains through hydrophobic interactions. For the bilayer comprised by DMPC:SM:Chol [7:1.5:1.5] similar values of K_{SV} were obtained for both fluorescent probes, which means that doxorubicin has an identical accessibility to DPH and TMA-DPH in the model membranes with cholesterol present.

Effect of Doxorubicin on membrane fluidity

Since doxorubicin interacts with membranes in order to exert its therapeutic action, it is important to understand how the compound can influence the physicochemical state of the phospholipid bilayer in order to explain its pharmacological action. Furthermore, alterations of membrane fluidity can severely affect the cell's functional properties that might be correlated with the drug's mechanism of action. In this context, membrane fluidity studies were performed by steady-state anisotropy[19] using fluorescent probes (DPH and TMA-DPH). The ability of doxorubicin to disturb the membrane structure in different regions can be assessed since these two probes report the microfluidity of those sites[20]. Alterations in membrane fluidity can be detected by changes in anisotropy, which reflects perturbations in the probe's rotational movement caused by the changes in the stiffness of its surrounding matrix[21].

The effect of temperature on the DPH and TMA-DPH fluorescence anisotropy of the different model membranes without doxorubicin is presented in Fig. 4. The results demonstrate that the anisotropy values in both model membranes decrease with increasing temperature to a greater extent in the acyl chain region (given by the probe DPH) than in the phospholipid head region (given by the TMA-DPH probe). In fact, it is well documented that the membrane is characterized by a fluidity gradient from the aqueous interface to the bilayer interior, where the acyl chain end shows increased disorder. Also, it is known that the presence of SM and Chol in membranes promotes the formation and maintenance of specific domains in the liquid-ordered phase, more condensed and organized[22], which results in higher anisotropy values.

Fig. 5 shows the influence of doxorubicin on the DPH and TMA-DPH fluorescence anisotropy as a function of temperature for each model system studied. Regarding the model membrane composed of DMPC:SM two distinct doxorubicin behaviors can be observed in the different regions of the membrane. In the acyl chains doxorubicin increased the membrane fluidity, in a more ordered phase of the bilayer, and decreased the membrane microviscosity, in a more fluid phase of the bilayer. A similar behavior as Chol possesses when in incorporated in the membrane, helping to prevent the membrane to become too fluid or too rigid, due to cholesterol' ability to increase the order chains of fluid phase-lipids, and to cause disordering of gel-phase lipids while still maintaining a high degree of orientational ordering. At the same time, doxorubicin decreased the membrane microviscosity in the phospholipid's head groups. These findings are, once again, consistent with the previous results of partition and location, where it has been observed that the anticancer drug establishes electrostatic interactions and hydrogen bonds with the negative phosphate group and hydrophobic interactions with the hydrocarbon chains of the phospholipids.

On the other hand, it can be observed that doxorubicin did not produced any alterations in the membrane fluidity of DMPC:SM:Chol model along the bilayer. This fact can be correlated with the disposition of the molecules within the phospholipid membrane. Doxorubicin is a molecule that possesses an aromatic

Table 2: Fluorescence quenching parameters (K_D, dynamic constant; K_S, static constant; K_{SV}, Stern–Volmer constant) determined from the quenching of DPH and TMA-DPH by doxorubicin in the different mimetic models at pH 7.4 and 37°C. [a]

MODEL	Probe	K_D (M^{-1})	K_S (M^{-1})	K_{SV} (M^{-1})
DMPC:SM	DPH	7.4* ± 0.2	6.4 ± 0.3	13.8* ± 0.4
	TMA-DPH	6* ± 1	6.0 ± 0.3	11* ± 1
DMPC:SM:Chol	DPH	3.2* ± 0.4	3.2* ± 0.2	6.4 ± 0.2
	TMA-DPH	2.5* ± 0.2	4.2* ± 0.2	6.6 ± 0.1

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other probe, for each fluorescence quenching parameter.

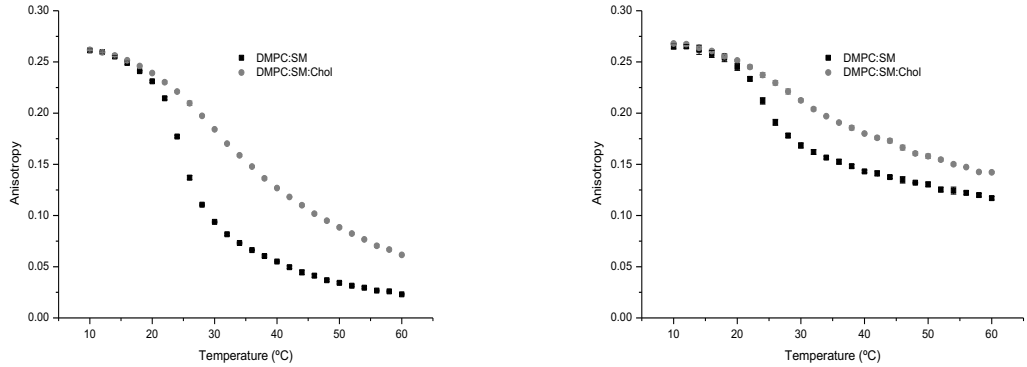


Fig. 4. Steady-state anisotropy of DPH (left) and TMA-DPH (right) as a function of temperature in DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] membrane models. Results present the mean of at least three independent assays.

ring wherein its planar structure bears resemblance to the structure of cholesterol. According to the location studies, doxorubicin has an identical accessibility to both DPH and TMA-DPH probes. Considering that the presence of Chol reduces the membrane fluidity, it is possible that doxorubicin does not cause significant alteration in the overall fluidity in both superficial and deeper regions of phospholipids. Furthermore, due to interactions between NH^{3+} of doxorubicin and the phosphate group in the polar head of the phospholipids, the further access of doxorubicin into the membrane might be impaired. The presence of SM together with Chol form within the membrane, liquid-ordered domains that are characterized by a low density and rigid environment, as shown through anisotropy measurements. It has been demonstrated by several structure–activity studies that hydrophobicity and the presence of planar aromatic regions, as in the case of doxorubicin, favor the drug-P-gp interaction.[23] Furthermore, it was also reported that increase of membrane fluidity can lead to an inhibition of P-gp function[24]. Since our results demonstrate that doxorubicin did not cause any changes in the fluidity of the DMPC:SM:Chol model membrane and that the drug's binding sites on the P-gp are via the lipid membrane[25], it is possible to correlate the gathered outcomes with the resistance problem associated with doxorubicin administration.

Molecular Dynamics simulation results: Interaction of doxorubicin with membrane bilayers

We used MD simulation with all atom resolution to study the partitioning of doxorubicin into the DMPC:SM and DMPC:SM:Chol membrane bilayers. The average distance between the z-coordinate of the center of mass of the doxorubicin molecule and the nearest phosphate group layer are calculated using results from the entire equilibrated trajectory as shown in Fig. 6A. This analysis shows that the doxorubicin molecule partitions closer to the lipid-water interface in the presence of cholesterol, in good agreement with the partition and location studies. Furthermore, the angle of distribution of the doxorubicin molecule with the membrane normal (shown in red in Fig. 6B and C) shows that the orientation of the anticancer drug in the presence of cholesterol is significantly altered. As seen in Fig. S1A (*Supporting Information*), in the DMPC:SM membrane bilayer, the doxorubicin cyclic group (dihydroanthraquinone residue) is perpendicular to the membrane normal, however for the DMPC:SM:Chol membrane, the presence of cholesterol increases the degree of membrane ordering, resulting in an orientation change such that the average angle of doxorubicin to the membrane normal becomes 22 degrees. Such a difference in the doxorubicin orientation, concerning the two model membranes, might help to explain why alterations in membrane fluidity are more pronounced in the DMPC:SM bilayer. Furthermore, the increase in membrane ordering that results from the presence of cholesterol is evident in the increase in membrane thickness, as shown in the Fig. S1B (*Supporting Information*). Also, the addition of cholesterol decreases the number of non-bonded contacts of lipids and cholesterol with doxorubicin as shown in Fig. S2 (*Supporting Information*). However, in the presence of cholesterol, the number of hydrogen bonds between

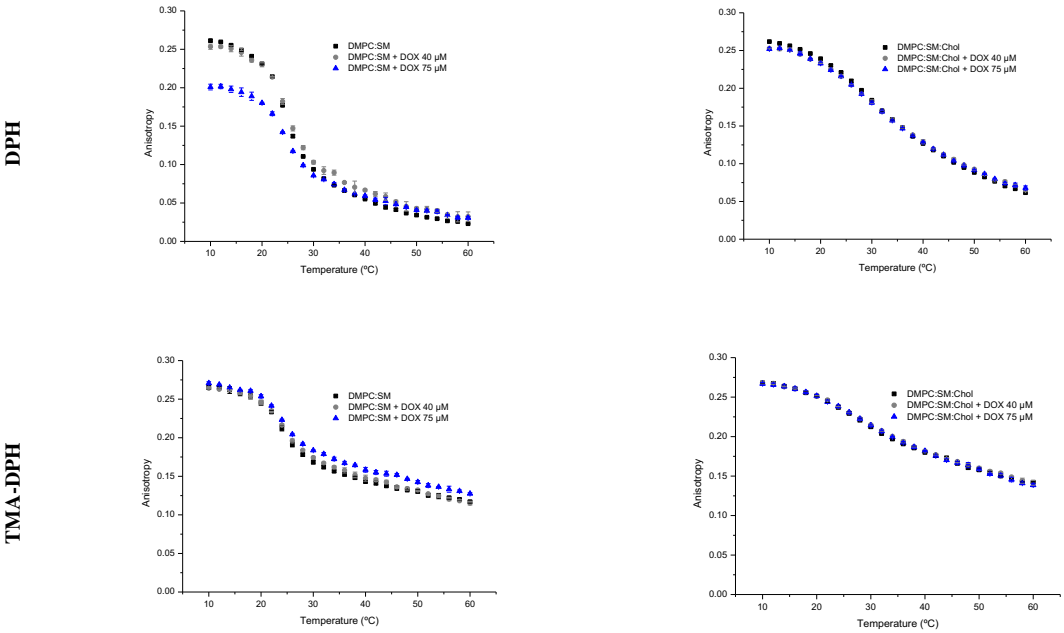


Fig. 5. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of doxorubicin 40 μM (●) and 75 μM (▲). Results present the mean of at least three independent assays.

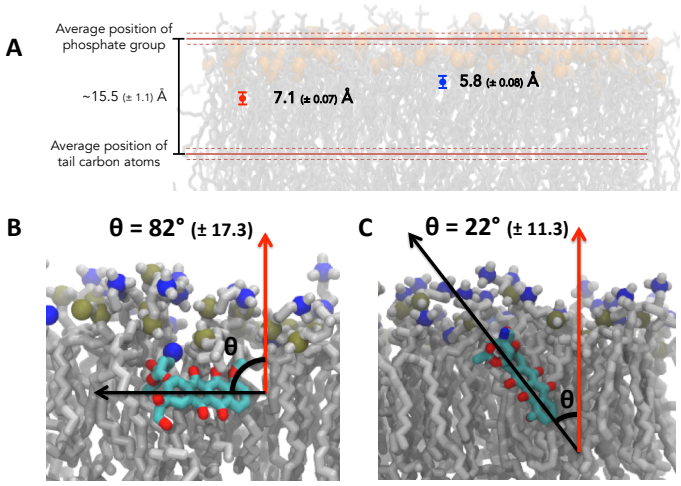


Fig. 6. (A) Partitioning of doxorubicin into the membrane bilayer models. The dots indicate the average distance of the doxorubicin molecule from the phosphate head groups of the lipids in the top leaflet of the membrane bilayer. The red and blue dots represent the position of the doxorubicin molecule in the DMPC:SM and DMPC:SM:Chol membranes respectively. (B) The Angle distribution of doxorubicin in DMPC:SM (left) and DMPC:SM:Chol (right) membranes. The red arrow represents the membrane normal.

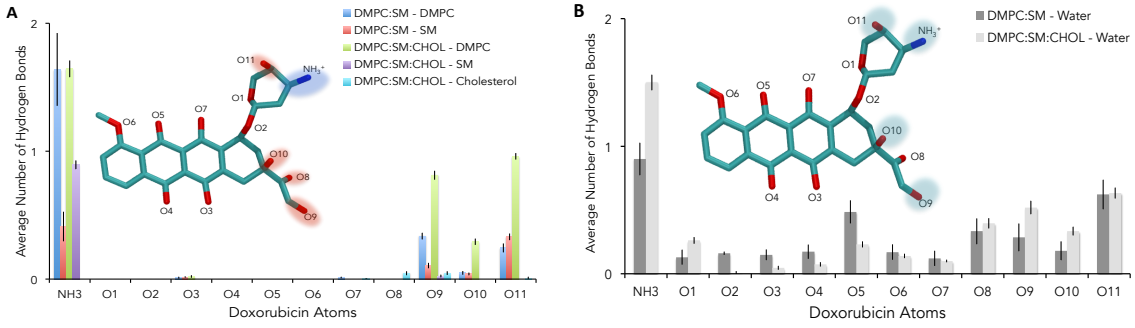


Fig. 7. Molecular interactions of doxorubicin with individual components of the membrane bilayers (A) Number of hydrogen bonds of doxorubicin with lipids. (B) Number of hydrogen bonds between doxorubicin and the water molecules, highlighted atoms of doxorubicin show the part of the molecule involved in hydrogen bonding.

doxorubicin and both the lipids and cholesterol increases, as presented in Fig. 7. Also in Fig. 7, we display the specific groups of doxorubicin involved in hydrogen bonding with lipids and both cholesterol (A) and water (B).

The high partitioning of doxorubicin with the two models can be explained by its ability to interact with the lipid tails as demonstrated in Fig. S2 (*Supporting Information*) and to form hydrogen bonds with lipid headgroups (Fig. 7).

Although it is observed that in the presence of cholesterol 1) the orientation of doxorubicin molecule in membrane bilayer is altered, 2) the number hydrogen bonds with the membrane is increased and 3) hydrophobic interactions is decreased (Fig. S2, *Supporting Information*), it is interesting to note that, the extent of direct interaction between cholesterol and doxorubicin can be observed is very small, as seen in Fig. S3 (*Supporting Information*). This might be explained by the fact that the OH group of cholesterol is masked by the polar head of sphingomyelin in a typical "umbrella" effect[26] and, therefore, is not available to interact with the doxorubicin molecule. In Fig. S3 (*Supporting Information*) the distribution of the measured distances between doxorubicin and DMPC, SM and Chol is shown. From this plot, it is clear that doxorubicin preferentially locates in close proximity to both the SM and DMPC molecules in the membrane. Hence the effect of cholesterol on the behavior of the membrane is significant even though this effect is not the result of direct interaction, but rather the change in the membrane structure that results from the presence of cholesterol.

Conclusions

The present work investigated the molecular interactions and the behavior of an extensively used drug, doxorubicin, with model membranes composed of DMPC and SM, both in the presence (ordered) and absence (liquid-disordered) of CHOL using *in vitro* and *in silico* models. Our goal was to shed light on the difference in behavior of doxorubicin in cholesterol rich ordered rafts from the rest of the biomembrane; the interaction of doxorubicin with a lipid membrane, i.e. how it positions in the membrane, was found to be strongly affected by the ordering effect of cholesterol. In all cases doxorubicin located within our model membranes, driven by electrostatic but also hydrophobic interactions, as observed by both *in vitro* and *in silico* results. Furthermore, in the presence of cholesterol, the doxorubicin 1) preferentially collocates within the bilayer closer to the lipid-water interface and 2) displays an increased number of hydrogen bonds to both lipid and water molecules. Cholesterol, however, was found to not interact directly with the drug: the effect of the presence of cholesterol was solely due to the ordering effect cholesterol has on membrane structure. Collectively the *in vitro* and *in silico* results combine to build this picture, where it was possible to demonstrate that the presence of cholesterol does not reduce doxorubicin membrane partition, but it influences the drug's ability to alter membrane fluidity. Furthermore, computational results show that cholesterol alters the behavior of the doxorubicin in the membrane without itself interacting with the doxorubicin: the effect is mediated through how it alters the membrane structure. This result can shed light on the doxorubicin chemoresistance problem as follows: 1) P-gp, the protein responsible for removal of xenobiotics like doxorubicin, preferential locates to lipid rafts and 2) our results now indicate that doxorubicin also preferentially locates to lipid rafts rich in cholesterol[11], thus increasing their bioavailability to the efflux pump probably responsible for the reduced drug concentration inside the cell.

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Materials and Methods

In this work, we have studied synthetic models (liposomes) composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were used in order to mimic specific membrane characteristics such as the presence of lipid rafts. Phosphatidylcholines are among the most abundant phospholipids of natural plasma membranes4 and were, for that reason, included in the mimetic models as the dominant constituent (DMPC). Sphingomyelin and cholesterol are important elements of the cell membrane that undergo tight packing with phosphatidylcholines, forming the so called dynamic lipid rafts/nano-domains. These structures also control membrane fluidity and permeability, crucial for the preservation of its organization and, consequently, contributing to the cell membrane's physiology and function.[27] Thereby, the ability of doxorubicin to partition into lipid bilayers (assessed by calculating the coefficient partition, Kp, using derivative spectrophotometry), its location within the membrane (by fluorescence quenching) and its effect in the membrane fluidity (through fluorescence anisotropy) were investigated. Molecular simulations were also performed, using the same lipid composition, in order to gain further insight regarding the interactions of doxorubicin with the studied model membranes.

Reagents. Doxorubicin (DOX) was obtained from LC Laboratories (Woburn, Massachusetts, USA). The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). All reagents were used without further purification. Drug solutions were prepared with Hepes buffer (10 mM, pH 7.4) and the ionic strength was adjusted with NaCl (I=0.1 M) to mimic physiological conditions. The buffer was prepared using double-deionized water (conductivity less than 0.1 μS cm^{-1}).

Liposomes Preparation. Liposomes were prepared according to the adapted classic thin film hydration

method.[28] Briefly, the lipids DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] were dissolved in chloroform/methanol (3:2 v/v) and the organic solvents were evaporated under a stream of nitrogen in a rotative evaporator (Buchi R-200, equipped with a thermostated bath Buchi R-490, Switzerland). The obtained dried lipid film was dispersed in Hepes (pH 7.4) and the mixture was vortexed to yield multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were obtained by extruding the MLVs eleven times (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) using policarbonate filters with a diameter pore of 100 nm (Nucleopore) at 60°C (temperature above the main phase transition temperature of the lipids). Labeled liposomes were identically prepared, except that the fluorescence probe (DPH or TMA-DPH) was co-dissolved with lipids in the organic solvents mixture to give a probe/lipid molar ratio of 1:300 (prevent changes in the membrane's structure).

Determination of Partition Coefficients by Derivative Spectrophotometry. Partition coefficient (Kp) of doxorubicin between liposomes/buffer systems was assessed by derivative spectrophotometry, as described in previous works[21,28,29]. For these studies, a set of samples containing a fixed concentration of doxorubicin (40 μM) and increasing concentrations of lipids (in the range of 0–1250 μM) were prepared. The equivalent references were identically prepared but without the drug. All suspensions were incubated during 30 min at 37°C. The absorption spectra of all solutions were recorded at 37°C using a multidetection microplate reader (Synergy™ HT, BioTek Instruments Inc, Winooski, VT, USA). The Kp values were calculated from the third derivative spectra, using a developed routine the *Kp Calculator* [30], by fitting equation 1 to the experimental data (D_T vs [L]) using a nonlinear regression method:

$$D_T = D_W + \frac{(D_m - D_w)K_p[L]V_m}{1 + K_p[L]V_m} \qquad (1)$$

where, D_T is the third derivative intensity ($D=d^nAbs/d\lambda^n$) obtained from the absorbance values of the total amount of doxorubicin (D_T), D_m and D_w, the doxorubicin distributed in the lipid membrane phase and in the aqueous phase respectively. [L] represents the lipid concentration (in M) and V_m is the lipid molar volume, calculated from the weighted molecular mean and the reported volume of lipids, for the biomimetic membranes (DMPC:SM and DMPC:SM:Chol) were 0.682 and 0.633 $L.mol^{-1}$, respectively. The Kp values obtained are dimensionless and presented as the mean ± standard deviation calculated from at least three independent assays.

Drug Location Studies Evaluated by Fluorescence Quenching. Doxorubicin location within the model systems was assessed by fluorescence studies that included steady-state and time-resolved measurements, according to an already described protocol31. These experiments were conducted by incubation of the anticancer-drug with DPH and TMA-DPH labeled liposomes. The samples containing a fixed concentration of lipid (500 μM) and increasing concentrations of doxorubicin (0, 40 and 75 μM), were incubated at 37°C during 30 min in the dark. The fluorescence measurements were carried out at 37°C, at the excitation and emission wavelengths defined as 357 nm and 429 nm, and 359 nm and 429 nm, for DPH and TMA-DPH, respectively. Fluorescence steady-state measurements were performed in a Jasco FP-6500 spectrofluorometer (Jasco, Great Dunmow, UK) equipped with a constant temperature cell holder and all data were recorded in a 1 cm path length cuvette. For each measurement, the fluorescence emission was automatically acquired during 30 s. The fluorescence intensity values obtained were corrected for inner filter effects (quencher absorbance) at the excitation wavelength32. The fluorescence time-resolved measurements were performed using a Fluorolog Tau-3 Lifetime system. The modulation frequencies were acquired between 10 and 200 MHz, with an integration time of 10 s. The fluorescence emission was detected with a 90° scattering geometry. All measurements were made using Ludox as a reference standard (τ=0.00 ns).

Membrane Fluidity Studies Evaluated by Fluorescence Anisotropy. The doxorubicin's effect on the membrane microviscosity, in the different model membranes, was assessed by fluorescence anisotropy studies. Samples were prepared with a fixed concentration of lipid (500 μM), labeled with DPH or TMA-DPH, and increasing concentrations of doxorubicin (0, 40 and 75 μM), and incubated during 30 min at 37°C in the dark. The steady-state fluorescence anisotropy values (r_s) were recorded between 10 and 60°C with intervals of 2°C. The experiments were performed in a Jasco FP-6500 spectrofluorometer with polarizers inserted (excitation/emission wavelengths were set as described for each fluorescent probe). The results obtained are presented as the mean ± standard deviation calculated from at least three independent assays.

Molecular Dynamics (MD) simulations. All-atom molecular dynamics simulations of doxorubicin molecule were performed with 2 membrane bilayers, with the following lipid compositions, DMPC:SM [8:2] DMPC:SM:Chol [7:1.5:1.5]. For lipid molecules Slipids forcefield parameters were used[33-35]. For water molecules we used the TIP3P model compatible with the Slipids forcefield set. Periodic boundary conditions with minimum image convention were used in all directions. Covalent bond lengths were preserved through the linear constraint solver (LINCS) algorithm[36]. For all systems the temperature was controlled using the Nosé-Hoover thermostat with solvent and solute controlled independently[37,38]. Pressure was controlled using the Parrinello-Rahmann barostat with semi-isotropic control[39]. Lennard-Jones interactions were cut off at 1.0 nm and for the electrostatic interactions the particle mesh Ewald method (PME)[40] was used. For all simulations physiological pressure and temperature were used (1 Bar and 37 °C). The GROMACS open source software package, version 4.6.5, was used[41] for all Molecular Dynamics (MD) simulations, along with all quantitative analysis. Visualization of the trajectories was performed using the Visual Molecular Dynamics (VMD) package[42]. For doxorubicin, the partial charges were derived with RESP[43] procedure implemented in ANTECHAMBER[44]. All the simulation systems were simulated for 1.2 microsecond. The first 200 ns were considered as an equilibration phase, and hence all the analysis was performed on the latter 1 microsecond trajectories.

Statistical analysis. Statistical analysis was performed using IBM® SPSS® Statistics software (v.20.0.0.0, IBM, Armonk, NY, USA). The measurements were repeated at least three times and data was expressed as mean ± standard deviation (SD). Data was statistically analyzed through the one-way analysis of variance (ANOVA) method and differences between groups were compared by Bonferroni and Tukey post-hoc tests in which a p value lower than 0.05 ($p<0.05$) was considered statistically significant.

1. Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. & Gianni, L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56,** 185–229 (2004).
2. Triton, T. R. & Yee, G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* **217,** 248–250 (1982).
3. Tritton, T. R. & Hickman, J. A. in *Experimental and Clinical Progress in Cancer Chemotherapy* **24,** 81–131 (Springer US, 1985).
4. Escribá, P. V. *et al.* Membranes: a meeting point for lipids, proteins and therapies. *J. Cell. Mol. Med.* **12,** 829–875 (2008).
5. Escribá, P. V. *et al.* Role of lipid polymorphism in G protein-membrane interactions: nonlamellar-prone phospholipids and peripheral protein binding to membranes. *Proceedings of the National Academy of Sciences* **94,** 11375–11380 (1997).
6. Escribá, P. V., Sastre, M. & García-Sevilla, J. A. Disruption of cellular signaling pathways by daunomycin through destabilization of nonlamellar membrane structures. *Proceedings of the National Academy of Sciences* **92,** 7595–7599 (1995).
7. Giorgione, J., Epand, R. M., Buda, C. & Farkas, T. Role of phospholipids containing docosahexaenoyl chains in modulating the activity of protein kinase C. *Proceedings of the National Academy of Sciences* **92,** 9767–9770 (1995).
8. London, E. Insights into lipid raft structure and formation from experiments in model membranes. *Curr. Opin. Struct. Biol.* **12,** 480–486 (2002).
9. George, K. S. & Wu, S. Lipid raft: A floating island of death or survival. *Toxicol. Appl. Pharmacol.* **259,** 311–319 (2012).
10. Holthuis, J. C. M., van Meer, G. & Huitema, K. Lipid microdomains, lipid translocation and the organization of intracellular membrane transport
(Review). *Molecular Membrane Biology* **20,** 231–241 (2003).
11. Eckford, P. D. W. & Sharom, F. J. Interaction of the P-glycoprotein multidrug efflux pump with cholesterol: effects on ATPase activity, drug binding and transport. *Biochemistry* **47,** 13686–13698 (2008).
12. Broxterman, H. J., Giaccone, G. & Lankelma, J. Multidrug resistance proteins and other drug transport-related resistance to natural product agents. *Curr Opin Oncol* **7,** 532–540 (1995).
13. Gottesman, M. M., Fojo, T. & Bates, S. E. MULTIDRUG RESISTANCE IN CANCER: ROLE OF ATP-DEPENDENT TRANSPORTERS. *Nat. Rev. Cancer* **2,** 48–58 (2002).
14. Shen, F. *et al.* Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J. Pharmacol. Exp. Ther.* **324,** 95–102 (2008).
15. Seydel, J. K. & Wiese, M. *Drug-Membrane Interactions: Analysis*. (Drug Distribution, 2002).
16. Lakowicz, J. R. *Quenching of Fluorescence, in Principles of Fluorescence Spectroscopy.* (Springer, 2006).
17. Kaiser, R. D. & London, E. Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth. *Biochemistry* **37,** 8180–8190 (1998).
18. Illinger, D. *et al.* A comparison of the fluorescence properties of TMA-DPH as a probe for
plasma membrane and for endocytic membrane. *Biochim. Biophys. Acta* **1239,** 58–66 (1995).
19. Lakowicz, J. R. in 366–375 (Springer, 2006).
20. Lúcio, M., Ferreira, H., Lima, J. L. F. C. & Reis, S. Interactions between oxicams and membrane bilayers: an explanation for their different COX selectivity. *Med Chem* **2,** 447–456 (2006).
21. Nunes, C. *et al.* Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality. *J. Phys Chem. B* **115,** 12615–12623 (2011).
22. Barenholz, Y. Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications. *Subcell. Biochem.* **37,** 167–215 (2004).
23. Seelig, A. & Landwojtowicz, E. Structure-activity relationship of P-glycoprotein substrates and modifiers. *Eur. J. Pharm. Sci.* **12,** 31–40 (2000).
24. Orlowski, S., Martin, S. & Escargueil, A. P-glycoprotein and 'lipid rafts': some ambiguous mutual relationships (floating on them, building them or meeting them by chance?). *Cellular and Molecular Life Sciences* **63,** 1038–1059 (2006).
25. Modok, S., Heyward, C. & Callaghan, R. P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment. *J. Lipid Res.* **45,** 1910–1918 (2004).
26. Róg, T., Pasenkiewicz-Gierula, M., Vattulainen, I. & Karttunen, M. Ordering effects of cholesterol and its analogues. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788,** 97–121 (2009).
27. Wydro, P. L. Sphingomyelin/phosphatidylcholine/cholesterol monolayers -- analysis of the interactions in model membranes and brewster angle microscopy experiments. *Colloids Surf., B* **93,** 174–179 (2012).
28. Nunes, C., Lopes, D., Pinheiro, M., Pereira-Leite, C. & Reis, S. In vitro assessment of NSAIDs-membrane interactions: significance for pharmacological actions. *Pharm. Res.* **30,** 2097–2107 (2013).
29. Pereira-Leite, C., Nunes, C., Lima, J. L. F. C., Reis, S. & Lúcio, M. Interaction of celecoxib with membranes: the role of membrane biophysics on its therapeutic and toxic effects. *J. Phys Chem. B* **116,** 13608–13617 (2012).
30. Magalhães, L. M. *et al.* High-throughput microplate assay for the determination of drug partition coefficients. *Nat Protoc* **5,** 1823–1830 (2010).
31. Brittes, J., Lúcio, M., Nunes, C., Lima, J. L. F. C. & Reis, S. Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects. *Chem. Phys. Lipids* **163,** 747–754 (2010).
32. Coutinho, A. & Prieto, M. Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrylamide: A laboratory experiment for undergraduate students. *Journal of Chemical Education* **70,** 425 (1993).
33. Jämbeck, J. P. M. & Lyubartsev, A. P. An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J Chem Theory Comput* **8,** 2938–2948 (2012).
34. Jämbeck, J. P. M. & Lyubartsev, A. P. Another Piece of the Membrane Puzzle: Extending Slipids Further. *J Chem Theory Comput* **9,** 774–784 (2012).
35. Jämbeck, J. P. M. & Lyubartsev, A. P. Derivation and systematic validation of a refined all-atom force field for phosphatidylcholine lipids. *J. Phys Chem. B* **116,** 3164–3179 (2012).
36. Hess, B., Bekker, H. & Berendsen, H. LINCS: a linear constraint solver for molecular simulations. *Journal of computational …* (1997).
37. Nosé, S. A unified formulation of the constant temperature molecular dynamics methods. *The Journal of Chemical Physics* **81,** 511–519 (1984).
38. Hoover, W. G. Canonical dynamics: Equilibrium phase-space distributions. *Phys. Rev. A* **31,** 1695–1697 (1985).
39. Parrinello, M. & Rahman, A. Polymorphic transitions in single crystals: A new molecular dynamics method. *Journal of Applied Physics* **52,** 7182–7190 (1981).
40. Essmann, U. *et al.* A smooth particle mesh Ewald method. *The Journal of Chemical Physics* **103,** 8577–8593 (1995).
41. Pronk, S. *et al.* GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* **29,** 845–854 (2013).

42. Humphrey, W., Dalke, A. & Schulten, K. VMD: Visual molecular dynamics. *Journal of Molecular Graphics* **14,** 33–38 (1996).
43. Bayly, C. I., Cieplak, P., Cornell, W. & Kollman, P. A. A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys Chem.* **97,** 10269–10280 (2002).
44. Wang, J., Wang, W., Kollman, P. A. & Case, D. A. Automatic atom type and bond type perception in molecular mechanical calculations. *Journal of Molecular Graphics and Modelling* **25,** 247–260 (2006).

Supporting Information

Fluorophore quenching behaviour

The drug efficiency to quench the fluorophore is related to its proximity to the probe. In this sense, because the fluorophore is inserted in the membrane, only the drug that partitions into the lipid phase will be able to act as a quencher. In order to determine the doxorubicin quenching efficiency, it is important to calculate its membrane concentration ($[Q]_m$) that is given by the equation 1:

$$[Q]_m = \frac{K_p[Q]_T}{K_p\alpha_m + (1 - \alpha_m)} \quad (1)$$

where α_m is the volume fraction of membrane phase ($\alpha_m = V_m/V_T$; V_m and V_T represent the volumes of the membrane and water phase, respectively) and Q_T represents the total drug concentration used.

The ability of doxorubicin to quench DPH and TMA-DPH probes was analyzed according to the modified Stern-Volmer equation (2):

$$\frac{I_0}{I} = K_{SV}[Q]_m + 1 = \frac{\tau_0}{\tau} \quad (2)$$

where I and I_0 are the steady state fluorescence intensities with and without the quencher (doxorubicin), respectively; K_{SV} is the Stern-Volmer constant; ($[Q]_m$) is the doxorubicin membrane concentration; and τ_0 and τ are the fluorophore lifetime in the absence and presence of doxorubicin, respectively. The molecular contact between the probe (fluorophore) and the drug (quencher) can be due to different molecular interactions and can be observed by the decrease in the fluorescence intensity (fluorescence quenching). Furthermore, the quenching process can result from collisional encounters between the fluorophore and quencher, and is therefore called collisional or dynamic quenching, or can be due to complex formation, which is called static quenching.[31] In order to understand which type of quenching mechanism occurs, the dynamic quenching constant (K_D) was calculated from the slope of the Stern-Volmer plots obtained by lifetime fluorescence measurements (τ_o/τ) versus the doxorubicin membrane concentration ($[Q]_m$) (equation 3):[31]

$$\frac{\tau_0}{\tau} = K_D[Q]_m + 1 \quad (3)$$

The static quenching constant (K_S) was also determined from the following relation between the K_{SV} and K_D constants (equation 4):[31]

$$K_{SV} = K_S + K_D \quad (4)$$

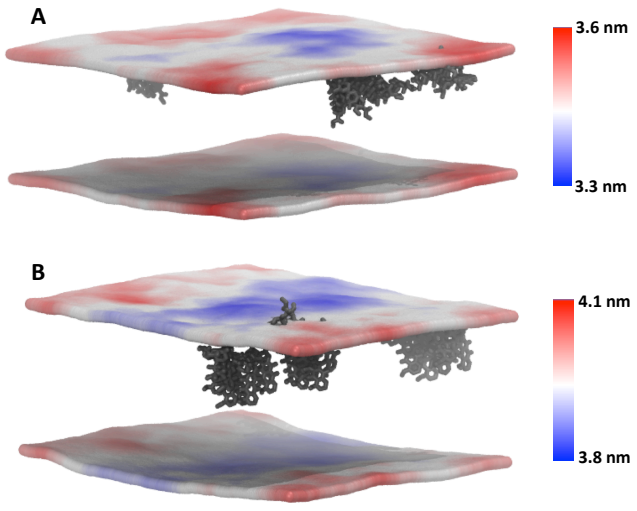


Fig. S1. Membrane thickness of (A) DMPC:SM and (B) DMPC:SM:Chol and average positioning of doxorubicin conformation sampled over 1 microsecond of simulation trajectory.

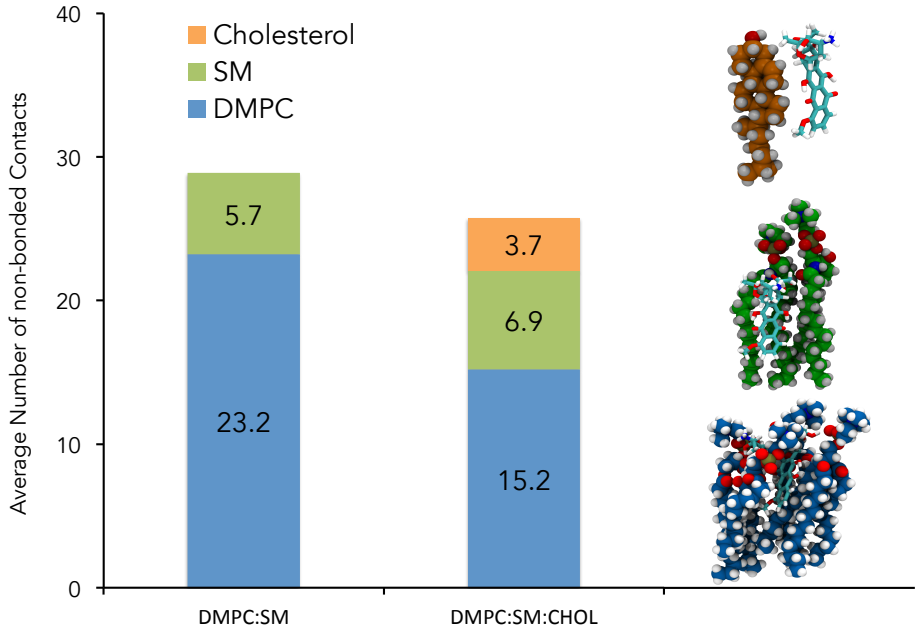


Fig. S2. Number of non-bonded contacts of doxorubicin with DMPC, SM and Chol. These atoms are involved in electrostatic and hydrophobic interactions with the doxorubicin molecule.

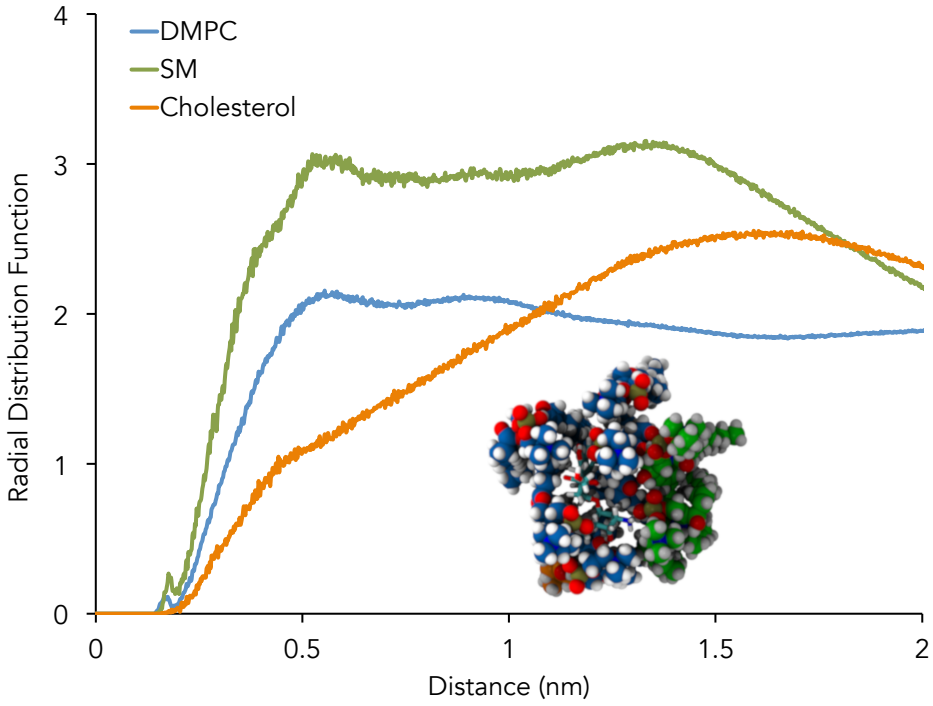
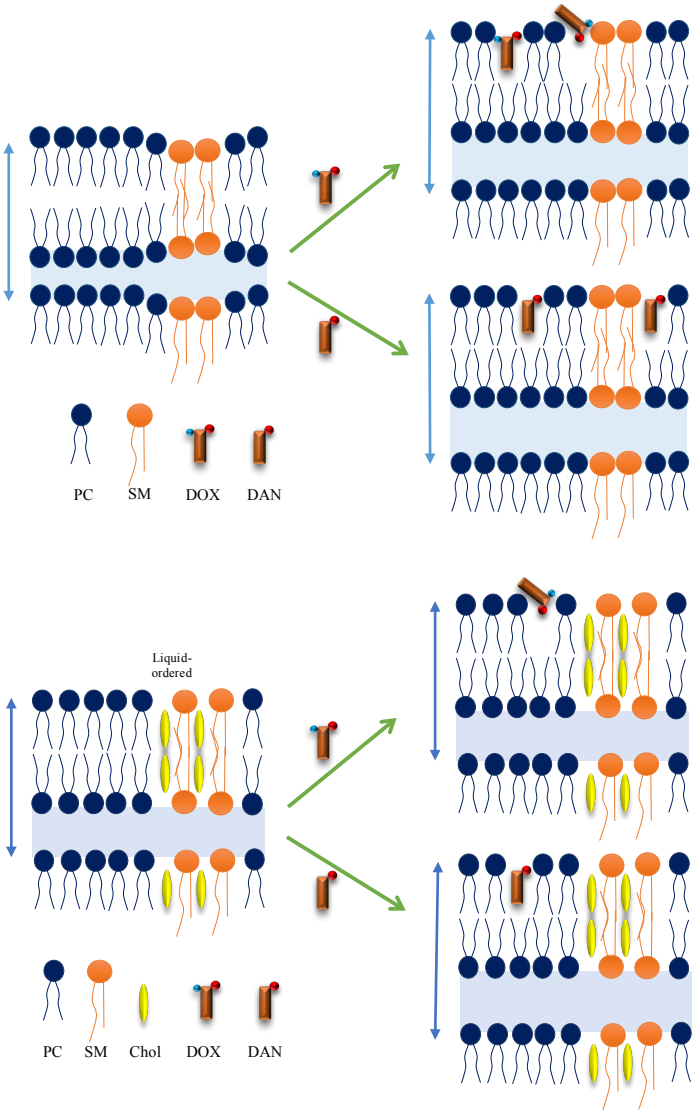


Fig. S3. Radial distribution function of doxorubicin Distance distribution of DMPC, SM and cholesterol from doxorubicin molecule. The plot shows the distance distribution of center of mass of doxorubicin molecule with DMPC, SM and cholesterol over 1 microsecond trajectory.

3. A synchrotron X-ray assessment regarding the nature of anthracyclines–lipid interactions



A synchrotron X-ray assessment regarding the nature of anthracyclines–lipid interactions

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The anthracyclines drug-membrane interactions play a crucial role in the molecules' bioavailability and the drug-induced toxicity process. In fact, the cell boundary is involved in several biological activities that lead to cell survival, differentiation and death. Thus, structural changes in the lipid phase induced by these drugs can disturb the membrane function and indirectly modulate membrane proteins. Structural information of DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] bilayers was accessed by small angle and wide angle X-ray measurements (SAXS and WAXS) that are powerful techniques to investigate the anthracycline drugs' influence on the long range bilayer order and the hydrocarbon chain packing, respectively.

At a physiological relevant temperature (37°C), DAN increases the bilayer and/or the water layer distance in both model membranes, however such effect was more pronounced in the membrane without Chol. On the other hand, DOX also caused an increased in the long-distances of DMPC:SM but in a less extend compared with DAN. The in-plane organization is also disturbed by both drugs, which confirms their high interaction with the studied model membranes. Nevertheless, DOX also demonstrate to induce a slight increase in the distance between the lipids head groups. This distinct behavior, in both model membranes, is probably related to the structural difference between the compounds, which modulates their location within the membrane and, for that reason, DAN is able to penetrate deeper while DOX remains more in the interface membrane-water.

Overall, the gathered data points to an anticancer drug's behavior that causes significant alteration in the membrane organization, to which extend depends not only on the chemical structure of the drug, but also on the bilayer' lipid composition and physical state.

SAXS | WAXS | anthracyclines | drug-membrane interactions

The anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DAN) are the most frequently prescribed anticancer agents (Fig. 1). These compounds display a lipophilic flat anthraquinone moiety coupled to an amino sugar and differ only by their side chain: the side chain of DOX ends with a primary alcohol, whereas that of DAN ends with a more lipophilic methyl. This small structural difference has relevant consequences on their spectrum of activity and pharmacokinetics (1). Thus, while DAN shows activity in acute lymphoblastic or myeloblastic leukemia's (2), DOX is used in the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas (1).

The most prevalent opinion is that their cytotoxic activity is mainly due to a direct interplay with nucleic acids, leading to DNA damage and inhibition of DNA synthesis. However, this still represents a matter of considerable debate, since there is also strong evidence suggesting that interactions with cell membranes plays an important role in their activity (3). For example, in 1982 Tritton *et al.* have demonstrated that anthracyclines, without entering the cell, could exert their cytotoxic effect only through their interplay with the membrane (4). Even considering that the main mechanism is the interaction with nucleic acids, in order to reach the target, anthracyclines must pass through a variety of other structures. First, the drugs must overcome the selective barrier to entry into the cell that is the plasma membrane and, finally, the nuclear membrane. For this reason, interaction with lipid membranes is an unavoidable step in the anticancer drugs activity, independently of the main mechanism of action.

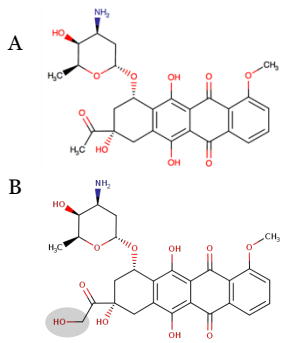


Fig. 1. Doxorubicin (A) and daunorubicin (B) chemical structure.

Drug-membrane interactions produce alterations in the physical properties of the cell membrane, as a consequence of the behavior of the drug within the membrane. How the drug orients in the membrane, which components of the lipid membrane the drug interacts with and what interactions within the membrane the drug disrupts, are all factors that alter the biophysical characteristics of the membrane (5). This can, in turn, lead to modifications in cell signaling and function of membrane proteins, such as transporters and ion channels (6). It has been demonstrated, for example, that DAN causes alteration of both G-proteins and protein kinase C-associated signaling pathways, through destabilizing the non-lamellar membrane structures that are involved in their location and activity (7, 8). Since these proteins participate in fundamental functions of the cell, including proliferation and differentiation, their modulation could be responsible for the antitumor activity of some drugs (7).

In light of all the aforementioned reasons, it becomes clear that understanding the interactions between cell membranes and anticancer drugs is a fundamental field of research due to the direct relationship with the drug's pharmacological activity. In fact, the role of the cell membrane extends beyond that of physical barrier and, consequently, the interplay of anticancer drugs with these structures are expected to influence the drug transport, activity and toxicity (3).

In this work, small- and wide-angle X-ray scattering (SAXS and WAXS), which measure the membrane thickness and the crystalline packing of lipids in the membrane plane, were performed to assess the influence of DOX and DAN on the structural organization of the membrane models at the molecular level. Multilamellar vesicles (MLVs) of different ratio of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were chosen to mimic the plasma membranes. Phosphatidylcholines are the most abundant phospholipids in the cell membrane along with two other categories of lipids: high melting temperature lipids, in particular SM, and Chol (9). Besides, SM is involved in several cellular functions (10) and Chol is one of the most important regulators of lipid organization (11), membrane fluidity and permeability (12) and is also known to be able to induce the formation of lipid domains (13). Furthermore, microdomains enriched in cholesterol and sphingomyelin have received a huge attention in the last past years, because they are thought to be involved in several cellular processes, including signal transduction, lipid trafficking and membrane protein activity (14). These domains exist in a liquid-ordered state (L_o), which is characterized by a tight acyl-chain packing and relatively extended acyl-chains (similar to the gel phase) but also by a high lateral mobility in the bilayer (similar to the liquid-disorder phase). In addition, evidence demonstrate that cholesterol interacts more favorably with both saturated sphingomyelins and phosphatidylcholines, compared to their unsaturated counterparts because unsaturation in the phospholipid acyl chain introduces kinks that limits the formation of attractive van der Waals forces with cholesterol. In fact, studies with model membrane systems have shown that a cholesterol-dependent lateral segregation occurs, wherein the planarity of the rigid sterol ring favors interaction with straighter, stiffer hydrocarbon chains of saturated lipids and disfavors interaction with the more bulky unsaturated lipid species (15).

In this context, the present work aims to simulate the L_o state by the use of MLVs containing saturated lipids (DMPC and SM) and cholesterol and to better understand, how the properties of this important lipid phase influences the anthracyclines-membrane interaction.

SIGNIFICANCE

Albeit the DNA has held the center stage as primary target, it has been demonstrated that biological membranes are also involved in the anthracyclines' mechanism of action. The efficiency of drugs to interplay with the membranes represents one of the most important pharmacological features and plays a key role in their biological action. Through the use of X-ray scattering measurements is was possible to demonstrate the influence of two anthracyclines, daunorubicin and doxorubicin, in model membranes composed of DMPC:SM and DMPC:SM:Chol. Results suggest that both chemical modification in the drug's structure, which has a significant impact in the structural organization of the bilayer models, and the lipid membrane composition contribute to the modulation of the drug-membrane interaction.

Results and Discussion

The structural effect of DAN and DOX on the order and packing of DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] bilayers at pH 7.4 was assessed by employing X-ray scattering techniques (SAXS and WAXS). SAXS measurements allow one to obtain information regarding the distances between the thickness of the bilayer plus the water layer and, thus, on the long range bilayer organization, while WAXS measurements permits to assess information about the packing of the lipid acyl chains, providing information about the short range order of the system (16). The experiments were performed at 10°C, 37° and 50°C in order to study the drugs effect on the different lipid phases, once the order and packing of lipids are temperature-dependent (17).

Anthracyclines influence on the structure of DMPC:SM bilayers

The small angle X-ray diffraction patterns of DMPC:SM in Hepes buffer (pH 7.4) and in Hepes buffered solutions containing 10 mol% of anthracyclines (DAN or DOX) at a physiological relevant temperature (37ºC), along with the schematic representation of the drugs' proposed effect in membrane model structure, are presented in Figure 2. The repeat distance, d, deduced from the SAXS and WAXS patterns of DMPC:SM as well as the respective correlation length between the bilayers (ξ) of the first-order Bragg peaks for all temperatures studied are depicted in Table 1 and 2, respectively.

The presence of 20 mol% SM in DMPC bilayers led to a lipid phase separation between the two components at 10ºC, as demonstrated by the low-angle diffraction pattern that contained reflections with two main different lamellar repeated distances. The observed lipid immiscibility for lower temperatures may be the result of phase separation of molecular species with different acyl chain lengths (18). Accordingly, while DMPC contains only saturated acyl chains with 14C, milk SM comprises a mixture with several acyl chain lengths species (C16:0, C22:0, C23:0, C24:0 >80% of milk-SM fatty acids).

Analysing the obtained SAXS results at 10 ºC, it is possible to verify that each main d-spacing (66 Å and 76 Å) corresponds to the repeat periods observed for separate samples in excess water of DMPC and SM, respectively. In fact, it has been described in the literature that the first order Bragg peaks of DMPC appear at 61.3 Å ($L\beta$), 66.8 Å ($P\beta'$) and 62.6 Å ($L\alpha$) (19). Confronting the gathered outcomes with the literature data, it is possible to observe that probably DMPC was still in the ripple phase at 10ºC (66 Å) but where both untilted and tilted gel phases coexist, as evidenced by the appearance of a shoulder at 62.9 Å in the peak diffraction. This probably happened because the incubation time at low temperature was too short and did not allow the transition to the gel phase that is characteristics at this temperature (20). This observation is consistent with Tristram-Nagle et al. work, which demonstrated that the pre-transition from the gel to the ripple phase of DMPC occurs at 13ºC (21), and with Lewis et al., which proposed that with temperature increase the DMPC crystalline phase (Lc) once formed, directly converts into the ripple phase and that this pre-transition is not reversible, allowing to explain the obtained mesophase structure of DMPC in the complex lipid system containing SM. Furthermore, according to

Table 1: Long-range distances (d) and correlation lengths (ξ) of DMPC:SM in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C, 37°C and 45°C and pH 7.4.

[Drug]	T	Long distances (Å)							
(mol%)	(°C)	d_1	d_2	d_3	d_4	ξ_1	ξ_2	ξ_3	ξ_4
0	10	62.9 ± 0.5	65.8 ± 0.5	72.4 ± 0.5	76.0 ± 0.5	8066 ± 10	8333 ± 10	6579 ± 10	3841 ± 10
	37	55.6 ± 0.5	57.1 ± 0.5	59.7 ± 0.5	64.0 ± 0.5	5800 ± 10	9860 ± 10	4970 ± 10	11470 ± 10
	50	54.3 ± 0.5	56.3 ± 0.5	59.7 ± 0.5	64.4 ± 0.5	10630 ± 10	8810 ± 10	2720 ± 10	12270 ± 10
DAN 10	10	71.6 ± 0.5	--	--	--	4180 ± 10	--	--	--
	37	73.5 ± 0.5	--	--	--	1260 ± 10	--	--	--
	50	73.0 ± 0.5	--	--	--	730 ± 10	--	--	--
DAN 20	10	70.6 ± 0.5	--	--	--	3600 ± 10	--	--	--
	37	91.9 ± 0.5	--	--	--	2970 ± 10	--	--	--
	50	90.2 ± 0.5	--	--	--	980 ± 10	--	--	--
DOX 10	10	75.6 ± 0.5	--	--	--	1590 ± 10	--	--	--
	37	65.2 ± 0.5	--	--	--	3430 ± 10	--	--	--
	50	66.5 ± 0.5	--	--	--	2554 ± 10	--	--	--
DOX 20	10	74.7 ± 0.5	--	--	--	939 ± 10	--	--	--
	37	66.9 ± 0.5	--	--	--	931 ± 10	--	--	--
	50	68.7 ± 0.5	--	--	--	872 ± 10	--	--	--

Shaw et al. (22) for lower temperatures, milk SM presents a layer spacing of 75 and 72 Å, corresponding to the untilted and tilted gel phases, respectively, that is once more consistent with the obtained results in this work. As suggested by the authors, this complex behavior indicates that there is significant chain interdigitation in SM, probably favored by the molecular heterogeneity in the acyl chain length of milk-SM species that leads to different packing states in the gel phase (23).

Table 2: Short-range distances (d) and correlation lengths (ξ) of DMPC:SM in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C and 37°C and pH 7.4.

[Drug]	T	Short distances (Å)	
(mol%)	(°C)	*d*	ξ
0	10	4.17 ± 0.05	520 ± 10
	37	4.43 ± 0.05	170 ± 10
DAN 10	10	4.17 ± 0.05	530 ± 10
	37	4.46 ± 0.05	260 ± 10
DAN 20	10	4.16 ± 0.05	510 ± 10
	37	4.43 ± 0.05	250 ± 10
DOX 10	10	4.17 ± 0.05	440 ± 10
	37	4.45 ± 0.05	210 ± 10
DOX 20	10	4.16 ± 0.05	480 ± 10
	37	4.52 ± 0.05	140 ± 10

Concerning the WAXS diffraction patterns, it has been shown that the DMPC ripple phase is characterized by a hexagonal lattice of the acyl chain. The same occurs for milk SM, where below the Tm (35ºC), the diffraction peak is significantly sharper and centered around 4.15 Å (23). The abovementioned information is in good agreement with our results, since a single peak at wide angles (d=4.17 Å), characteristic of an hexagonal packing was observed for the lipid mixture.

WAXS profile shows a broad diffraction peak centered on 4.43 Å characteristic of a fluid lamellar (Lα) phase, which is in good agreement with the literature data obtained for both lipid components. In fact, at temperatures above the Tm, each lipid adopts a fluid lamellar phase. Therefore, the mix bilayer presents an intermediate d-value (ca. 64 Å) to that obtained for each of the individual lipids (ca. 62 Å for DMPC (19) and ca. 66 Å for milk SM (24)). Nevertheless, several shoulders can be observed in the main diffraction peak, which might be attributed to the heterogeneous milk SM composition regarding the length of the fatty acid chains. Furthermore, the decrease in the DMPC:SM bilayers thickness, from a more ordered towards a more disorder phase upon heating, can be explained by the variation in the water layer thickness between the bilayers and also by the difference in the melting of the SM' fatty acid chains (24).

The addition of DAN and DOX to the DMPC:SM model system led to some changes in the X-ray diffraction patterns. The ratio of d-spacing between two consecutive orders of Bragg peaks, clearly indicate a lamellar structure as a result of the interaction with both the anticancer drugs. The first consideration obtained from WAXS profiles is that that the compounds did not change the in-plane molecular organization at 10ºC, but for higher temperatures (37ºC) 20 mol% of DOX induced a slight increase in the distance between the lipids chains.

At the same time SAXS results demonstrate that the lipid phase separation between DMPC and SM, observed at 10ºC, disappears in the presence of both anticancer drugs, which convert the four first-order Bragg peaks into only one diffraction peak. These results clearly indicated that the drug molecules penetrated into the phospholipid bilayer structure and led to a more homogeneous lipid distribution, evidencing the high interaction of these compounds with the studied bilayers. Furthermore, the disturbing anthracyclines' effect on the membrane structure was also confirmed by the decrease in the correlation length of the DMPC:SM bilayer and also by the broader peaks obtained in SAXS diffraction patterns. This behavior can be attributed to both the loss of the tilt angle in the lipids and increase in the water layer, since the molecular organization characteristic of a hexagonal phase was not influenced. In this lipid phase, it is possible to verify that DOX had a more

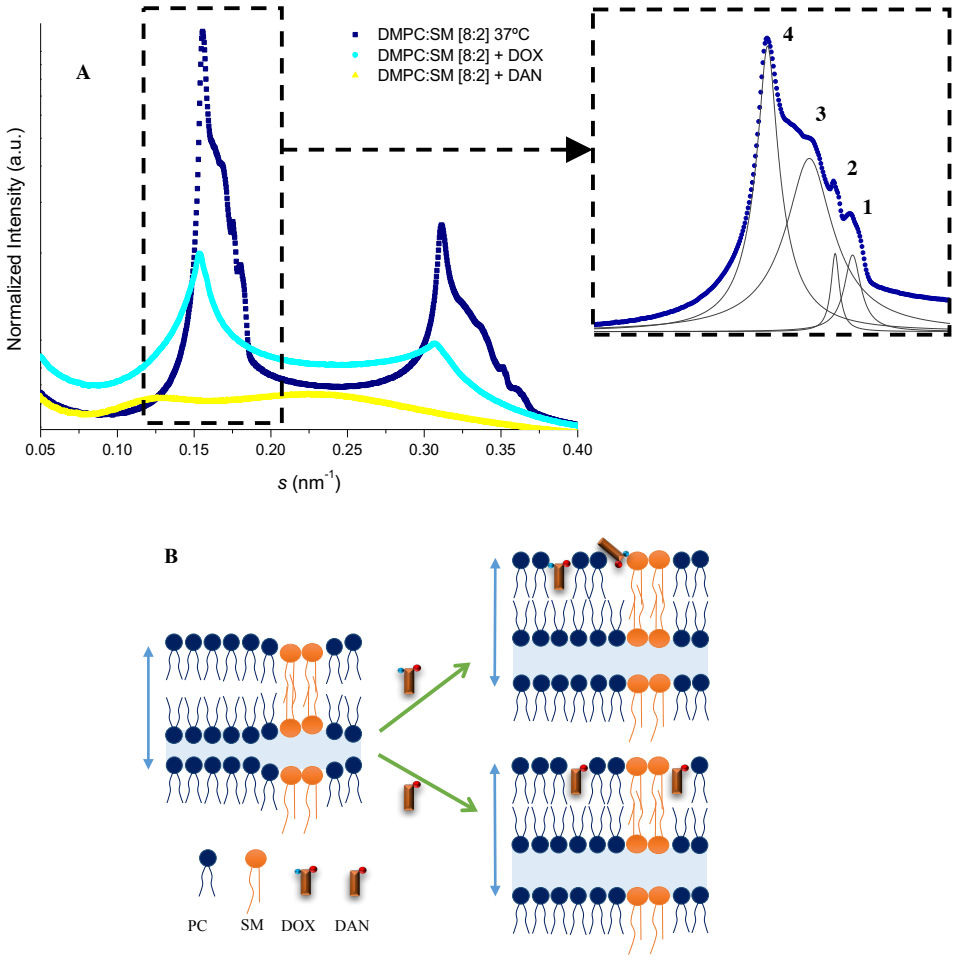


Fig. 2. (A) Small-angle X-ray diffraction profiles of DMPC:SM [8:2] with 10 mol% DAN or DOX at 37ºC. (B) Schematic representation of the proposed anthracyclines' effect in the membrane model structure.

pronounced impact in increasing the long-range distances of the bilayer when compared to DAN.

Additionally, Table 1 reveals that anthracyclines had different effects on the order of the DMPC:SM bilayer for higher temperatures (37 and 50ºC). The perturbing effect of these drugs was confirmed by the significant decrease in the correlation length of the bilayers and the presence of broader peaks in SAXS diffraction patterns. Although both anticancer drugs induce an increase in the d-spacing values as a function of concentration at both 37 and 50ºC, DAN had a much more pronounced effect in the DMPC:SM membrane. The distinct influence is probably associated to the different drugs' location within the membrane. In fact, the pKa value, determined using Marvin sketch calculator software, predict that nearly 80% of both anticancer drug molecules are in the cationic form at pH 7.4, which means that the drug-membrane interactions are be driven by electrostatic interaction with the polar headgroups of the phospholipids. Nonetheless, these compounds are amphiphilic and penetration within the bilayer is also expected to occur, and the drug's dihydroanthraquinone residue may interplay with the lipid fatty acid chains through hydrophobic interactions.

Despite anthracyclines molecules are able to interact with the bilayer lipids by electrostatic and hydrophobic forces, DOX presents an extra OH group that might change its membrane orientation and location when compared with DAN and, thus, have a distinct impact in the bilayer' molecular organization. Therefore, one possible hypothesis for the higher d-values obtained in the presence of DAN is that these molecules penetrate deeper into the membrane, which lead to a higher increase in the bilayer thickness and water layer. On the other hand, DOX might adopt a more superficial position in the bilayer, anchored near the head region, where the influence has a smaller impact in the membrane long-distances. In fact, WAXS corroborate this proposed explanation, since higher distances between the head of lipids were seen in the presence of 20 mol% DOX, especially for higher temperatures.

Anthracyclines influence on the structure of DMPC:SM:Chol bilayers

The SAXS diffraction patterns of DMPC:SM:Chol in Hepes buffer (pH 7.4) and in Hepes buffered solutions containing 10 mol% of anthracyclines (DAN or DOX) at a physiological relevant temperature (37ºC), along with the schematic representation of the drugs' proposed effect in membrane model structure, are presented in Figure 3. Table 3 displays the long- and short-range repeat distances (d) and the respective correlation length

Table 3: Long-range and short-range distances (d) and correlation lengths (ξ) of DMPC:SM:Chol in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C, 37°C and 50°C and pH 7.4.

[Drug]	T	Long distances (Å)				Short distances (Å)	
(mol%)	(°C)	d1	d 2	ξ1	ξ2	d	ξ
0	10	69.3 ± 0.5	--	8130 ± 10	--	4.27 ± 0.05	210 ± 10
	37	64.7 ± 0.5	--	8330 ± 10	--	4.53 ± 0.05	190 ± 10
	50	62.9 ± 0.5	--	6810 ± 10	--	4.60 ± 0.05	140 ± 10
DAN 10	10	68.9 ± 0.5	--	4980 ± 10	--	4.26 ± 0.05	190 ± 10
	37	66.5 ± 0.5	--	8080 ± 10	--	4.54 ± 0.05	150 ± 10
	50	63.7 ± 0.5	76.1 ± 0.5	4940 ± 10	1510 ± 10	4.61 ± 0.05	140 ± 10
DAN 20	10	69.7 ± 0.5	--	5090 ± 10	--	4.30 ± 0.05	200 ± 10
	37	66.6 ± 0.5	--	6600 ± 10	--	4.56 ± 0.05	150 ± 10
	50	62.8 ± 0.5	87.1 ± 0.5	5690 ± 10	2160 ± 10	4.59 ± 0.05	130 ± 10
DOX 10	10	69.9 ± 0.5	--	4430 ± 10	--	4.31 ± 0.05	200 ± 10
	37	64.8 ± 0.5	--	7580 ± 10	--	4.57 ± 0.05	160 ± 10
	50	64.5 ± 0.5	--	6790 ± 10	--	4.67 ± 0.05	130 ± 10
DOX 20	10	69.7 ± 0.5	95.1 ± 0.5	4510 ± 10	6410 ± 10	4.25 ± 0.05	200 ± 10
	37	64.4 ± 0.5	83.8 ± 0.5	6090 ± 10	1050 ± 10	4.58 ± 0.05	150 ± 10
	50	63.1 ± 0.5	--	5550 ± 10	--	4.66 ± 0.05	140 ± 10

(ξ) of each first-order Bragg peak at all the investigated temperatures.

The Bragg reflection peaks on the scattering curves for DMPC:SM:Chol appear at 1/69.3, 1/64.7, 1/62.9 Å-1. The two series of reflections in SAXS results reveal the presence of lamellar structures at all temperatures studied. However, the addition of 15 mol% cholesterol induced a structural reorganization of DMPC:SM bilayer below the phase transition temperature. In fact, this lipid mixture is characterized by a SAXS diffraction pattern with only one narrow peak. Therefore, in the presence Chol, the lipid phase separation previous observed disappears and the system becomes homogeneous and the lipids uniformly distributed, where d1 value corresponds to the thickness of the DMPC:SM:Chol bilayer plus the water layer. In fact, Kahya et al. demonstrated that cholesterol has poor ability in sorting short-chain saturated glycerophospholipids (DMPC) and SM (25) and, thereby, it is distributed similarly by the whole membrane.

Cholesterol has an important role in the cell membrane. The modification of membrane lipid fluidity induced by this molecule is thought to reduce the difference between fluid and gel states of the phospholipid acyl chains, which may be expected to favor the mixing of membrane lipids at intermediate temperatures (26). Furthermore, according to the umbrella model, the small polar head group of Chol associates with large-head group lipids (such as PC or SM), in order to protect its bulky hydrophobic body (27), where, beneath the umbrella, the acyl chains and the sterol rings become tightly packed. In addition, Chol interacts preferentially with phospholipids containing saturated chains rather than those with unsaturated chains, because the later poorly pack with Chol and, consequently, confer a poor coverage of the sterol molecules (28).

In this work, the studied model comprises lipids with saturated chains (DMPC and SM) able to provide full coverage to the bulky hydrophobic body of Chol. For that reason, the sterol molecules are distributed along the bilayer, interacting with both DMPC and SM and inducing an homogenizing effect that is evidenced by the SAXS narrow peak.

Besides contributing to the maintenance of the membrane homeostasis, Chol is also thought to interact specifically with high melting temperature sphingolipids (26) and to promote the formation of liquid-ordered (Lo) lamellar phase. This assumption can help to clarify the obtained results, which demonstrate that the WAXS peak associated with the gel phase (seen in the DMPC:SM bilayer) progressively broadened and decreased in intensity in the presence of Chol. Although the d-spacing (4.27 Å) was very similar to the one usually seen for the gel phase (4.2 Å), the latter is much sharper because it arises from an ordered packing of the hydrocarbon chains into a two-dimensional hexagonal lattice. This can be interpreted as a Lo phase (29), an intermediate phase between the gel and fluid phase, induced by Chol. In fact, evidence show that the association of SM and Chol in complex mixtures of membrane lipids creates domains of Lo phase, characterized by a single peak as detected by both SAXS and WAXS (30).

It is also possible that the incorporation of Chol into DMPC:SM bilayers led to a decrease in the angle tilt of the acyl chains and, therefore, a reorientation of the lipids' hydrocarbon chains so they become fully extended in the Lo phase. In fact, appearance of a reflection at 4.27 Å arises from the fully extended acyl chains organized with rotational disorder in a hexagonal lattice. This is good agreement with previous studies, where it has been shown that for DMPCs located near Chol molecules the number of the chain tilt is decreased (31). Since Chol interacts with both DMPC and SM, the same assumption can be made regarding the sterol effect in reducing the SM chain tilt. This hypothesis can also help to explain the elimination of the phase separation between DMPC and SM observed at 10ºC.

The disordering of the hydrocarbon chains of the DMPC:SM:Chol bilayer is associated with a progressive decrease in d-spacing of the lamellar repeat that reaches a value of 62.9 Å at 50ºC. Furthermore, the wide-angle peak shifts to higher values (4.53 at 37ºC and 4.60 Å at 50°C) and becomes even broader, as the temperature

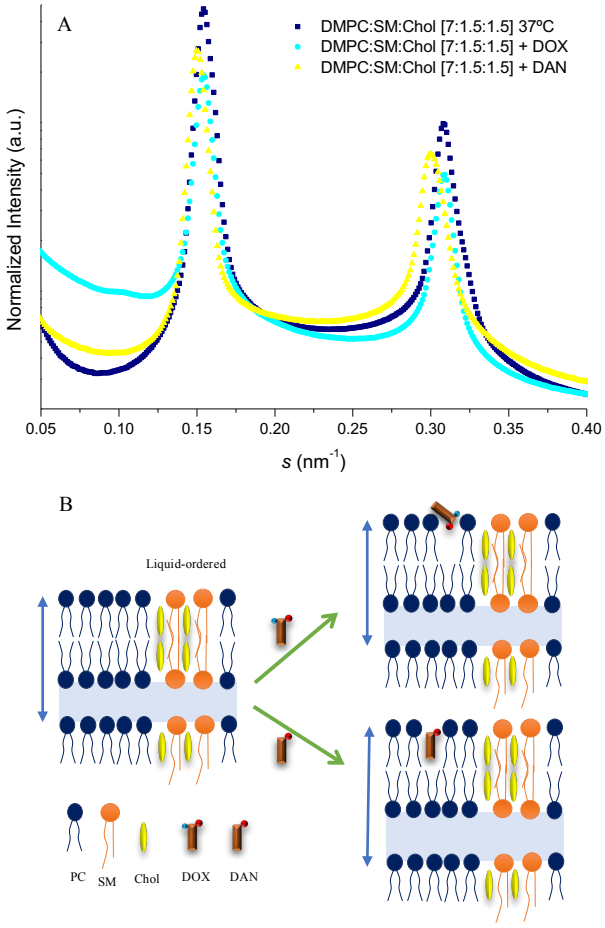


Fig. 3. (A) Small-angle X-ray diffraction profiles of DMPC:SM:Chol [7:1.5:1.5] with 10 mol% DAN or DOX at 37ºC. (B) Schematic representation of the proposed anthracyclines' effect in the membrane model structure.

increases, indicating that the in-plane packing of the molecules within the bilayer is disordered, as in the fluid lamellar Lα phase. This also shows that there is a large increase in lateral intermolecular spacing with temperature. Furthermore, when comparing with the previous model membrane, it is also possible to conclude that the presence of cholesterol led to an increase in the distances between the phospholipids head groups, as evidenced by the higher values obtained for the DMPC:SM:Chol model membrane for all investigated temperatures.

The addition of DAN and DOX to the DMPC:SM:Chol model system causes alterations in both SAXS and WAXS diffraction patterns, depending on the chemical structure and concentration of the anthracycline drug studied. From the gathered data, it is possible to conclude that the presence of anthracyclines in the bilayers greatly reduced the main peak correlation length at all temperatures, which indicates that both drugs have a disturbing effect on the membrane structure. Nevertheless, the drugs' action on the order and packing is quite different between them but also depending on the physical state of the membrane.

At 10ºC, the main effect of DAN at both concentrations was the decrease of cooperativity, as evidenced by the smaller values of ξ and by the broader peaks obtained in SAXS and WAXS diffraction patterns. The same was observed for 10 mol% of DOX. In contrast, the presence of 20 mol% DOX led to a deconvolution of the first order Bragg peak due to the existence of an asymmetric Bragg peak that indicates a lipid phase separation. Therefore, a non-influenced DMPC:SM:Chol phase (the d1 values are not significantly altered comparing with the bulk bilayer) and a mixed DMPC:SM:Chol phase with higher d2 values could be observed. This means that the anticancer drug was not homogeneous distributed in the studied membrane and, probably, formed lipid domains composed of DOX. In addition, by analyzing the WAXS diffraction patterns, the bilayers chain packing is greatly influenced by both drugs, as demonstrated by the broader peaks. Nevertheless, the characteristic hexagonal chain packing is maintained in the presence of anthracyclines, even though the peaks are less sharp and intense.

At 37ºC, the addition of DAN caused an increase in the bilayer thickness and/or water layer in a concentration dependent manner, as indicated by the higher d-spacing values. However, with further temperature increase (50ºC), it is possible to verify the existence of an asymmetric Bragg peak indicating a lipid phase separation in both drug concentrations tested. Thus, a non-influenced DMPC:SM:Chol phase (the d1 values are not significantly altered comparing with the bulk bilayer) and a mixed DMPC:SM:Chol phase with higher

d2 values can be observed. It is also interesting to observe that the addition of two time more DAN, the difference in the distances between the two phases was also two times higher. One possible explanation for this DAN effect is that, as the lipid molecules become more fluid and, therefore, with more space between them, the drug concentration in the bilayer increases. As a consequence, the compound ceases to be homogeneous distributed in the membrane and forms domains together with other lipids. Furthermore, WAXS diffraction patterns show that the d-spacing is not influenced by DAN, probably due to the planar structure of anthracyclines and the adopted orientation within the membrane, positioning itself between the lipids closer to the acyl chains. Nevertheless, the decrease in the correlation length between the bilayers supports its high disturbance influence of DAN in the DMPC:SM:Chol bilayer. The opposite can be observed with DOX, where the formation of the lipid phase separation disappears with the temperature increase. Therefore, despite the presence of 10 mol% DOX did not significantly altered the X-ray diffractions, increasing DOX concentration (20 mol%) led to a lipid phase separation, where a non-influenced DMPC:SM:Chol phase (the d1 values are not significantly altered comparing with the bulk bilayer) and a mixed DMPC:SM:Chol phase with higher d2 values can be observed at 37ºC.

Nevertheless, the phase separation was less pronounced than the one observed at 10ºC. Upon heating to 50ºC, the molecules of DOX present at the interface are now able to penetrate the membrane, interplay with both head and acyl chains regions and causing an increase in the bilayer thickness and/or water layer in a concentration dependent manner, as indicated by the higher d-spacing values. The structural ability of DOX to penetrate the lipid bilayer in more fluid a membrane, i.e. with more space for the drug molecules, causes a decrease in the effective area requirement of the headgroups by changing the hydration behavior and/or their orientation. Besides, the fact that the wide-angle peak is broad indicates that the in-plane packing of the molecules within the bilayer is disordered, as in the fluid lamellar Lα phase. Finally, the decrease in the correlation length between the bilayers supports a high membrane disturbance effect caused by DOX.

Overall, the incorporation of anthracyclines in the DMPC:SM:Chol bilayers led to an alteration of the bilayer and/or water layer thickness depending on the membrane lipid phase and drug studied. Nevertheless, the two series of reflections in SAXS results reveal that the anthracyclines did not disrupted the lamellar structures at all temperatures studied. Both anticancer drugs induced a decrease in the cooperativity as indicated by the broader peaks obtained in X-ray diffraction patterns, which reveal a disturbing effect of DAN and DOX. Nonetheless, the results point towards a different drug interplay with the DMPC:SM:Chol probably due to a distinct drug position and orientation within the membrane. In fact, the obtained difference in SAXS and WAXS results can be explained by the anthracyclines' structural features. Therefore, DOX probably position itself in a more interfacial region and, for that reason, at all studied temperatures, the effect in the lipid packing was more pronounced for DOX than DAN, as demonstrated by the appearance of significantly less intense and broader peaks in the WAXS diffraction patterns. In this scenario, two situations can occur: one involving the amino-sugar moiety, where the bulky ring remained outside the bilayer, in the aqueous medium and the second, tough in a smaller extent, involving hydrophobic interaction arising from the intercalation of the bulky ring between the lipid acyl chains within the bilayer. When the temperature is low (10ºC) the interactions forces are too high between the lipids constituents in the liquid-ordered phase, which may prevent a higher drug penetration and, consequently, a lipid phase separation occurs due to the drug molecules present in the water layer. Since the molecule is positively charged at pH 7.4, probably only electrostatic interactions with the lipids polar regions can take place. As the temperature increases, the membrane becomes more disordered and there is more space between the lipids in which the compound can be located. Furthermore, the lipid phase separation becomes less pronounced until one point that ceases to exist, as can be seen for 50ºC, and the bilayer becomes more homogenous (a similar state to the one observed for DAN at 37ºC). In this scenario both electrostatic and hydrophobic interactions can occur between the compound and the membrane lipids, which probably leads to a reduction in the area requirement of the headgroups and, thus, to an increase of the bilayer thickness, due to the increase penetration in the bilayer of DOX molecules that were at the interface. In fact, a slightly increase between the lipids' heads distance occurs at 50ºC, because the drug molecules act as spacers between the bilayer components.

In contrast, DAN penetrates deeper towards the acyl chains even at lower temperatures and, for that reason its influence is restricted to the hydrophobic part of the bilayer although in a smaller extent. Upon heating, the drug molecules start to accumulate in the membrane, increasing the long-range distance; and when the temperature is too high, the drug-concentration becomes even higher so that it forms domains, regions enriched in DAN.

Conclusions

Several reports increasingly point to the involvement of cell membranes in the cytotoxic mechanism of action of anthracyclines, which apparently modulate membrane properties and functions. Our results clearly indicate that DAN and DOX interact with membranes by incorporation into lipid bilayers. It appears that two types of interactions coexist and are responsible for the distribution of anthracyclines at the membrane level: electrostatic interactions between the positive group of the anticancer drug and the negative pole of the phospholipid head group and hydrophobic interactions between the drug's dihydroanthraquinone residue and the lipid fatty acid chains. Nevertheless, the presence of the hydroxyl group in DOX might be responsible for a distinct drug's influence on the structural organization of membranes when compared to DAN. In this context, the deeper penetration of DAN causes a higher increase in the bilayer plus the water layer distances, as observed in both DMPC:SM and DMPC:SM:Chol membranes. On the other hand, the presence of DOX in a more superficial region might be responsible for the higher impact in the in-plane structural organization, as demonstrated by the appearance of broader peaks in the WAXS diffraction patterns.

Furthermore, the obtained data also point to a differential interaction depending on the lipid composition. In fact, it was possible to observe that the presence of cholesterol, besides altering the bilayer' structural organization it also modulates how anthracyclines interplay with the membranes. On the other hand, the different lipid state, mimic here by the use of several temperatures, was also a determinant factor in the anthracyclines-membrane interactions. The gathered results demonstrate that the drug's influence on the membranes' order and packing was more pronounced for higher temperatures, i.e. when the bilayer was more fluid probably because anthracyclines penetration is easier at this physical state.

The present work provides a motivation for further studies involving more complex models with other lipids to better understand the anthracyclines-membrane interactions. The knowledge acquired can help the development of more effective and less toxic anthracyclines. In fact, drug-membrane interactions cannot be overlooked during drug design and synthesis.

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Materials and Methods

Materials and reagents. Daunorubicin (DAN) was obtained from Biovision (Milpitas, California) and doxorubicin (DOX) from LC Laboratories (Woburn, Massachusetts, USA). The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). All compounds were used without further purification. Drug solutions were prepared with HEPES buffer (10 mM, pH 7.4). The buffer was prepared with double deionized water (conductivity inferior to 10 μScm−1) and ionic strength adjusted to mimic physiological conditions with NaCl (I=10 M).

Multilamellar vesicles (MLVs) preparation. The several molar fractions of doxorubicin (DOX) and daunorubicin (DAN) (0, 10 and 20 mol%) were mixed with DMPC:SM [8:2] or DMPC:SM:Chol [7:1.5:1.5] in a chloroform/methanol mixture (3:1 v/v). Lipid films were formed by drying the samples under nitrogen stream and left overnight under reduced pressure to remove all traces of the organic solvents. After that, the lipid films were hydrated with Hepes buffer (pH 7.4) and vortexed, at 60°C (well above the main phase transition temperature of the lipid mixtures). The lipid dispersions were transferred into glass capillaries of 1.5 mm diameter and the flame-sealed capillaries were stored at 4°C until the X-ray measurements.

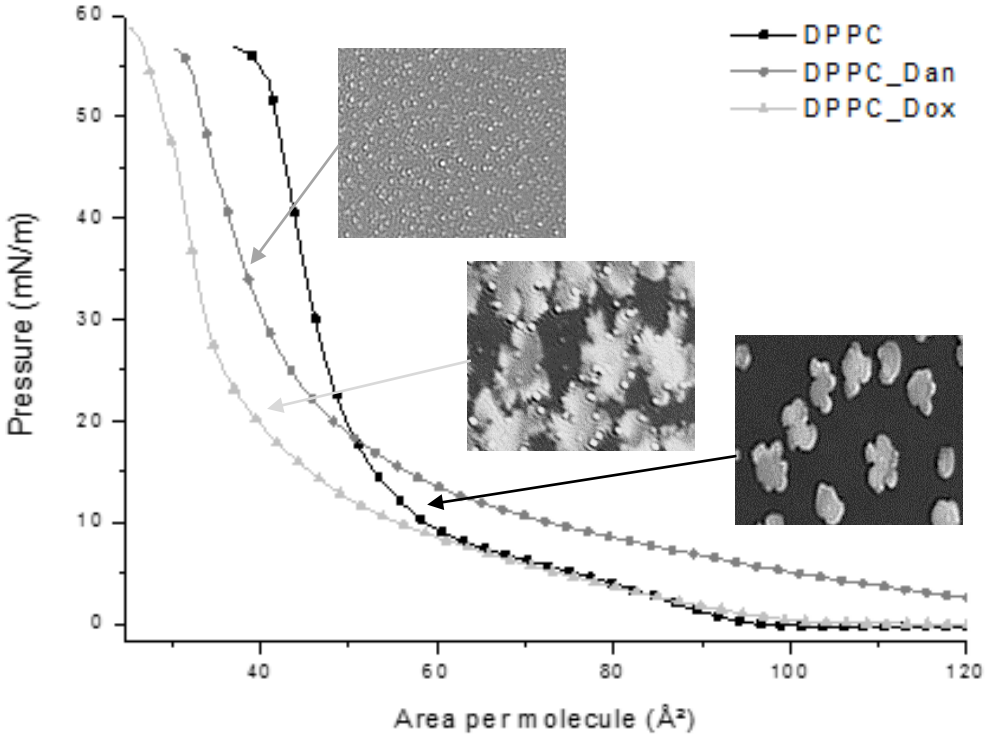
X-ray scattering measurements. The assessment of the long-range membrane order and the hydrocarbon chain packing by X-ray scattering measurements were performed as described elsewhere (32, 33). Small-angle and wide-angle X-ray scattering (SAXS and WAXS) experiments were done at the Austrian SAXS beamline in the electron storage ring Elettra (Trieste, Italy) (34). A monochromatic radiation with a wavelength of 1054 nm was used and the SAXS and WAXS detectors Pilatus (Dectris, Baden Switzerland) were calibrated with silver behenate powder (*d*-spacing =58.376 Å) (35) and with p-bromo benzoic acid with *d*-spacing taken from Ohkura *et al*. (36), respectively. Measurements were performed at several temperatures (10°C, 37°C and 50°C) using a custom-made sample cell with a water bath Unistat (Huber, Offenburg, Germany) for cooling. Each diffraction pattern is presented as normalize scattering intensity in arbitrary units (a.u.) in function of the reciprocal spacing in nm^{-1}, s ($s = (2 \sin \theta) / \lambda$, where θ is the diffraction angle and λ is the X-ray wavelength). The obtained X-ray diffraction patterns was then subjected to detailed analysis using a Peak Fitting tool of OriginPro 8.5.1 Software (OriginLab Corporation), where each Bragg peak was fitted with Lorentzians. The positions of maximum intensities (s) were determined and used to calculate the repeat distances – the lipid bilayer plus the water layer distances, d ($d = 1/s$), while the widths at half maximum (w) were used to calculate the correlation lengths between the lipid bilayers, ξ ($\xi = 2\pi/w$).

1. Weiss, R. B., The anthracyclines: will we ever find a better doxorubicin? *Semin Oncol* **1992,** *19* (6), 670-86.
2. Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L., Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* **2004,** *56* (2), 185-229.
3. Tritton, T. R.; Hickman, J. A., Cell Surface Membranes as a Chemotherapeutic Target. In *Experimental and Clinical Progress in Cancer Chemotherapy*, Muggia, F. M., Ed. Springer US: Boston, MA, 1985; pp 81-131.
4. Tritton, T. R.; Yee, G., The Anticancer Agent Adriamycin Can Be Actively Cyto-Toxic without Entering Cells. *Science* **1982,** *217* (4556), 248-250.
5. Escriba, P. V.; Sastre, M.; Garciasevilla, J. A., Disruption of Cellular Signaling Pathways by Daunomycin through Destabilization of Nonlamellar Membrane Structures. *P Natl Acad Sci USA* **1995,** *92* (16), 7595-7599.
6. Escriba, P. V., Membrane-lipid therapy: a new approach in molecular medicine. *Trends Mol Med* **2006,** *12* (1), 34-43.
7. Escriba, P. V.; Gonzalez-Ros, J. M.; Goni, F. M.; Kinnunen, P. K.; Vigh, L.; Sanchez-Magraner, L.; Fernandez, A. M.; Busquets, X.; Horvath, I.; Barcelo-Coblijn, G., Membranes: a meeting point for lipids, proteins and therapies. *J Cell Mol Med* **2008,** *12* (3), 829-75.
8. Goormaghtigh, E.; Chatelain, P.; Caspers, J.; Ruysschaert, J. M., Evidence of a specific complex between adriamycin and negatively-charged phospholipids. *Biochim Biophys Acta* **1980,** *597* (1), 1-14.
9. Karczmar, G. S.; Tritton, T. R., The interaction of adriamycin with small unilamellar vesicle liposomes. A fluorescence study. *Biochim Biophys Acta* **1979,** *557* (2), 306-19.
10. van Meer, G.; Voelker, D. R.; Feigenson, G. W., Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **2008,** *9* (2), 112-24.
11. Slotte, J. P., Biological functions of sphingomyelins. *Prog Lipid Res* **2013,** *52* (4), 424-437.
12. Maxfield, F. R.; Tabas, I., Role of cholesterol and lipid organization in disease. *Nature* **2005,** *438* (7068), 612-621.
13. Wydro, P., Sphingomyelin/phosphatidylcholine/cholesterol monolayers - analysis of the interactions in model membranes and Brewster Angle Microscopy experiments. *Colloids Surf B Biointerfaces* **2012,** *93*, 174-179.

14. Lingwood, D.; Simons, K., Lipid Rafts As a Membrane-Organizing Principle. *Science* **2010,** *327* (5961), 46-50.
15. Nunes, C.; Brezesinski, G.; Lopes, D.; Lima, J. L.; Reis, S.; Lucio, M., Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality. *J Phys Chem B* **2011,** *115* (43), 12615-23.
16. Wydro, P., Sphingomyelin/phosphatidylcholine/cholesterol monolayers--analysis of the interactions in model membranes and Brewster Angle Microscopy experiments. *Colloids Surf B Biointerfaces* **2012,** *93*, 174-9.
17. Mendelsohn, R.; Mao, G.; Flach, C. R., Infrared reflection-absorption spectroscopy: principles and applications to lipid-protein interaction in Langmuir films. *Biochim Biophys Acta* **2010,** *1798* (4), 788-800.
18. Vollhardt, D.; Fainerman, V. B., Characterisation of phase transition in adsorbed monolayers at the air/water interface. *Adv Colloid Interface Sci* **2010,** *154* (1–2), 1-19.
19. Matyszewska, D.; Brzezinska, K.; Juhaniewicz, J.; Bilewicz, R., pH dependence of daunorubicin interactions with model DMPC:Cholesterol membranes. *Colloids Surf B Biointerfaces* **2015,** *134*, 295-303.
20. Ramin, M. A.; Le Bourdon, G.; Daugey, N.; Bennetau, B.; Vellutini, L.; Buffeteau, T., PM-IRRAS investigation of self-assembled monolayers grafted onto SiO2/Au substrates. *Langmuir* **2011,** *27* (10), 6076-84.
21. Nunes, C.; Brezesinski, G.; Pereira-Leite, C.; Lima, J. L.; Reis, S.; Lucio, M., NSAIDs interactions with membranes: a biophysical approach. *Langmuir* **2011,** *27* (17), 10847-58.
22. Buehler, L. K., *Cell Membranes*. Garland Science Taylor & Francis Group: New York, 2015.
23. Feng, S.-S.; Gong, K.; Chew, J., Molecular Interactions between a Lipid and an Antineoplastic Drug Paclitaxel (Taxol) within the Lipid Monolayer at the Air/Water Interface. *Langmuir* **2002,** *18* (10), 4061-4070.
24. Vitovic, P.; Nikolelis, D. P.; Hianik, T., Study of calix[4]resorcinarene-dopamine complexation in mixed phospholipid monolayers formed at the air-water interface. *Biochim Biophys Acta* **2006,** *1758* (11), 1852-61.
25. Duncan, S. L.; Larson, R. G., Comparing Experimental and Simulated Pressure-Area Isotherms for DPPC. *Biophys J* **2008,** *94* (8), 2965-2986.
26. Duncan, S. L.; Dalal, I. S.; Larson, R. G., Molecular dynamics simulation of phase transitions in model lung surfactant monolayers. *Biochim Biophys Acta* **2011,** *1808* (10), 2450-65.
27. Jiao, X.; Keating, E.; Tadayyon, S.; Possmayer, F.; Zuo, Y. Y.; Veldhuizen, R. A., Atomic force microscopy analysis of rat pulmonary surfactant films. *Biophys Chem* **2011,** *158* (2-3), 119-25.
28. Zhang, H.; Fan, Q.; Wang, Y. E.; Neal, C. R.; Zuo, Y. Y., Comparative study of clinical pulmonary surfactants using atomic force microscopy. *Biochim Biophys Acta* **2011,** *1808* (7), 1832-42.
29. Matyszewska, D.; Bilewicz, R., Interactions of daunorubicin with Langmuir–Blodgett thiolipid monolayers. *Electrochim Acta* **2015,** *162*, 45-52.
30. Gaber, M. H.; Ghannam, M. M.; Ali, S. A.; Khalil, W. A., Interaction of Doxorubicin with phospholipid monolayer and liposomes. *Biophys Chem* **1998,** *70* (3), 223-9.
31. Blaudez, D.; Turlet, J.-M.; Dufourcq, J.; Bard, D.; Buffeteau, T.; Desbat, B., Investigations at the air/water interface using polarization modulation IR spectroscopy. *J Chem Soc Faraday Trans* **1996,** *92* (4), 525-530.
32. Mattjus, P.; Slotte, J. P., Does cholesterol discriminate between sphingomyelin and phosphatidylcholine in mixed monolayers containing both phospholipids? *Chem Phys Lipids* **1996,** *81* (1), 69-80.
33. Somasundaran, P., *Encyclopedia of Surface and Colloid Science*. Taylor and Francis Group, LLC: New York, 2006; Vol. 5.
34. Alonso, C.; Alig, T.; Yoon, J.; Bringezu, F.; Warriner, H.; Zasadzinski, J. A., More than a monolayer: relating lung surfactant structure and mechanics to composition. *Biophys J* **2004,** *87* (6), 4188-202.
35. McConlogue, C. W.; Vanderlick, T. K., A Close Look at Domain Formation in DPPC Monolayers. *Langmuir* **1997,** *13* (26), 7158-7164.
36. Castro, C. M.; Pinheiro, M.; Lucio, M.; Giner-Casares, J. J.; Camacho, L.; Lima, J. L.; Reis, S.; Segundo, M. A., Insights about alpha-tocopherol and Trolox interaction with phosphatidylcholine monolayers under peroxidation conditions through Brewster angle microscopy. *Colloids Surf B Biointerfaces* **2013,** *111*, 626-35

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4. Daunorubicin and doxorubicin molecular interplay with 2D membrane models



Daunorubicin and doxorubicin molecular interplay with 2D membrane models

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The anthracyclines daunorubicin and doxorubicin are widely used antineoplastic agents due to their therapeutic activity against a broad variety of human cancers. Although, the classical model to explain anthracyclines' cytotoxicity has been based in the direct interference with nucleic acid function, evidence suggest that the plasma membrane is also involved in the drug's mechanism of action.
In this work, the interaction of these drugs with two-dimensional membrane models were studied in order to gain further insights at the molecular level regarding anthracyclines membrane interactions. For that purpose, Langmuir monolayers composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), sphingomyelin (SM) and cholesterol (Chol) were used, since these are the most common lipids found in biological membranes. Several biophysical techniques were employed: surface pressure (π)-area (A) isotherms measurements were used to investigate the adsorption and penetration of drugs, polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS) to acquire structural information and Brewster angle microscopy (BAM) to record images of the monolayers on the micrometer scale.
The interactions of anthracyclines were assessed by alterations in the monolayers' shape, characteristic parameters (C_s^{-1} values and area per lipid molecule at 30 mN.m^{-1} and under maximum packing conditions) and morphology of each 2D model studied. The presence of the drugs in the interface led to the production of less ordered monolayers, as evidenced by the decrease in the compressibility modulus. In addition, the drugs' effect on the membrane organization is related with their chemical structure and depends on the membranes´ phase. For lower surface pressures, both electrostatic and hydrophobic interactions led to significant modifications in the monolayer order. With further compression, the impact of such interactions is reduced, resulting in the squeezing-out of some drug molecules from the interface. Furthermore, BAM images showed a clear anticancer drug interplay with the lipid monolayer by changes in the domains shape and appearance of bright dots, which are located in the frontier between the condensed and expanded lipid phases.

DPPC:SM:Chol | monolayer | anthracyclines | membrane interactions

Anthracyclines are considered one of the most effective group of anticancer drugs ever developed[1], wherein daunorubicin (DAN) and doxorubicin (DOX) where the first isolated antibiotics from cultures of *Streptomyces peucetius*[1]. DAN and DOX (Fig. 1) share a quinone containing a rigid planar aromatic ring structure (dihydroxyanthraquinone) bound to an amino sugar moiety and the only difference between these two molecules lies in the end of the side chain: DOX ends with a hydroxyl group, whereas DAN ends with a methyl group. This small structural difference has relevant consequences on their spectrum of activity and pharmacokinetics.[1] Thus, while DAN shows activity in acute lymphoblastic or myeloblastic leukemia's[2], DOX is used in the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas.[1]

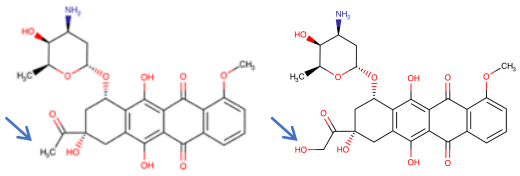


Fig. 1. Structure of daunorubicin (left) and doxorubicin (right): same dihydroxyanthraquinone and sugar moieties, but doxorubicin ends with a hydroxyl group instead of the methyl group that is present in daunorubicin.

Although, the mechanism of anthracyclines' cytotoxicity may involve multiple pathways, the DNA has held the center stage as primary target. In fact, anthracyclines have been thought to act either by directly binding to nucleic acids or by interfering with the enzymes that are involved in their biosynthesis.[3] Nevertheless, growing evidence has contributed to the idea that the cell membrane can itself be a target for these drugs and can also be involved in their mode of action.[4-5,6] For example, in 1982 Tritton *et al*. have demonstrated that anthracyclines, without entering the cell, could exert their cytotoxic effect only through their interplay with the membrane.[4]
The cell membrane is not just an lipid bilayer in which proteins are embedded. Such cell boundary is involved in several biological activities that lead to cell survival, differentiation, cell death, among others.[7] Therefore, alterations of its general lipid organization and structure, like the ones induced by chemotherapeutic compounds, can initiate several processes implicated in the anticancer cytotoxic effects (such as apoptosis). For this reason, the goal of this study was to perform a systematic comparison between the degree of penetration of DAN and DOX, and their ability to induce changes in the biophysical properties of membranes.
Besides, the interaction of DAN and DOX with membranes is inevitable, since they must pass across membranes in order to exert their cytotoxic effects through interplay with DNA or other intracellular components (taking in consideration that the DNA is the primary target of anthracyclines). Several studies have demonstrated that anthracycline' molecules interact specifically with negatively charged membrane lipids, which have been implicated in the cardiotoxicity caused by these drugs.[8-9] Finally, evidence also shown that this type of chemotherapeutic agents has the ability to alter the membrane structure, by reducing the formation of the non-lamellar hexagonal (H_{II}) phase propensity and just by interacting with membrane phospholipids.[5] Because

the H_{II} phase is involved in the location and activity of several proteins (such as, the G-protein and the kinase C) important for cell signaling, various cellular events implicated in the signal transduction are indirectly affected by these drugs.[5]

Taking in consideration all the abovementioned reasons and since the efficacy of drugs depends on complex drug-membrane interactions, the study of anticancer drug-membrane interactions is fundamental to better understand the drugs' bioavailability and the drug-induced toxicity process. Therefore, two-dimensional (Langmuir monolayers) model systems composed of different ratio of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), sphingomyelin (SM) and cholesterol (Chol) were chosen to mimic the plasma membranes and explain the drug's impact on the structure-packing behavior and molecular organization of the model membranes. The lipids choice is related to the fact that such lipids are the major components of the outer layer of human membranes.[10] In fact, phosphatidylcholines are the most abundant phospholipids and DPPC is commonly present in natural cell membranes. SM is the major sphingolipid found in membranes, being involved in many cellular functions.[11] Chol is also a major component of the membrane and one of the most important regulators of lipid organization.[12] Chol has also the ability to control membrane fluidity and permeability, which is important for the preservation of membrane' organization, contributing to the cell membrane's physiology and function.[13] In addition, Chol and SM form liquid-ordered domains in a more fluid matrix, the so called lipids rafts, crucial for membrane organization and where several proteins and other biomolecules are placed.[14] The use of monolayers at the air/liquid interface allows one to assess the adsorption and penetration of DAN and DOX at the membrane surface without the interference of trans-bilayer events.[15] Furthermore, the combination of Langmuir monolayer with other methods provides valuable information regarding the monolayer morphology, domains structure, packing properties, phase coexistence[16], structural organization (such as chain conformation and hydration[17-18]); and about the effects of the anticancer drugs on such properties, important to better understand the drug's cytotoxic mechanisms. In this regard, different biophysical techniques were performed using Langmuir monolayers: pressure-area isotherm measurements, polarization modulation infrared reflection-absorption spectroscopy (PM-IRRAS) and Brewster angle microscopy (BAM).

Materials and Methods

Materials and reagents. Daunorubicin (DAN) was obtained from Biovision (Milpitas, California) and doxorubicin (DOX) from LC Laboratories (Woburn, Massachusetts, USA). The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), sphingomyelin (SM) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). All compounds were used without further purification. Drug solutions were prepared with HEPES buffer (10 mM, pH 7.4). The buffer was prepared with double deionized water (conductivity inferior to 0.1 μScm^{-1}) and ionic strength adjusted with NaCl (I=0.1 M).

Langmuir isotherms. The surface pressure area isotherms (π/A) experiments were carried out in a KSV NIMA Langmuir balance. The system was equipped with two barriers, used in compression isotherms assays, and a Wilhelmy microbalance with filter paper plate (accuracy superior to 0.1 mN m^{-1}), used as a surface pressure sensor. Monolayers of DPPC, DPPC:SM [8:2] and DPPC:SM:Chol [7:1.5:1.5] were prepared by spreading dropwise the lipid/chloroform solutions (1 mM) using a Hamilton syringe on the Hepes subphase (pH 7.4), with or without anticancer drug (DAN or DOX, 10 μM). The drug concentration choice was based on Matyszewska *et al*. work, which demonstrated that concentrations of DAN above 10−5 M did not affect the DMPC:Chol monolayer to a greater extent due to a limiting concentration of drug in the subphase.[19] For all Langmuir trough measurements, the volume of the aqueous subphase was kept constant at 420 mL. The monolayers were equilibrated during 20 minutes to allow the complete chloroform' evaporation and were compressed at a rate of 5 $Å^2$/molecule/min to obtain the isotherms. Before each measurement, the trough was thoroughly cleaned with ethanol and double-deionized water, until the compression of the water subphase resulted in a zero surface pressure. Each compression isotherm was performed at least three times and all measurements were carried out at 20 ± 1 °C.

Polarization-modulation infrared reflection-absorption spectroscopy. Polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) experiments were conducted in a PM-IRRAS KSV NIMA. The polarization modulation allows one to eliminate the environmental signal' interference, such as water and CO_2, which provides a highly sensitive and suitable technique for monolayer analysis.[20] The angle of incidence of the infrared beam with respect to the surface normal was 80°. The measurements conditions were set as previously described except for the trough size, which was filled with 45 mL of aqueous subphase. The spectrum of each subphase (Hepes buffer with or without anticancer drug) was recorded as a background and subtracted in each sample experiment. Each spectrum was recorded with a spectra resolution of 8 cm^{-1} during 300 seconds and with a rate of 10 scans per second. All measurements were performed at 20 ± 1 °C.

Brewster angle microscopy. The morphology of the monolayers at the air–water interface was recorded using a KSV NIMA Brewster angle microscope (BAM) with a lateral resolution of 2 μm. The system was coupled to a Langmuir trough that provided a real-time visualization of the monolayer at several pressures. A KSV NIMA Langmuir trough was used, containing two symmetric barriers and a Wilhelmy microbalance with a filter paper plate with an accuracy superior to 0.1 $mN.m^{-1}$. Similar conditions as those in π/A measurements were employed (drug solution of 10 μM in a trough of 420 mL). The brightness of each image was scaled to improve contrast. All images were recorded at 20 ± 1 °C.

RESULTS AND DISCUSSION

Langmuir isotherms

The determination of the surface pressure/area (π/A) isotherms allows one to obtain insights about the phases

and the type of phase transitions of a lipid monolayer.[21] Langmuir isotherms also provide information regarding the interactions between drugs and lipids at the membrane surface that are important to understand the drug's ability to penetrate the lipid layer and its influence in the monolayer's order and permeability of the monolayer, as a consequence of drug´s incorporation. In this regard, monolayers composed of DPPC, DPPC:SM and DPPC:SM:Chol were used as simple 2D models to study the effect of DAN and DOX at the membranes' surface level.

The π/A isotherms of each DPPC, DPPC:SM [8:2] and DPPC:SM:Chol [7:1.5:1.5] films on Hepes buffer are shown in Fig. 2. Since the anthracycline compounds studied are amphiphilic molecules their penetration and/or adsorption into the lipid monolayers is expected to take place and was assessed by comparing the π/A isotherms of each model in the absence and presence of drugs (Fig. 3, 4, 5). From the π/A isotherms obtained it was possible to calculate the area per lipid molecule (A) and the compressibility modulus (C_s^{-1}) of each monolayer, which were also used to evaluate the anthracyclines effects. The area occupied per lipid can be determined by extrapolating the tangent to the isotherm in the steepest part (under maximum packing conditions) to zero surface pressure[22] (and is called minimum area A_0) or at a certain surface pressure (A_π). On the other hand, the compressibility modulus (C_s^{-1}), which reflects the compressibility properties of Langmuir monolayers, can be calculated from each π/A isotherms by the following equation:

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)$$

where A is the area per lipid molecule and π is the surface pressure. C_s^{-1} describes the relationship between the surface pressure increase and the area per molecule decrease[23], giving information about the state in which the monolayer is at a given surface pressure. Therefore, a higher value of C_s^{-1} is indicative of a less compressible monolayer. In this work, the lipid monolayers were compared with respect to the maximum compressibility modulus value but also to the one obtained at surface pressure of 30 mN.m^{-1} due its biological relevance.[22] It is described in the literature that compressibility modulus values between 0 to 12.5 mN.m^{-1} refer to the gas phase; from 12.5 to 50 mN.m^{-1} to the liquid-expanded (LE); from 100 to 250 mN.m^{-1} to the liquid-condensed (LC) and values above 250 mN.m^{-1} are typical of solid films.[24] These values were used to characterize the studied lipid films. In Table 1 are depicted the obtained values of minimum area per lipid molecule and the maximum compressibility modulus, along with the respective values at 30 mN.m^{-1} for each monolayer and anticancer drug studied.

As can be seen at Fig. 2, the isotherm obtained for DPPC spread on aqueous Hepes buffer (pH 7.4) presents similar characteristic shape as reported in other studies[21, 25], with a marked plateau between 63 and 81 Å^2 per molecule at surface pressure of ca. 3–8 mN.m^{-1} that corresponds to the first order transition of LE to LC phase. In addition, the A_0 (54 Å^2) and the maximum compressibility modulus C_s^{-1} (217 mN.m^{-1}) are also in agreement with values described in the literature (Table 1).

The DPPC monolayer containing SM suffered an increase in the minimum area per molecule (60 Å^2), probably due to the SM higher head group' size, and a decrease in the maximum compressibility modulus C_s^{-1} (209 mN.m^{-1}), indicating that the lipid monolayer becomes more compressible. In fact, compared with the other studied lipid monolayers, this is the one with the smaller value compressibility modulus and, as such, the most flexible one. Furthermore, the plateau corresponding to LE/LC phase transition of DPPC is still present when SM was included (Fig. 2A) and can also be observed in the C_s^{-1}/π plot – minimum value obtained (Fig. 2B).

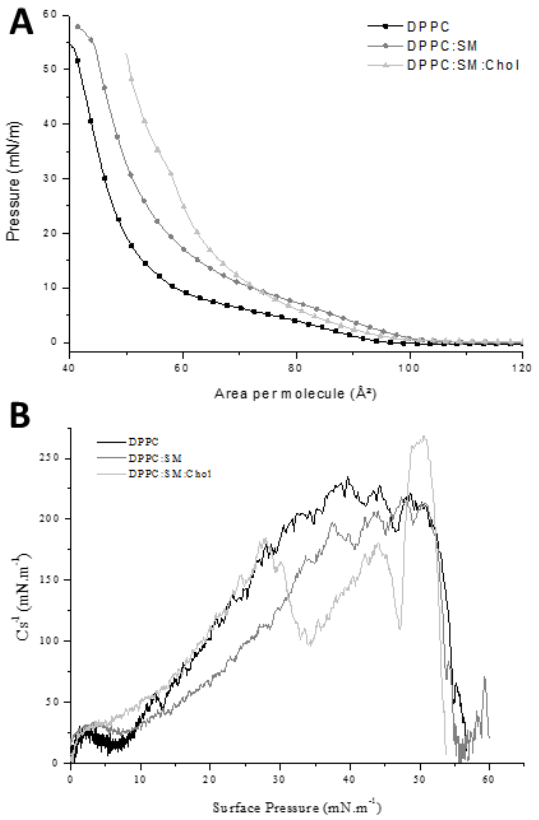


Fig. 2. A) Surface pressure/area per molecule (π/A) isotherms. B) compressibility modulus/surface pressure (C_s^{-1}/π) of DPPC, DPPC:SM [8:2] and DPPC:SM:Chol [7:1.5:1.5] monolayers on buffer subphase at pH 7.4 and 20°C.

On the other hand, the addition of SM:Chol to DPPC monolayer caused an increase in the minimum area per molecule (61 Å^2) and increase in the maximum compressibility modulus C_s^{-1} (274 mN.m^{-1}) (Fig. 2A and B). Compared to DPPC and DPPC:SM films, the presence of SM:Chol provided a less compressible monolayer, with the highest value of C_s^{-1}. Nevertheless, as shown in Fig. 2B, the compressibility modulus/surface pressure plot of DPPC:SM:Chol film presents two peaks with C_s^{-1} values corresponding to the liquid-condensed phase (~184 mN.m^{-1}), followed by a maximum peak corresponding to a solid phase (274 mN.m^{-1}). It is possible that both lipid components induce the formation liquid-ordered (LO) domains that appears after the LE phase and before the LC regime. In fact, it has been described that DPPC monolayers containing Chol display an intermediate phase between the LE and the LC phases[26], which are referred to the LO phase[27-28]. It has also been reported that cholesterol has preferential affinity for the interface between LE and LC phases[26], which corroborates the acquired results.

Table 1 Characteristic parameters of DPPC, DPPC:SM [8:2] or DPPC:SM:Chol [7:1.5:1.5] monolayers on the buffer subphase with or without 10 μM of daunorubicin (DAN) or doxorubicin (DOX) at pH 7.4.

	DPPC			DPPC:SM			DPPC:SM:Chol		
	Buffer	**DAN**	**DOX**	**Buffer**	**DAN**	**DOX**	**Buffer**	**DAN**	**DOX**
A_0 (Å2)	54 ± 1	50 ± 3	43 ± 2	60 ± 1	66 ± 1	66 ± 1	61 ± 1	63 ± 1	67 ± 2
Max C_s^{-1} (mN.m^{-1})	217 ± 8	139 ± 8	121 ± 7	209 ± 6	185 ± 3	150 ± 4	274 ± 8	256 ± 8	221 ± 7
A_{30} (Å2)	47 ± 1	41 ± 2	35 ± 1	52 ± 1	56 ± 1	59 ± 1	59 ± 1	60 ± 1	62 ± 2
C_s^{-1} at π= 30 (mN.m^{-1})	177 ± 7	115 ± 4	116 ± 2	122 ± 1	108 ± 3	70 ± 4	158 ± 9	112 ± 5	123 ± 3

When Hepes subphase was substituted by a Hepes solution of DAN or DOX at 10 μM, significant alterations in the π/A isotherms profile (Fig. 3) of each lipid monolayer and in the characteristic parameters (Table 1) were found. In fact, in the DPPC monolayer, the presence of DAN caused an isotherm expansion until the surface pressure of 16 mN.m^{-1}, from which there is a crossover showing that the monolayer can be compressed to smaller areas. At the same time, the A_0 and the C_s^{-1} decreased (50 Å^2 and 139 mN.m^{-1}, respectively). On the other hand, the presence of DOX did not cause any significant alteration in the shape of the monolayer until about 10 mN.m^{-1}, from which the monolayer is shifted towards smaller areas. In addition, both A_0 and the C_s^{-1} decreased (43 Å^2 and 121 mN.m^{-1}, respectively) with DOX. The presence of the phase transition in DPPC isotherm, represented by the minimum on the compressibility modulus/surface pressure plot (Fig. 3B), disappears when both DAN and DOX were present in the interface. The results
suggest that, in the condensed phase of the DPPC monolayer, the combined effect of both drugs in decreasing the minimum area per lipid molecule and decreasing the compressibility modulus, both drugs are squeezed-out of the monolayer dragging along lipid molecules. Furthermore, the obtained lower A_0 indicate that the anticancer drugs are able to reduce the steric and/or dipolar repulsions between the phospholipids' headgroups. This may happen due to an alteration in the orientation and/or hydration of the lipids and can only happen if the drugs establish strong interaction with the DPPC molecules.

The isotherms of the mixed DPPC:SM and DPPC:SM:Chol monolayers are shifted towards larger areas per molecule in the presence of both DAN and DOX in the interface, that can be explained by the penetration of both anticancer drugs into the monolayers. At the same time, the addition of DAN and DOX caused a decrease in the maximum C_s^{-1} values, more significant in the case of the monolayer composed of DPPC:SM than in DPPC:SM:Chol. Furthermore, a higher difference in the area per lipid caused by the anthracyclines could also be observed in the case of DPPC:SM, when comparing these two isotherms (Fig. 4A and 5A). It is clear that the adsorption of anthracyclines to the lipid films produced less condensed monolayers as a consequence of drug–lipid interactions, which were more pronounced for DOX (smaller values of C_s^{-1}). These findings are more evident for lower pressures where the differences in the area per molecule are superior. However, as the surface pressure increases, the differences in the pressure-area became smaller and the isotherms of the monolayers containing the anthracyclines got closer to the isotherms without drugs, which means that some anticancer drug molecules are probably being squeezed-out from the interface. In fact, the difference in the area per lipid (obtained by reading the value of area per molecule at a given surface pressure) between the values for DPPC:SM without and with anthracyclines is: ~16 and 31 at 10 mN.m^{-1} and 4 and 7 at 30 mN.m^{-1} with DAN and DOX, respectively. In the case of DPPC:SM:Chol this difference is: ~13 and 18 at 10 mN.m^{-1} and 1 and 3 at 30 mN.m^{-1} with DAN and DOX, respectively. Once more, it can be found that DOX causes more pronounced effects as evidenced by the higher differences in the area per lipid for all surface pressures. Furthermore, the high values of A_0 obtained for DAN and DOX in these monolayers, reveal that some anticancer molecules are still incorporated at pressures above 30 mN.m^{-1} and, probably, act as spacers between the lipid molecules.

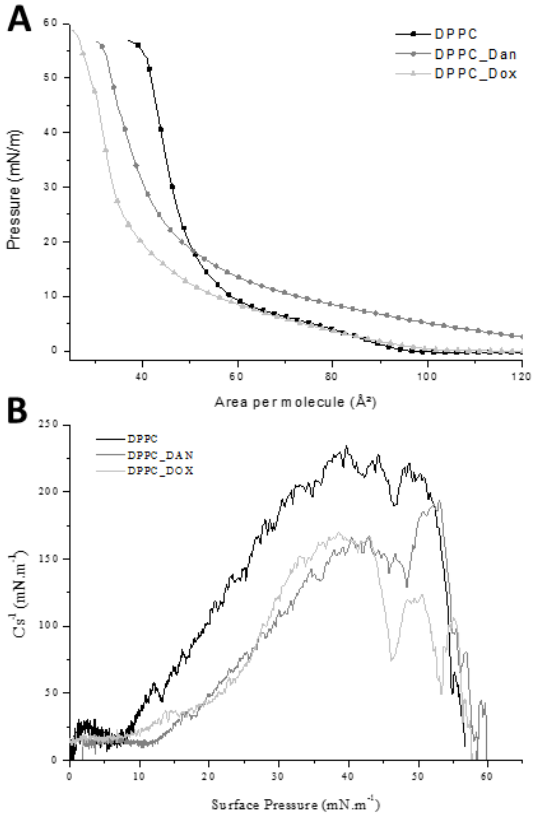


Fig. 3. A) Surface pressure/area per molecule (π/A) isotherms and B) compressibility modulus/surface pressure (C_s^{-1}/π) of DPPC monolayer on buffer and on 10 μM of daunorubicin (DAN) or doxorubicin (DOX) subphase at pH 7.4 and 20°C.

The interaction of DAN and DOX molecules with the studied monolayers are more pronounced for lower pressures, as evidenced by expansion to larger areas,

which suggests that their penetration into less-compressed lipid monolayers is easier and is in good agreement with some studies found in the literature.[29]
It is interesting to observe that the effect produced in the monolayer compressibility by the interaction of anthracyclines with the different films is more pronounced when cholesterol is not present. This probably happens because there is no competition between the anticancer drugs and cholesterol for the place in the monolayer. Consequently, their incorporation in the membrane without cholesterol is facilitated and produces higher modifications in its properties. This in in good agreement with previous studies where it has been demonstrated that interactions between compounds, possessing an aromatic ring and planar structure similar to the one found in cholesterol, with model lipid membranes containing cholesterol could be considered in terms of competition between cholesterol and drugs.[19] Gaber *et al*. have also demonstrated that the addition of Chol to the DPPC monolayer caused a membrane stabilization with consequent reduction of DOX' interaction.[30]

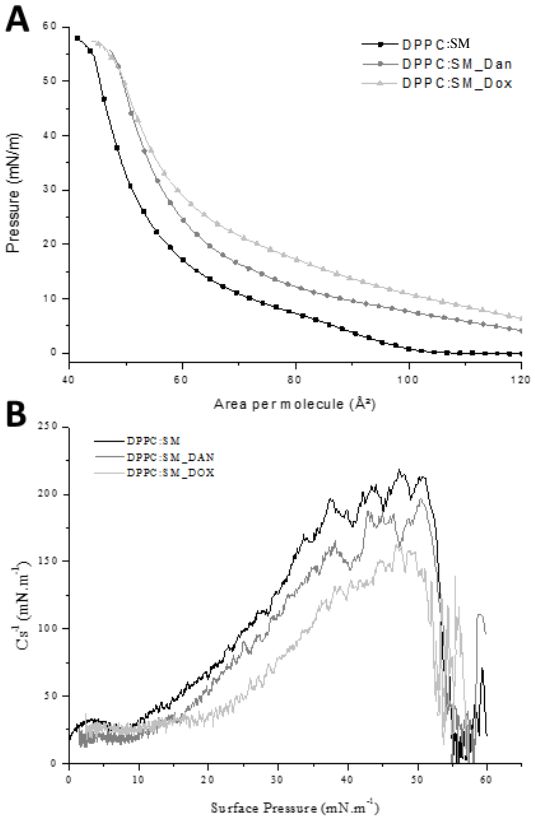


Fig. 4. A) Surface pressure/area per molecule (π/A) isotherms and B) compressibility modulus/surface pressure (C_s^{-1}/π) of DPPC:SM [8:2] monolayer on buffer and on 10 μM of daunorubicin (DAN) or doxorubicin (DOX) subphase at pH 7.4 and 20°C.

It is important to stress out that, despite monolayers containing cholesterol could reduce the anthracyclines' interaction with the acyl chains, these compounds can still interplay and produce alterations in the polar regions of the monolayer. For that reason, the observed high differences in the area per lipid for all films in the presence of anticancer drugs are due to the ability of these compounds to interact with the lipids at the interface through electrostatic interactions. In fact, the area per lipid is mostly influenced by the head group size present at the air/water interface; therefore, alterations in this region cause modifications in such characteristic parameter.
Comparing the values of area per lipid at 30 mN.m^{-1} and the elastic modulus of the DPPC:SM:Chol monolayer on the buffer subphase with the values obtained for the DPPC:SM monolayer on the subphase containing DOX, it appears that they are very similar. This may indicate that DOX presents a similar behaviour to Chol.
Another important observation is that, as shown in Table 1, all the investigated monolayers are characterized by maximum compressibility modulus values higher than 121 mN.m^{-1}, which lie in the typical range of liquid-condensed state (100-250 mN.m^{-1})[24] and for DPPC:SM:Chol monolayers witch values corresponds to the solid state (> 250 mN.m^{-1}). Notwithstanding, at a surface pressure of 30 mN.m^{-1} the compressibility modulus of all monolayers are still in the liquid-condensed state. The presence of DAN and DOX tend to decrease the C_S^{-1} values, suggesting that the compounds produced more flexible (i.e. more compressible) monolayers. This reduction in the compressibility modulus was more noticeable for DOX in all lipid films. Overall, the analysis of the Langmuir isotherms regarding the area expansion and the compressibility modulus values revealed that the anticancer drugs present a strong interaction with the lipid membrane models studied.

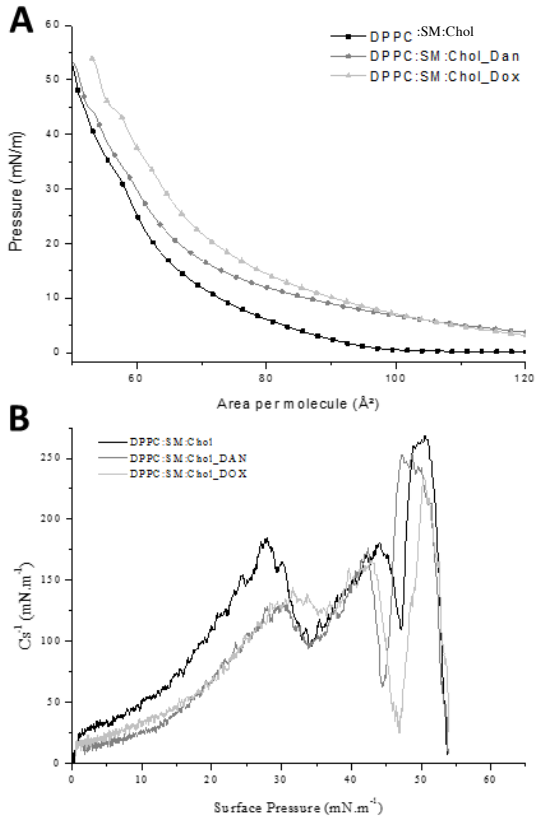


Fig. 5. A) Surface pressure/area per molecule (π/A) isotherms and B) compressibility modulus/surface pressure (C_s^{-1}/π) of DPPC:SM:Chol [7:1.5:1.5] monolayer on buffer and on 10 μM of daunorubicin (DAN) or doxorubicin (DOX) subphase at pH 7.4 and 20°C.

Infrared reflection-absorption spectroscopy measurements

In order to complete the information obtained with the Langmuir isotherms, regarding chain conformation, hydrogen bonding and ionic interactions, polarization-modulation infrared reflection-absorption (PM-IRRAS) experiments were performed. The PM-IRRAS is an infra-red (IR) spectroscopic method adapted to the air–water interface that allows to obtain information regarding the molecular structure and orientation of the monolayer constituents[17] through the measurement of the frequencies (wavelength) and intensities of molecular vibrations. Further detail about the principle of PM-IRRAS can be found in Blaudez *et al*..[31]

Therefore, the band positions of the asymmetric phosphate (1220-1260), the carbonyl (1710-1740) and the methyl (2849-2854 and 2916-2924) stretching vibrations in all monolayers in the absence and presence of the anticancer drugs at a constant surface pressure of 10, 20 and 30 $mN.m^{-1}$, are gathered in Table 2. The target values of surface pressure were chosen in order to obtain monolayers at different organizational states as can be observed by the C_s^{-1}/π plot. Thereby, pressures from the liquid-expanded state that is characterized by low lipid packing and high configurational freedom to the liquid-condensed state, where the lipid molecules form a well-ordered layer as the surface pressure increases, as well as the liquid-ordered regime in the case of DPPC:SM:Chol, were chosen. Besides, as previous mention, the surface pressure of 30 $mN.m^{-1}$ is very relevant since it reflects the surface pressure of real cell membranes.The evaluation of the phosphate and carbonyl bands permits to monitor the interplay of anticancer drugs with the head group of phospholipids. These chemical groups are also sensitive to the hydration state of the monolayer and a shift towards smaller wavenumbers indicates the formation of new H-bonds or an increase in the local hydration.[21] At the same time, the wavenumbers of the methylene stretching vibrations can be used to characterize the state of the conformation order in the lipid monolayer and to monitor the average trans/gauche isomerization in the systems.[15]

The values depicted in Table 2 demonstrate that for DPPC, with increasing surface pressure (from $\pi = 10$ to 30 $mN.m^{-1}$), occurs a deviation to smaller values in the wavenumbers of the methylene, due to an increase in the monolayer packing and, consequently, in the film's order. In fact, values around 2925 cm^{-1} indicate disorder in the acyl chain region (presence of *gauche* conformers) and values bellow 2920 cm^{-1} are typical of more ordered membrane (all-*trans* conformation).[21] Furthermore, it is also possible to observe that the presence of SM and SM:Chol, at $\pi = 30$ $mN.m^{-1}$, induce a decrease in the DPPC monolayer order as shown by the shift toward higher values in the stretching band of CH_2. This is in good agreement with the previous results of isotherms, where a decrease in the compressibility modulus for DPPC:SM and DPPC:SM:Chol monolayers was seen at the same surface pressure.

The phosphate stretching band of DPPC:SM monolayer exhibits a pronounced asymmetry (data not shown): it consists of two overlapping bands, one at 1225 cm^{-1} for $\pi = 10$ $mN.m^{-1}$ (corresponding to the dihydrated phosphate) or 1235 cm^{-1} for $\pi = 20$ and 30 $mN.m^{-1}$ (corresponding to the monohydrated phosphate) and another at 1265 cm^{-1} (corresponding to the unhydrated phosphate). As can be observed, with pressure increase, the IR intensity is transferred from the dihydrated to the monohydrated phosphate form, which indicates a structural alteration due to the presence of SM that changes the accessibility of water molecules to the phosphate groups. In the case of DPPC:SM:Chol monolayer, the phosphate wavenumber is shifted towards smaller values for lower surface pressures (until 10 $mN.m^{-1}$), demonstrating that an increase in the hydration of the phosphate groups is occurring in the presence of cholesterol. This probably happens because Chol has preferential affinity for SM and increases the accessibility of water to the phosphate in the DPPC. In fact, using model membranes, it has been demonstrated that cholesterol interacts more favorably with sphingomyelin than with phosphatidylcholine [32] and according to the umbrella model, the head groups of SM molecules protect the large nonpolar structures of cholesterol from the aqueous environment. However, with surface pressure augmentation the PO^{2-} wavenumber start to increase, i.e. the head groups become less hydrated, which might reflect the increase in the monolayer order, probably to the formation of LO domains.

On the other hand, the C=O stretching band of DPPC shifts to smaller wavenumbers with SM, because SM molecules contain different functional groups, such as amide and free hydroxyl groups, which allow them to form new H bonds. Therefore, no alteration of the C=O wavenumbers in the case of DPPC:SM:Chol can be detected, because the amide group of SM is interacting with the hydroxyl group of Chol.

Structural alterations could be observed in the lipid monolayers studied when the anticancer drugs were present at the interface. In fact, due to anthracyclines chemical structure, modifications in both head group and/or acyl chains could be found depending on the 2D model membrane studied. The drugs did not cause any significant deviation in the PO^{2-} stretching band of DPPC monolayer, which can be explained by the fact the compounds are buried in the hydrophobic part of DPPC where they stablish strong interactions with the acyl regions (as can show by the increase in the CH_2 wavenumber). As explained in the isotherms results, the consequence of such interaction is the drugs' squeezing-out along DPPC molecules, as the monolayer is compressed. A shift towards smaller values with surface pressure increase occurred in the case of mixed DPPC:SM:Chol film, indicating that for pressures above 20 $mN.m^{-1}$ the anticancer drugs lead to an increase in the number of H bonds or hydration in the phospholipids polar region of this model. It is possible to infer that as the lipid film is compressed, the molecules that were inserted with the dihydroxyanthraquinone ring system between the acyl chains are being squeezed out and interact with the monolayer mainly with the negatively charged phosphate group through the positively charged amino sugar moiety. This fact is in good agreement with the decrease in the area per molecule' difference observed with the monolayer compression. For DPPC:SM, it can be seen that for surface pressure until 20 $mN.m^{-1}$ only DAN cause an increase in the hydration or increase in the number of H-bonds formed with the phospholipids' PO^{2-} group. Analyzing the compressibility results (Fig. 4B) it appears that the DAN influence did not lead to a significant decrease in the C_s^{-1} values (monolayer order), which means that most of its

Table 2 Vibrational wavenumbers of DPPC (A), DPPC:SM [8:2] (B) and DPPC:SM:Chol [7:1.5:1.5] (C) monolayers in absence or in presence of daunorubicin (DAN) or doxorubicin (DOX) at 10 μM, pH 7.4 and for π = 10, 20 and 30 mN.m^{-1}.

		π=10 mN.m-1			π=20 mN.m-1			π=30 mN.m-1		
		Buffer	DAN	DOX	Buffer	DAN	DOX	Buffer	DAN	DOX
A	ν_{as} (PO^{2-})	1265	1265	1260	1265	1265	1265	1265	1265	1265
	ν (CO)	1736	1744	1728	1736	1744	1728	1736	1744	1728
	ν_{as} (CH_2)	2922	2924	2924	2921	2924	2924	2917	2924	2924
B	ν_{as} (PO^{2-})	1265	1262	1265	1265	1262	1265	1265	1265	1265
	ν (CO)	1733	1744	1728	1739	1744	1721	1739	1744	1723
	ν_{as} (CH_2)	2924	2924	2924	2924	2924	2924	2924	2924	2924
C	ν_{as} (PO^{2-})	1258	1265	1265	1273	1265	1265	1273	1265	1265
	ν (CO)	1736	1744	1721	1728	1744	1721	1736	1744	1721
	ν_{as} (CH_2))	2924	2924	2924	2924	2924	2924	2924	2924	2924

interaction is taking place in the headgroups region and helps to explain the observed outcomes with PM-IRRAS.

An interesting observation can be made regarding the anthracyclines effect in the C=O group for all lipid monolayers. As so, while the wavenumber of the carbonyl increases with DAN, it decreases with DOX. This means that there is an increase of H-bonds or an increase in the local hydration with DOX (contrary to what happens with DAN), which might be related to the fact that DOX possess an OH group in the end of the side chain (absent in DAN).

Furthermore, when the anticancer drugs were incorporated in the interface a decrease in the wavenumbers of the methylene group occurred in the DPPC monolayer. Although the values did not change for the others lipid films, they were around 2924 cm^{-1} which are characteristic of disorder in the acyl chain region (presence of *gauche* conformers) and are in good agreement with the compression isotherms measurements, where a smaller value of C_s^{-1} was observed in the presence of the anticancer drugs for all investigated monolayers. In addition, from the compressibility/surface pressure plots it is clear that the compound's influence in the compressibility parameter were more pronounced in the case of DPPC film and, probably, for that reason it was only possible to depict alterations with PM-IRRAS technique in this monolayer. It is also important to stress out that usually an isotherm' expansion can indicate an increase in membrane fluidity or a drug penetration in the monolayer. In this case, the obtained results with PM-IRRAS clearly confirm that the observed isotherm shift to higher areas occurs as a consequence of penetration of both anticancer drugs into the monolayers.

Daunorubicin and doxorubicin are weak acids with pK_a values of 9.9 and at pH 7.4 are positively charged. Accordingly, both hydrophobic and electrostatic interactions are expected to take place in these anticancer drugs-membrane interactions. In fact, for lower surface pressures the anthracyclines interplay mainly through hydrophobic interactions between the drugs' hydrophobic moiety and acyl chains of the lipids, especially because the monolayer is less compressed and the compounds penetrate deeper into the phospholipids.

As surface pressures increases the monolayer becomes more condensed and the compounds have more difficult to penetrate the lipid film. Certainly, at this point the anthracyclines interplay with the phospholipids heads and, therefore, electrostatic interactions also occur between the drugs' positively charged amino group and phospholipids' negatively charged phosphate group. Although it was only possible to observe with this technique significant modifications in the phosphate group in the case of DPPC:SM:Chol and the main alteration occurred in the carbonyl group for all lipid films. Therefore, as a consequence of electrostatic and hydrophobic interactions, alterations in both C_s^{-1}values and area per molecule were observed.

Brewster Angle microscopy (BAM)

Brewster Angle microscopy (BAM) is an important microscopic technique to study thin film topography directly at the air/water interface.[33] The principle of BAM is based on the alteration of the reflectivity of polarized light reflected from films between two isotropic media with distinct refractive index.[33] Therefore, BAM allows the visualization of domains' coexistence of different phases (fluid and condensed) along with their shape and size.[18] As such, in BAM images the dark background corresponds to the fluid phase and the light grey areas to the condensed domains.[34]

In Fig. 6 are depicted the BAM pictures acquired during the first compression of all monolayers, both without and with anticancer drugs. The morphology of the DPPC monolayer is well described in the literature[35-36] and can be characterized by the appearance of light gray structures upon compression that evolve into bean and two-lobed shaped domains against a black background. With increasing surface pressure, these organized condensed domains become bigger and acquire irregular shapes with smoother contours. The incorporation of anthracyclines in the interface clearly changes such morphology, causing alterations in the size and shape of the DPPC domains. In fact, is possible to observe that DAN is able to impair the formation of the lobed domains and small white dots emerge for higher π. In the presence of DOX the lipid domains are bigger, more rounded and present a more irregular shape, containing small bright points that correspond to the anticancer

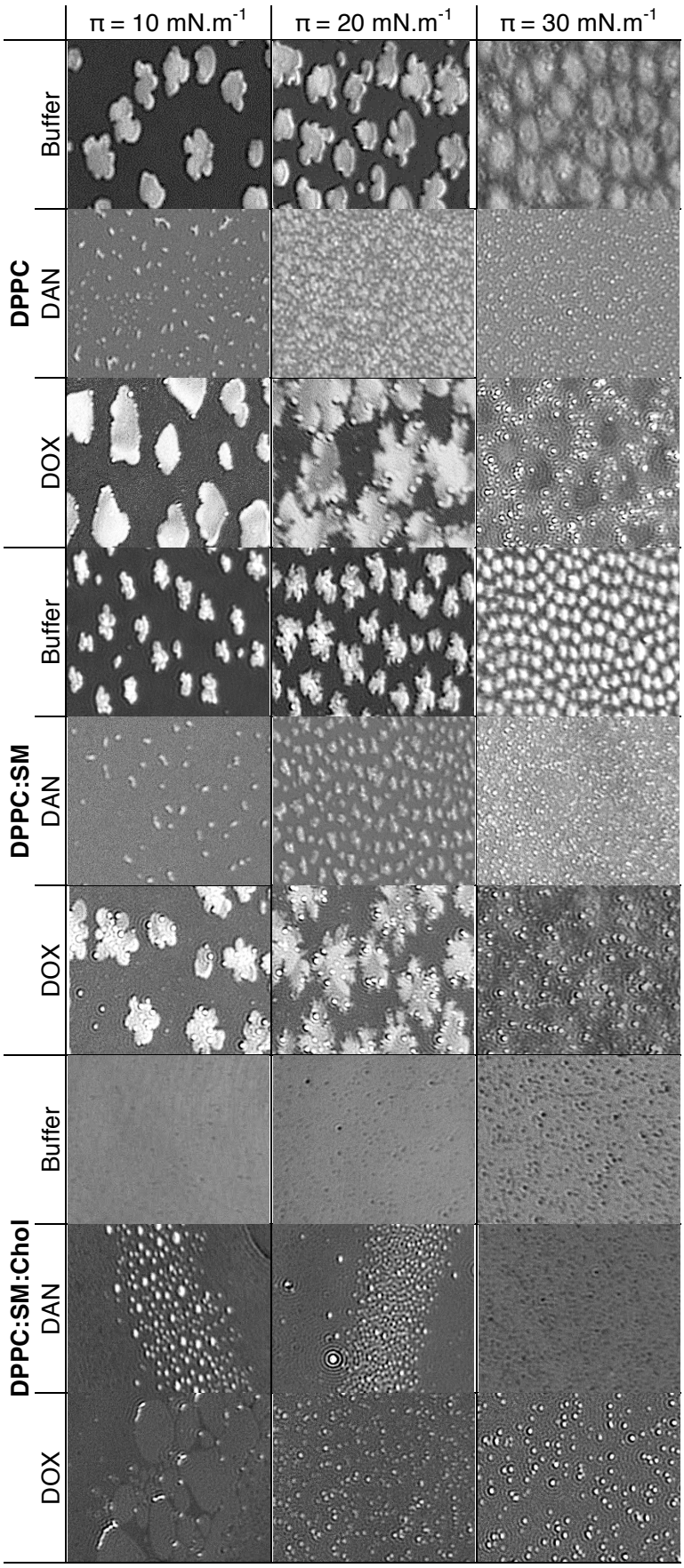


Fig. 6. Brewster angle microscopic images of Langmuir monolayers of DPPC, DPPC:SM [8:2] and DPPC:SM:Chol [7:1.5:1.5] on a Hepes buffer subphase and on a subphase containing 10 μM of daunorubicin (DAN) or doxorubicin (DOX), for π = 10, 20 and 30 $mN.m^{-1}$ at pH 7.4.

drug. With compression, the condensed domains become less organized and with higher number of white dots. It is possible that the detected bright regions are associated with DAN or DOX molecules, which exist in the form of aggregates with a high refractive index.

To the best of our knowledge, this was the first time that the lipid mixtures of DPPC:SM [8:2] and DPPC:SM:Chol [7:1.5:1.5] were characterized by this microscopic technique. BAM images demonstrate that, in the case of DPPC:SM monolayer spread on Hepes buffer, small round gray structures appeared and progressed toward more complex, multilobed structures that became less organized and formed small white aggregates above 20 $mN.m^{-1}$. With the addition of SM, the typical condensed domains of DPPC are still present but are smaller and with irregular borders. This is in good agreement with the isotherms results, where the DPPC monolayer became more flexible in the presence of SM. On the other hand, with the addition of DAN, the formation of the previous mentioned structures was not observed and, with surface pressure increase, several small round white dots emerged. When DOX was incorporated in the interface, similar structures appeared as for DPPC:SM monolayer spread on buffer, along with small white aggregates that, with compression, increased in number. In fact, as previous demonstrated, DOX molecules did not cause any significant alteration in the shape of the monolayer until about 10 $mN.m^{-1}$, which is in good agreement with the observed lobed structures. For higher pressures, less organized structures with several small round white dots can be seen. In fact, the isotherms outcomes have shown that the lipid film becomes more flexible, as demonstrated by the increase in the A_{min} and decrease in the C_s^{-1} values. BAM results clearly corroborate such observations since an alteration in the complexity of the LC domains was observed upon anticancer drugs addition to the interface.

For DPPC:SM:Chol monolayer spread on Hepes buffer, the formation of the characteristic DPPC domains was not seen. As abovementioned, the presence of Chol induce the formation of another lipid phase, the LO, which appears between the LE and the LC (see Fig. 2B) and can be confirmed by BAM pictures where, instead of the multilobed domains, an uniform grey phase with LE character in coexistence with dark dots characteristic of LO domains was observed. In the presence of the anticancer drugs, small white aggregates can be seen, which increase in number and size upon further compression. The BAM images of DPPC:SM:Chol monolayer containing anthracyclines in the interface present a heterogeneous organization, which is consistent with the condensed-liquid phase coexistence, where more compact density states that are usually thicker and show higher refraction index (bright domains) coexist with low molecular ones (dark domains).

BAM results show that the anticancer drugs clearly interact with all monolayers studied, causing alterations in the domain shape and appearing in the form of white aggregates. Furthermore, it is really interesting to observe that the investigated anthracyclines had different impact in the monolayer morphology. Thus, in the presence of DAN the condensed organized domains are completely absent; while in the presence of DOX these structures acquire a similar shape, but become bigger and more irregular.

CONCLUSIONS

In this work, the influence of anthracyclines, daunorubicin and doxorubicin, on the properties of 2D model membranes was investigated by employing Langmuir isotherms, BAM and PM-IRRAS techniques. From the provided complementary information, it can be concluded that the membrane composition and the lipid phase can determine the fate of drug-membrane interactions. At the same time, these drug molecules greatly affect the properties of the different lipid films studied. In fact, previous studies have demonstrated that DAN and DOX highly interact with membrane phospholipids and that interaction can lead to modifications in the lipid organization, which are believed to play an important function in their anticancer action. The gathered outcomes shown that the presence of DAN or DOX at 10 μM caused significant alterations in the π/A isotherms profile of each lipid monolayer and in the characteristic parameters. The most evident effect was the monolayer' expansion to higher molecular areas with consequent increase in the film compressibility as a result of anticancer drugs interaction. In fact, the studied compounds present a structure that allows them to penetrate into the hydrophobic region of the lipid film, which, consequently, led to the production of more flexible monolayers.

This intercalation between the lipid components can also help to explain the obtained higher areas per molecule when the anthracyclines were present in the interface. At the same time, is possible to observe that these findings were more evident for lower pressures where the differences in the area per molecule were bigger, which means that the drug penetration in the monolayer takes place more easily when it is less condensed. Taking into consideration the chemical structure of the studied compounds, it is expected that both hydrophobic and electrostatic forces take place in the lipid films. Therefore, for lower surface pressures the penetration of anthracyclines into lipid monolayers is easier because the monolayers are less compacted. However, with surface pressure augmentation, the molecules that were inserted with the dihydroxyanthraquinone ring system between the acyl chains are being "squeezed out" as a result of film order' increase, and the interaction becomes essentially electrostatic.

Furthermore, the obtained results pointed to different interactions with the monolayers that can be associated to the differences in the chemical structures of the anthracyclines molecules. Actually, these compounds had an opposite effect in the C=O group for all lipid monolayers that can be attributed to the presence of OH group in the end of the side chain of DOX molecules (absent in DAN). As a consequence of this distinct feature, DOX is able to establish more interactions in both polar and hydrophobic regions of the monolayer constituents, a behavior similar to cholesterol, which explains its higher impact in the monolayer properties (in both A and C_s^{-1}).

Another interesting observation is related to the fact that the effect produced in the films' compressibility by the interaction of anthracyclines was higher when cholesterol was absent. This probably happens because the anticancer drugs and cholesterol compete for the place in the monolayer. Consequently, their incorporation in the membrane without cholesterol is easier and produces higher modifications in its properties, as demonstrated by the Langmuir isotherms

results. Besides, when the monolayer was composed of only DPPC, the interactions between the lipid and the drug molecules were so strong that, with compression, were squeezed out from the film.

Additionally, the modification of the molecular packing was also confirmed by BAM images, where the typical lobed DPPC structures, which also appear in DPPC:SM film, could not be observed in the presence of DAN and acquired a similar shape, but become bigger and more irregular with DOX.

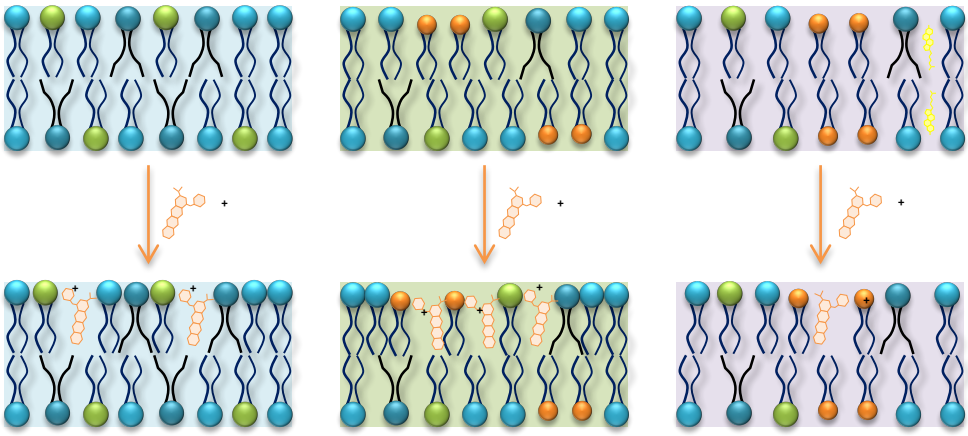
The knowledge acquired with this investigation about the structure-activity relationship is important to better understand the mechanism of action at the molecular level, which in turn is crucial to produce more effective and safer anticancer drugs. In fact, the clarification of the type of forces involved in these anticancer drug-membrane interactions might provide a deeper understanding of their action on cells.

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1. Weiss, R. B., The anthracyclines: will we ever find a better doxorubicin? Semin Oncol 1992, 19 (6), 670-86.
2. Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L., Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 2004, 56 (2), 185-229.
3. Tritton, T. R.; Hickman, J. A., Cell Surface Membranes as a Chemotherapeutic Target. In Experimental and Clinical Progress in Cancer Chemotherapy, Muggia, F. M., Ed. Springer US: Boston, MA, 1985; pp 81-131.
4. Tritton, T. R.; Yee, G., The Anticancer Agent Adriamycin Can Be Actively Cyto-Toxic without Entering Cells. Science 1982, 217 (4556), 248-250.
5. Escriba, P. V.; Sastre, M.; Garciasevilla, J. A., Disruption of Cellular Signaling Pathways by Daunomycin through Destabilization of Nonlamellar Membrane Structures. *P Natl Acad Sci USA* **1995,** *92* (16), 7595-7599.
6. Escriba, P. V., Membrane-lipid therapy: a new approach in molecular medicine. *Trends Mol Med* **2006,** *12* (1), 34-43.
7. Escriba, P. V.; Gonzalez-Ros, J. M.; Goni, F. M.; Kinnunen, P. K.; Vigh, L.; Sanchez-Magraner, L.; Fernandez, A. M.; Busquets, X.; Horvath, I.; Barcelo-Coblijn, G., Membranes: a meeting point for lipids, proteins and therapies. *J Cell Mol Med* **2008,** *12* (3), 829-75.
8. Goormaghtigh, E.; Chatelain, P.; Caspers, J.; Ruysschaert, J. M., Evidence of a specific complex between adriamycin and negatively-charged phospholipids. *Biochim Biophys Acta* **1980,** *597* (1), 1-14.
9. Karczmar, G. S.; Tritton, T. R., The interaction of adriamycin with small unilamellar vesicle liposomes. A fluorescence study. *Biochim Biophys Acta* **1979,** *557* (2), 306-19.
10. van Meer, G.; Voelker, D. R.; Feigenson, G. W., Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **2008,** *9* (2), 112-24.
11. Slotte, J. P., Biological functions of sphingomyelins. *Prog Lipid Res* **2013,** *52* (4), 424-437.
12. Maxfield, F. R.; Tabas, I., Role of cholesterol and lipid organization in disease. *Nature* **2005,** *438* (7068), 612-621.
13. Wydro, P., Sphingomyelin/phosphatidylcholine/cholesterol monolayers - analysis of the interactions in model membranes and Brewster Angle Microscopy experiments. *Colloids Surf B Biointerfaces* **2012,** *93*, 174-179.
14. Lingwood, D.; Simons, K., Lipid Rafts As a Membrane-Organizing Principle. *Science* **2010,** *327* (5961), 46-50.
15. Nunes, C.; Brezesinski, G.; Lopes, D.; Lima, J. L.; Reis, S.; Lucio, M., Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality. *J Phys Chem B* **2011,** *115* (43), 12615-23.
16. Wydro, P., Sphingomyelin/phosphatidylcholine/cholesterol monolayers--analysis of the interactions in model membranes and Brewster Angle Microscopy experiments. *Colloids Surf B Biointerfaces* **2012,** *93*, 174-9.
17. Mendelsohn, R.; Mao, G.; Flach, C. R., Infrared reflection-absorption spectroscopy: principles and applications to lipid-protein interaction in Langmuir films. *Biochim Biophys Acta* **2010,** *1798* (4), 788-800.
18. Vollhardt, D.; Fainerman, V. B., Characterisation of phase transition in adsorbed monolayers at the air/water interface. *Adv Colloid Interface Sci* **2010,** *154* (1–2), 1-19.
19. Matyszewska, D.; Brzezinska, K.; Juhaniewicz, J.; Bilewicz, R., pH dependence of daunorubicin interactions with model DMPC:Cholesterol membranes. *Colloids Surf B Biointerfaces* **2015,** *134*, 295-303.
20. Ramin, M. A.; Le Bourdon, G.; Daugey, N.; Bennetau, B.; Vellutini, L.; Buffeteau, T., PM-IRRAS investigation of self-assembled monolayers grafted onto SiO2/Au substrates. *Langmuir* **2011,** *27* (10), 6076-84.
21. Nunes, C.; Brezesinski, G.; Pereira-Leite, C.; Lima, J. L.; Reis, S.; Lucio, M., NSAIDs interactions with membranes: a biophysical approach. *Langmuir* **2011,** *27* (17), 10847-58.
22. Buehler, L. K., *Cell Membranes*. Garland Science Taylor & Francis Group: New York, 2015.
23. Feng, S.-S.; Gong, K.; Chew, J., Molecular Interactions between a Lipid and an Antineoplastic Drug Paclitaxel (Taxol) within the Lipid Monolayer at the Air/Water Interface. *Langmuir* **2002,** *18* (10), 4061-4070.
24. Vitovic, P.; Nikolelis, D. P.; Hianik, T., Study of calix[4]resorcinarene-dopamine complexation in mixed phospholipid monolayers formed at the air-water interface. *Biochim Biophys Acta* **2006,** *1758* (11), 1852-61.
25. Duncan, S. L.; Larson, R. G., Comparing Experimental and Simulated Pressure-Area Isotherms for DPPC. *Biophys J* **2008,** *94* (8), 2965-2986.
26. Duncan, S. L.; Dalal, I. S.; Larson, R. G., Molecular dynamics simulation of phase transitions in model lung surfactant monolayers. *Biochim Biophys Acta* **2011,** *1808* (10), 2450-65.
27. Jiao, X.; Keating, E.; Tadayyon, S.; Possmayer, F.; Zuo, Y. Y.; Veldhuizen, R. A., Atomic force microscopy analysis of rat pulmonary surfactant films. *Biophys Chem* **2011,** *158* (2-3), 119-25.
28. Zhang, H.; Fan, Q.; Wang, Y. E.; Neal, C. R.; Zuo, Y. Y., Comparative study of clinical pulmonary surfactants using atomic force microscopy. *Biochim Biophys Acta* **2011,** *1808* (7), 1832-42.
29. Matyszewska, D.; Bilewicz, R., Interactions of daunorubicin with Langmuir–Blodgett thiolipid monolayers. *Electrochim Acta* **2015,** *162*, 45-52.
30. Gaber, M. H.; Ghannam, M. M.; Ali, S. A.; Khalil, W. A., Interaction of Doxorubicin with phospholipid monolayer and liposomes. *Biophys Chem* **1998,** *70* (3), 223-9.
31. Blaudez, D.; Turlet, J.-M.; Dufourcq, J.; Bard, D.; Buffeteau, T.; Desbat, B., Investigations at the air/water interface using polarization modulation IR spectroscopy. *J Chem Soc Faraday Trans* **1996,** *92* (4), 525-530.
32. Mattjus, P.; Slotte, J. P., Does cholesterol discriminate between sphingomyelin and phosphatidylcholine in mixed monolayers containing both phospholipids? *Chem Phys Lipids* **1996,** *81* (1), 69-80.
33. Somasundaran, P., *Encyclopedia of Surface and Colloid Science*. Taylor and Francis Group, LLC: New York, 2006; Vol. 5.
34. Alonso, C.; Alig, T.; Yoon, J.; Bringezu, F.; Warriner, H.; Zasadzinski, J. A., More than a monolayer: relating lung surfactant structure and mechanics to composition. *Biophys J* **2004,** *87* (6), 4188-202.

35. McConlogue, C. W.; Vanderlick, T. K., A Close Look at Domain Formation in DPPC Monolayers. *Langmuir* **1997,** *13* (26), 7158-7164.
36. Castro, C. M.; Pinheiro, M.; Lucio, M.; Giner-Casares, J. J.; Camacho, L.; Lima, J. L.; Reis, S.; Segundo, M. A., Insights about alpha-tocopherol and Trolox interaction with phosphatidylcholine monolayers under peroxidation conditions through Brewster angle microscopy. *Colloids Surf B Biointerfaces* **2013,** *111*, 626-35.

5. The daunorubicin interplay with mimetic model membranes of cancer cells: a biophysical interpretation



Dan decreases the membrane fluidity in the phospholipid's hydrophobic region.

Dan's membrane **partition** and **location** and influence in membrane **fluidity.**



HIGHLIGTHS

- DAN-membrane interaction is driven by electrostatic and hydrophobic interactions.
- Presence of PE increases DAN's membrane partition while Chol has the opposite effect.
- DAN membrane location affects the lipid organization of both phospholipid' headgroups and tails.
- DAN increases the overall order of the membranes' deeper regions.
- Membrane interplay of DAN constitutes an additional drug' mechanism

The daunorubicin interplay with mimetic model membranes of cancer cells: a biophysical interpretation

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The present work aimed to study the interactions between the anticancer drug daunorubicin and lipid membrane mimetic models of cancer cells composed by their most representative classes of phospholipids, with different degrees of complexity.
Regarding these anticancer drug-membrane interactions, several biophysical parameters were assessed using liposomes (LUVs) composed of different molar ratios of DMPC, DOPC, DPPS, DOPE and Chol. In this context, daunorubicin's membrane concentration was determined by calculating its partition coefficient (Kp) between liposomes and water using derivative UV/vis spectrophotometry at 37°C and pH 6.3, a typical tumoral microenvironment. Characterization of the zeta potential of such model membranes, in both the absence and presence of the compound, was accomplished through Electrophoretic Light Scattering (ELS). Fluorescence quenching studies, which determine the location of the drug that interacts with the bilayer, were carried out using liposomes labeled with DPH and TMA-DPH, fluorescent probes with known membrane position. Temperature dependent steady-state anisotropy assays were also performed to measure the daunorubicin effect on the membranes' microviscosity.
The overall results obtained support that daunorubicin permeation depends on the phospholipid membrane composition and causes alterations in the biophysical properties of the bilayers, namely in the membrane fluidity. The interaction of daunorubicin with the studied phospholipids is mainly driven by electrostatic interactions and hydrophobic bonds.
These insights demonstrated that not only membranes can affect daunorubicin accumulation in cells but the compound can alter the properties of membranes. The changes produced by daunorubicin on the lipid structure may constitute an additional mechanism of action, which might lead to modifications in the location and, consequently, the activity of membrane signaling proteins.

cancer	phosphatidylethanolamine	phosphatidylserine
location	fluidity	partition

Anthracyclines are considered one of the most effective group of anticancer drugs ever developed [1, 2]. Daunorubicin, an anthracycline antibiotic, is widely used for the treatment of different types of cancer, including leukemia, breast cancer, ovarian cancer and lung carcinoma, as well as several sarcomas [3]. In addition to the development of multidrug resistance by cancer cells, which impairs therapy efficiency, cardiotoxicity also represents a major obstacle in the use of this class of cytotoxic agents [3].
Daunorubicin's mechanisms of cytotoxicity in cancer cells are diverse and include binding to DNA, which causes a stabilization of the DNA double strand, inhibiting the process of duplication and transcription of mRNA; DNA damage by the inhibition of topoisomerase II; direct membrane effect as it intercalates into biological membranes; and production of free oxygen radicals [3]. Beyond the overall mechanisms of action, abundant evidence suggests that the cell membrane can, itself, be a target for these types of agents [4-6]. Actually, biomembranes are one of the first structures encountered by drugs after their systemic administration. Furthermore, the cell membrane is involved in several biological functions [7], such as regulation of protein location and activity, and modifications of its biophysical properties could trigger different processes implicated in anticancer agents' cytotoxic effects. Therefore, it is not surprising that biological membranes are involved, and can even play a key role, in daunorubicin's biological action.
Even though the emergence of cancer cell may have a genetic link, significant data shows that cells, during malignant transformation, suffer alterations in their membrane's lipid profile and biophysical properties [8, 9]. In fact, under normal conditions, phosphatidylcholine (PC) and sphingomyelin (SM) are mainly found in the outer leaflet of the plasma membrane, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are confined to the inner one [10]. In contrast with this scenario, it has been observed that PS is exposed in the outer leaflet of cancer cells' membranes [11-13]. Since the distribution of PE and PS within the membrane is maintained by the same aminophospholipid translocases, it has been demonstrated that PE along with PS can also be localized in the external leaflet of tumor endothelial cells [14].
In addition to lipid content, membrane fluidity of cancer cells seems to be different from their normal counterparts. In fact, although no common pattern was evidenced, a number of studies have demonstrated a significant correlation between plasma membrane fluidity and cancer malignancy [15, 16]. It appears that some tumor cells exhibit higher membrane fluidity than normal cells [16-23] due to low levels of cholesterol and sphingomyelin [24, 25] and increased percentage of unsaturated fatty acids [26]. Other cancer cells present a decreased membrane fluidity, such as the hepatoma tumor cell line, as a result of a lower degree of fatty acid unsaturation and increased cholesterol content [27].
In view of the abovementioned, it becomes clear that cancer cell membranes are altered in many different aspects. From the pharmacological point of view, it is important to understand the molecular mechanisms through which drugs interplay with cell membranes. In fact, the different characteristics of membranes can, themselves, influence a drugs' pharmacological activity, being fundamental for their pharmacokinetics, efficacy and toxicity [28-30]. On the other hand, the interaction of drugs with membranes can modulate their biophysical properties, affect their function and integrity and,

Table 1. Lipid composition (in molar ratio) used to develop the mimetic model membranes.

MODEL MEMBRANE	Lipid Composition (molar ratio)				
	DMPC	DOPC	DPPS	DOPE	Chol
DMPC:DOPC:DPPS	6	2	2	--	--
DMPC:DOPC:DPPS:Chol	5	1.7	1.7	--	1.6
DMPC:DOPC:DPPS:DOPE	4	1.5	1.5	3	--
DMPC:DOPC:DPPS:DOPE:Chol	4	1	1	2.5	1.5

ultimately, modify protein activity [4, 7, 8]. Drug-membrane studies arise, therefore, as fundamental assays to unveil the biophysical mechanisms behind the therapeutic and toxic effects of daunorubicin. For that reason, the main goal of this work is to systematically characterize the influence of daunorubicin on the biophysical properties of membranes and further correlate these effects with some of their pharmacological actions. The use of membrane model systems, such as liposomes, allows one to more easily perform these studies. Actually, liposomes mimic biomembranes constitution and dimensionality and, at the same time, minimize their molecular complexity. In this context, different mimetic models were designed in order to simulate some of the abovementioned features encountered in cancer cells as lipid content is concerned (presence of PS and PE). Cholesterol has also been included since it represents an important component of biological membranes, being involved not only in the control of membrane fluidity but also in its structure [8]. The external pH conditions encountered by daunorubicin in the tumor microenvironment (acidic pH) were also taken into account. In fact, several studies reported that this acidic extracellular pH, caused by the high glucose consumption rates [31], is a typical intrinsic feature of tumor phenotype and benefits cancer cells once it promotes invasiveness.

Materials and Methods

Materials. Daunorubicin (DAN) was obtained from Biovision (Milpitas, California). The lipids: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC); cholesterol (Chol); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-L-serine (DPPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). All reagents were used without further purification. Tris maleate was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Drug solutions were prepared in Tris buffer (0.1 M, pH 6.3). The buffer was prepared using double-deionized water (conductivity lower than 0.1 μS cm^{-1}).

Liposomes Preparation. Liposomes were prepared according to the adapted classic thin film hydration method [32]. Briefly, the lipids (Table 1) were dissolved in chloroform/methanol (3:2 v/v) and then evaporated under a nitrogen stream in a rotative evaporator (Buchi R-200, equipped with a thermostated bath Buchi R-490, Switzerland). The lipid film was hydrated in Tris buffer (pH 6.3) and vortexed to yield multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were formed by extruding the MLVs eleven times (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) through policarbonate filters with a diameter pore of 100nm (Nucleopore) at 60°C (temperature above the main phase transition temperature of the lipids). The labelled liposomes were obtained by adding the fluorescence probe (DPH or TMA-DPH) in the organic phase to give a probe/lipid molar ratio of 1:300. Afterwards, labelled LUVs were similarly prepared by evaporation of the organic solvents, by dissolution of the dried lipid film and by extrusion of the MLVs suspension.

Table 1 presents the lipid composition used to develop the model membranes in order to mimic some of the external characteristics found in tumor membranes, namely the presence of PS and PE. It has been reported that PS and PE, which are usually absent from the plasma
membrane outer monolayer under normal conditions, are exposed in cancer cells [12, 14].

Zeta Potential characterization by Electrophoretic Light Scattering (ELS). The Zeta potential was assessed using a Brookhaven™ BI-MAS and Zeta-Pals (Brookhaven Instruments, Holtsville, NY, USA) at 37.0±0.1°C. Lipid concentration was kept constant at 500μM while daunorubicin concentrations ranged between 0 and 75μM. For each sample, the mean zeta potential values of six runs of 10 cycles each were calculated. The values obtained were calculated from at least three independent assays.

Determination of Partition Coefficients by derivative UV-Vis spectrophotometry. The partition coefficient (Kp) of daunorubicin between liposomes and an aqueous phase was assessed by derivative UV-Vis spectrophotometry. Tris buffered LUV suspensions containing a fixed concentration of the anticancer agent (40μM) and increasing concentrations of lipid solution (in the range of 0–1500μM) were prepared. The corresponding reference solutions were identically prepared but in the absence of drug. All samples were incubated for 30min at 37°C. The absorption spectra were recorded using a multidetection microplate reader (Synergy™ HT, BioTek Instruments Inc, Winooski, VT, USA) at 37°C. The Kp values are then determined as described in [33]. The V_m values for the mimetic systems studied (DMPC:DOPC:DPPS,

Table 2: Characterization of liposomes' zeta potential with increasing daunorubicin concentration. Studies conducted at 37°C and pH 6.3.

MODEL	ZETA (mV)			
	0 μM	5μM	40 μM	75 μM
DMPC:DOPC:DPPS	-42 ± 3	-41 ± 6	-38 ± 3	-31* ± 3
DMPC:DOPC:DPPS:Chol	-56 ± 4	-56 ± 3	-52 ± 3	-42* ± 4
DMPC:DOPC:DPPS:DOPE	-35 ± 2	-29* ± 2	-21* ± 2	-17* ± 2
DMPC:DOPC:DPPS:DOPE:Chol	-43 ± 3	-41 ± 2	-25* ± 3	-21* ± 2

DMPC:DOPC:DPPS:Chol, DMPC:DOPC:DPPS:DOPE, DMPC:DOPC:DPPS:DOPE:Chol) were 0.696, 0.646, 0.710 and 0.670 L mol^{-1}, respectively. The obtained V_m values were calculated using the specific volume of each lipid (DMPC [34], DOPC [35], DOPE [36], DPPS and Chol [37]) and taking into account the different lipid molar ratio used for each model membrane. The Kp values obtained are adimentional and presented as the mean ± standard deviation calculated from at least three different assays.

Drug Location Studies Performed by Fluorescence Quenching. The membrane location of daunorubicin was assessed by steady-state and lifetime fluorescence quenching measurements using DPH and TMA-DPH probes with a known membrane position and depth [38, 39]. Quenching experiments were conducted by incubation of samples containing a fixed concentration of lipid (500μM) and increasing concentrations of daunorubicin (0–75μM) at 37°C for 30min in the dark. The fluorescence measurements were performed at 37°C and pH 6.3 with excitation/emission wavelengths defined as 357/429 nm, and 359/429 nm, for DPH and TMA-DPH, respectively. Fluorescence steady-state measurements were carried out in a Jasco FP-6500 spectrofluorometer (Jasco, Great Dunmow, UK) equipped with a constant temperature cell holder and all data was recorded in a 1 cm path length cuvette. For each measurement, the fluorescence emission was automatically acquired during 30 s. The fluorescence intensity values obtained were corrected for inner filter effects (quencher absorbance) at the excitation wavelength [40].
The time-resolved fluorescence measurements were performed using a Fluorolog Tau-3 Lifetime system. The modulation frequencies were acquired between 10 and 200 MHz, with an integration time of 10 s. The fluorescence emission was detected with a 90° scattering geometry. All measurements were made using Ludox as a reference standard (τ=0.00 ns).

Membrane Fluidity Studies Performed by Fluorescence Anisotropy. Fluorescence anisotropy measurements with liposomes labelled either with DPH or TMA-DPH probes were used to study the effect of daunorubicin on membrane fluidity. Samples containing different concentrations of daunorubicin (0, 40 and 75μM) and a fixed concentration of lipid (500μM), labelled with DPH or TMA-DPH, were incubated for 30min at 37°C in the dark. The steady-state fluorescence anisotropy measurements was recorded between 10 and 60°C with intervals of 2°C, according with anisotropy equation presented in [41]. The experiments were performed in the Jasco FP-6500 spectrofluorometer with polarizers inserted (excitation/emission wavelengths were set as 357/429 nm for DPH and 359/429 nm for TMA-DPH). The results obtained are presented as the mean ± standard deviation calculated from three different assays.

Statistical analysis. Statistical analysis was performed using IBM® SPSS® Statistics software (v.21.0.0.0, IBM, Armonk, NY, USA). The measurements were repeated at least three times and data was expressed as mean ± standard deviation (SD). Data was statistically analyzed through the one-way analysis of variance (ANOVA) method and differences between groups were compared by Bonferroni and Tukey post-hoc tests in which a p value lower than 0.05 ($p<0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Zeta potential characterization

The influence of daunorubicin in model membranes' charge was analyzed and the results obtained are depicted in Table 2. Regarding zeta potential values, all membrane models present a negative surface charge due to the presence of the negatively charged phosphatidylserine. The presence of increasing drug concentration lead to an alteration of the membrane zeta potential towards a less negative surface charge. This outcome can be explained by the fact that daunorubicin molecules are positively charged at pH 6.3, thereby, interacting with the phospholipid's headgroups by electrostatic interactions.

Daunorubicin Partition Coefficients in mimetic model membranes

The drug's partition coefficient (Kp) between aqueous and lipid medium strongly influences its pharmacokinetic and pharmacodynamic properties. In fact, drugs need to cross several biological membranes, generally by passive diffusion processes, in order to be absorbed, distributed, metabolized, and eliminated. The determination of the partition coefficient (Kp) becomes, therefore, important to understand and explain the compounds biological activity [42] and should be the first step when studying drug-membrane interactions. The partition coefficient can be calculated through measurements of zeta potential or by derivative spectrophotometry, as explained in [43]. In this work, the daunorubicin partition coefficient was assessed in the several model membranes by derivative UV-Vis

Table 3. Experimental partition Coefficients (expressed as Kp and logD) of daunorubicin for the different model membranes at pH 6.3 and 37°C.[a]

MODEL	Kp	Experimental logD
DMPC:DOPC:DPPS	1565 ± 28*	3.19 ± 0.01*
DMPC:DOPC:DPPS:Chol	747 ± 21*	2.87 ± 0.01*
DMPC:DOPC:DPPS:DOPE	2164 ± 24*	3.34 ± 0.01*
DMPC:DOPC:DPPS:DOPE:Chol	582 ± 35*	2.76 ± 0.03*

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other models.

spectrophotometry due to the several advantages that the method presents.

Fig. 1 (A) shows, as an example, the third-derivative absorption spectra of daunorubicin with increasing lipid concentration at pH 6.3 and 37°C, which allowed the elimination of the background noise caused by light scattering of lipid vesicles. A shift in the λ_{max} with increasing lipid concentrations can be observed, indicating that daunorubicin partitions from the aqueous medium to the liposomes [44]. The best fit of equation from [33] to the third-derivative data was achieved at 546nm and is shown in Fig. 1 (B). Similar results were obtained for the remaining model membranes. The values of Kp and logD are listed in Table 3.

The values predicted for the octanol/water (0.91 at pH 6.3), calculated using Marvin sketch calculator software from Chemaxon, are significantly different from those obtained experimentally using liposomes. Such discrepancy is related to the type of interactions that are assessed by each technique: while the octanol/water can only take into consideration hydrophobic interactions between the compound and octanol; the liposome/water system also allow one to study electrostatic interactions, due to the amphiphilic nature of phospholipids. For that reason, liposomes provide additional information to that obtained from the classical biphasic octanol/water system. In fact, from the calculated pKa values (9.17), using Marvin sketch calculator, it was possible to predict that around 98% of daunorubicin molecules are positively charged at pH 6.3, which points towards a drug-membrane interaction mainly driven by electrostatic forces. Furthermore, it can be found that the compound's partition is highly dependent on the lipid composition of the mimetic models studied (Table 3), which highlights the importance of using the liposome/water system in order to obtain more realistic information about daunorubicin lipophilicity and, therefore, its *in vivo* membrane partition. On the other hand, the theoretical logD value is much smaller than the experimental one, since it only reflects the hydrophobic interactions of a very small amount of molecules that are in the neutral form at pH 6.3.

From the experimental gathered values (Table 3) it is possible to conclude that, not only the daunorubicin properties such as the ionization state but also the membrane lipid composition and its biophysical features are important factors that affect the drug concentration between the membrane and aqueous medium. Indeed, there is a higher daunorubicin partition in the presence of PE in models containing DMPC:DOPC:DPPS, probably due to the PE geometry. Phosphatidylethanolamines display a cone shape because of their small polar headgroups, producing a lower surface packing density and, as a consequence, the membrane surface becomes more easily accessible for the drug. On the other hand, the addition of Chol causes an increase in membrane complexity and a decrease in fluidity, which, in turn, reduces the amount of anticancer drug that can partition in these mimetic models.

Drug location studies using different fluorescent probes

Daunorubicin location within the different liposomes was assessed using fluorescence quenching of several probes with a known position within the membrane [45]. More specifically, while DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer [39], TMA-DPH is reported to be anchored in the polar head groups region of phospholipids due to its charged group [38].

The drug's efficiency to quench the fluorophore is related to its proximity to the probe. Since the probes used in this study are inserted in the bilayer, only the drug that partitions into the membrane will be able to act as a quencher. In this context, it is important to calculate daunorubicin membrane concentration $[Q]_m$ that is given by equation 2:

$$[Q]_m = \frac{K_p[Q]_T}{K_p\alpha_m + (1 - \alpha_m)} \quad (2)$$

where α_m is the volume fraction of membrane phase ($\alpha_m = V_m/V_T$; V_m and V_T represent the volumes of the membrane and water phase, respectively) and $[Q]_T$ represents the total daunorubicin concentration used. The Kp used to calculate the $[Q]_m$ were the experimental values obtained for each membrane model.

The ability of daunorubicin to quench the fluorescence of DPH and TMA-DPH probes was assessed by the determination of the Stern–Volmer constant (K_{SV}) from the slope of the Stern–Volmer plots obtained by steady-state fluorescence measurements (I_0/I) [45]. In order to differentiate the quenching mechanism (static, dynamic or both), the dynamic quenching constant (K_D) was calculated from the slope of the Stern–Volmer plots obtained by lifetime fluorescence measurements (τ_0/τ) [45]. From the gathered results, it is possible to conclude that daunorubicin molecules have effectively quenched all probes in all mimetic membranes. In Fig. 2, the Stern–Volmer plots of I_0/I -1 and τ_0/τ -1 in function of daunorubicin membrane concentration are shown, for DPH probe. Similar results were obtained for the TMA-DPH probe (Fig. S1 *Supporting Information*).

According to the aforementioned description of the quenching behavior, the quenching parameters, namely the dynamic constant (K_D), the static constant (K_S) and the Stern–Volmer constant (K_{SV}), obtained from this technique are presented in Table 4.

The observation of the obtained data leads to the conclusion that all probes were quenched by daunorubicin, albeit with different quenching efficiencies depending on the type of mimetic model membrane. It appears that the quenching process results

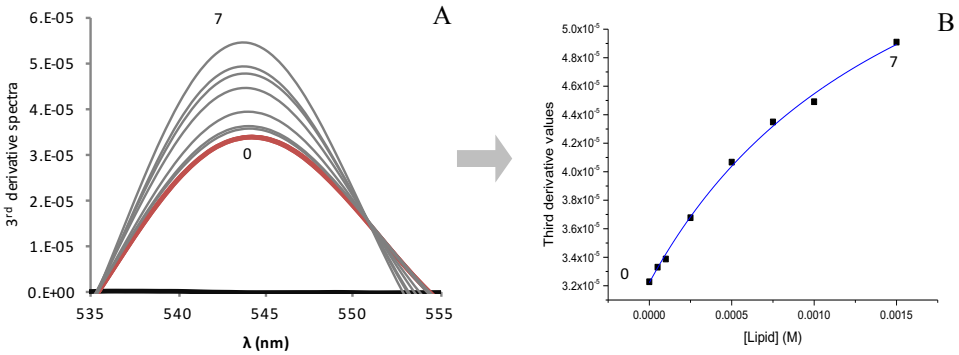


Fig. 1. Third-derivative absorption spectra (A) of daunorubicin (40μM) alone (red line, 0), incubated in DMPC:DOPC:DPPS model membrane with increasing lipid concentration (7, represents the maximum lipid concentration) at 37°C and pH 6.3. Plot (B) represents the best fitting curve to the experimental third-derivative spectrophotometric data (D_T vs. [L]) using a nonlinear regression method at a wavelength of 546nm.

from both collisional and static interactions, which mean that daunorubicin must diffuse to the fluorophore during the lifetime of the excited state but it also might form a non-fluorescent complex. Furthermore, daunorubicin induced a concentration dependent reduction of light emitted by both probes incorporated into liposomes, which indicates a broad distribution of the drug across the bilayer. This fact suggests that the drug is submerged inside the lipid bilayer but also near the phospholipid head. However, the decrease of fluorescence was more pronounced for DPH than for TMA-DPH in all model systems, as indicated by the higher values of K_{sv}. In fact, the membrane location of daunorubicin can be correlated with its structure, lipophilicity and also with the degree of ionization of the drug at pH 6.3. This is in good agreement with the occurrence of electrostatic interactions between the cationic drug and the phosphate group of the polar head groups of the phospholipids and hydrophobic interactions between the drug's dihydroanthraquinone residue and the lipid fatty acid chains. Furthermore, the gathered results are in good agreement with other studies where it has been demonstrated, using model membranes with lipid composition different from our model systems, that the glucosamine moiety of daunorubicin strongly interacts with the external surface of the bilayer [46] and the fluorophore moiety of the drug presents a deeper location in the membrane [46, 47].

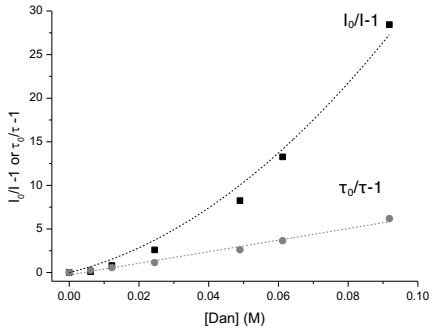


Fig. 2. Stern–Volmer plots of the probe DPH in the DMPC:DOPC:DPPS:DOPE model: square symbols represent the plot obtained by steady-state fluorescence measurements ($I_0/I-1$) and circle symbols represent the obtained by lifetime fluorescence measurements ($\tau_0/\tau-1$).

Influence of daunorubicin on membrane fluidity

Since anticancer drugs interact with membranes in order to reach their intracellular targets, it is crucial to understand how these compounds can induce perturbation in the physicochemical state of the phospholipid bilayer, as it can provide valuable information about their therapeutic and toxic effects. In fact, membrane fluidity plays a key role in the maintenance of most basic cellular functions [48]. Therefore, steady-state fluorescence anisotropy [41] of DPH and TMA-DPH probes was used to study the effect of daunorubicin on the membrane's fluidity. Due to the different probes' location within the membrane, it is possible to infer about daunorubicin's ability to disturb the membrane structure in different regions, since these probes report the microfluidity of those sites [49]. Such membrane alterations can be observed by changes in anisotropy that are the reflection of a probe's rotational movement caused by differences in the stiffness of its surrounding matrix [50].

The effect of temperature on the DPH and TMA-DPH fluorescence anisotropy of the different model membranes without daunorubicin is presented in Fig. S2 (*Supporting Information*).

The anisotropy values decrease with increasing temperature at higher rate in the acyl chain region (given by the probe DPH) than in the phospholipid head region (given by the TMA-DPH probe). In fact, it is well documented that the membrane is characterized by a fluidity gradient from the aqueous interface to the bilayer interior, where the acyl chain end causes an increased disorder [51].

The influence of daunorubicin on the DPH and TMA-DPH fluorescence anisotropy as a function of temperature for each model system studied is presented in Fig. 3 and Fig. 4, respectively. It becomes evident that daunorubicin produces a differential perturbation in membrane fluidity, depending on composition and on the region within the membrane (i.e. near the head groups or near the hydrocarbon tails).

Analyzing the results obtained for the DPH probe, it appears that increased drug concentration led to an increase in anisotropy values, except in the case of DMPC:DOPC:DPPS:Chol at higher drug

Table 4: Fluorescence quenching parameters (K_D, dynamic constant; K_S, static constant; K_{SV}, Stern–Volmer constant) calculated from the quenching of DPH and TMA-DPH by daunorubicin in the different mimetic models, at 37°C and pH 6.3. [a]

Model	Probe	K_D (M^{-1})	K_S (M^{-1})	K_{SV} (M^{-1})
DMPC:DOPC:DPPS	DPH	88 ± 5*	108 ± 9*	196 ± 4*
	TMA-DPH	53 ± 6*	52 ± 5*	105.1 ± 0.4*
DMPC:DOPC:DPPS:Chol	DPH	73 ± 1*	324 ± 29*	397 ± 30*
	TMA-DPH	94 ± 4*	60 ± 3*	155 ± 2*
DMPC:DOPC:DPPS:DOPE	DPH	34 ± 4	36 ± 2*	67 ± 5
	TMA-DPH	37 ± 3	23 ± 2*	58.5 ± 0.3
DMPC:DOPC:DPPS:DOPE:Chol	DPH	96 ± 6	130 ± 11	233 ± 27*
	TMA -DPH	90 ± 3	85 ± 4	175 ± 1*

[a] All values represent the mean ± standard deviation (n=3).

* $p < 0.05$ statistically different from the same parameter for the other probe.

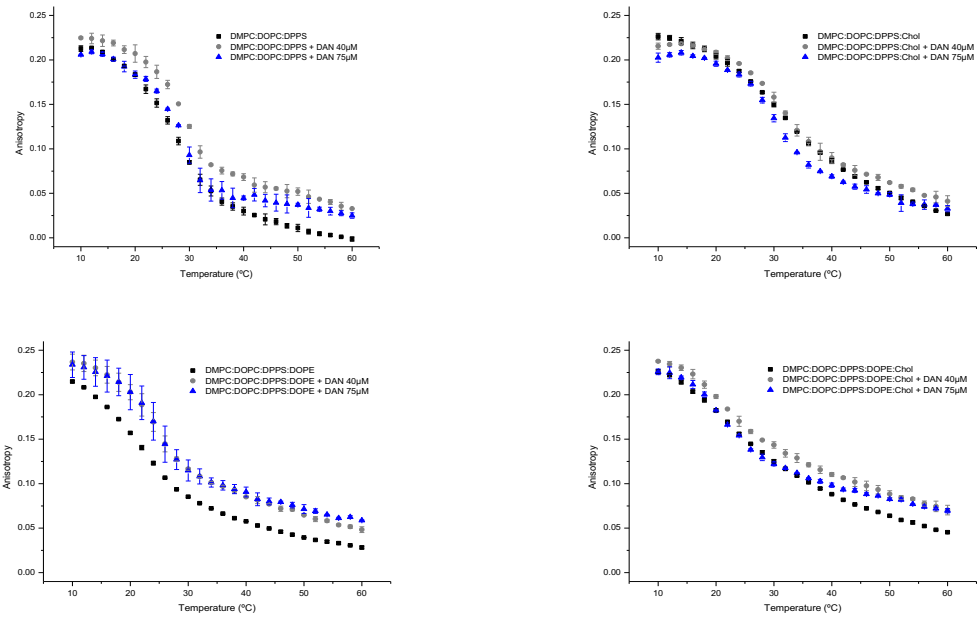


Fig. 3. Steady-state anisotropy of DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of DAN 40 μM (●) and 75 μM (▲), at pH 6.3. Results present the mean of at least three independent assays.

concentrations. This means that the anticancer drug induces a higher lipid packing of the models membrane in a deeper region, probably due to its intercalation in the hydrophobic region of the phospholipids. Nevertheless, this effect is less pronounced in models that contain Chol. In fact, as already mentioned, Chol decreases the fluidity and increases the lipid packing of membranes and, as a consequence, the bilayer becomes less accessible to the drug. These outcomes are consistent with the drug partition coefficient, which supports the notion that daunorubicin penetration within the membrane is less pronounced when Chol is present. Probably the increase in membrane fluidity for DMPC:DOPC:DPPS:Chol at higher DAN concentrations may be due to a different drug orientation within the membrane when compared to the other models membrane.

On the other hand, the results obtained for TMA-DPH show that there is a significant increase in membrane fluidity near the head groups in liposomes containing PE. Once more, these findings are in agreement with the previous partition outcomes, where it has been observed that, due to the shape of PE, daunorubicin can more easily permeate the membrane. Because daunorubicin establish electrostatic interactions with the negative phosphate groups, it must increase the distances between the several phospholipid headgroups in such regions (increase in membrane fluidity), due to the small head of PE.

Overall, the results are in close agreement with the partition and location studies, evidencing that daunorubicin behavior can be modulated depending on the type of lipid and molecular order used in the membrane. Indeed, specific membrane biophysical properties can benefit or impair the drug's interplay with these cell boundaries. Besides, due to its ionization state, daunorubicin has the ability to establish electrostatic interactions with the phospholipids heads. A more superficial location of daunorubicin is expected (shown by TMA-DPH) in model membranes and, as a consequence, changes in the lipid organization of such region are observed (shown by fluidity measurements). Nevertheless, the anticancer drug's molecular structure also allows it to interact through hydrophobic forces with the phospholipid tails (displayed by DPH location

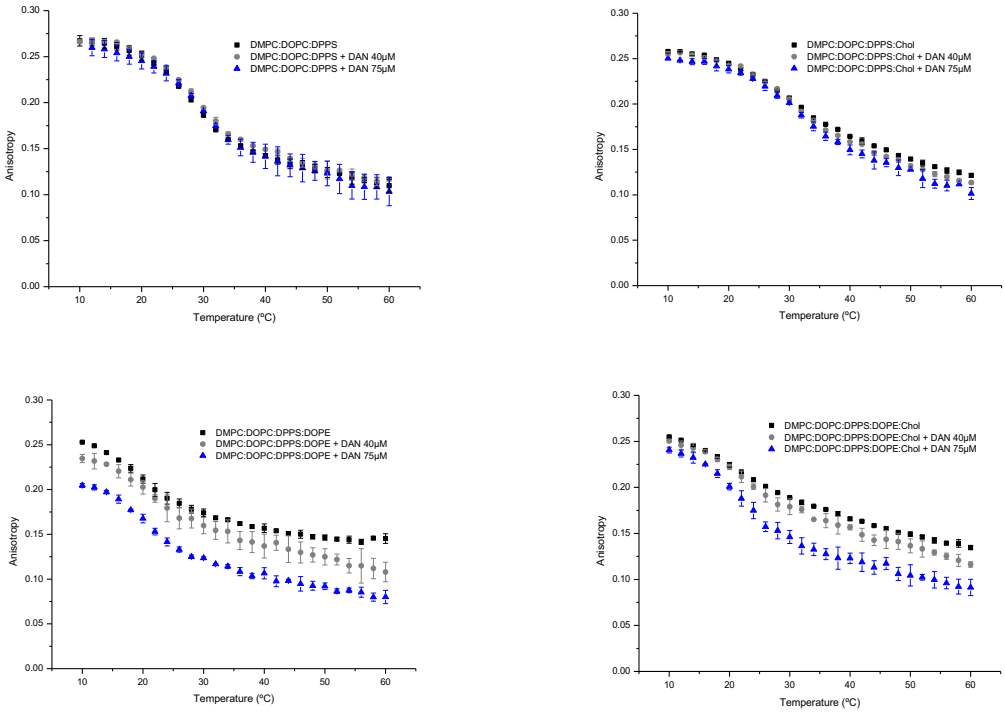


Fig. 4. Steady-state anisotropy of TMA-DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of DAN 40 μM (●) and 75 μM (▲), at pH 6.3. Results present the mean of at least three independent assays.

studies) and increase the order of the deeper region of the membranes (verified by fluidity measurements).

CONCLUSIONS

Different reports over the past years demonstrated that the membrane of cancer cells suffers several alterations in its lipid profile and properties, particularly in terms of fluidity, during their malignant transformation. For that reason, the present work aimed to develop different model systems to assess the effects of PS and PE exposure in the external monolayer and the influence of unsaturated chains and cholesterol on daunorubicin membrane-interactions, in order to understand how specific changes in lipid composition of the cancer membrane can be reflected in the drug's behavior. The overall results show that the partition of daunorubicin determined using liposome/water systems, an indicator of the drug's affinity towards a specific membrane, is influenced by the different characteristics of those models. This is the first point that demonstrates the importance of the membranes in daunorubicin's biological activity, because only the drug that can pass through this boundary structure can reach the intracellular target. Therefore, the several findings obtained can be correlated with daunorubicin's pharmacological effects. Regarding this, the high partition coefficient obtained for the anticancer drug in the mimetic models is consistent with its mechanism of action and its "selectivity" towards cancer cell membranes. Furthermore, the alteration of membrane' properties by the anticancer drug are expected to influence the location and activity of proteins embedded in the membranes. It is well described that phosphatidylethanolamines have the propensity to form nonlamellar membrane structures, favoring the association of different proteins (such as protein kinase C family and G proteins) to membranes that are involved in many signal transduction cascades [52, 53]. For that reason, such changes can lead to modifications in signaling cascades which might alter the cell function and can, therefore, be correlated with daunorubicin mechanism of cytotoxicity. In fact, Escriba *et al*. have demonstrated that daunorubicin influence several cellular signaling pathways through a direct interaction with membrane PE [5].

The use of two fluorescent probes with different membrane location allowed to confirm that daunorubicin is able to penetrate all membranes and is placed within the phospholipids, positioned between the polar region (where it establishes electrostatic interactions with the negative phosphate group) and the membrane core (where it establishes hydrophobic interactions with the acyl chains). Furthermore, due to its location, the compound induces alterations in membrane fluidity: it causes an increase in membrane fluidity near the phospholipid head groups, while it causes a decrease in membrane fluidity in the hydrophobic tails of the phospholipids.

To conclude, the interactions between daunorubicin and different mimetic systems are expressed as modifications in the overall membrane biophysical properties. These interactions are intrinsically dependent on the lipid composition and the membrane's physical state. Furthermore, such characteristics are key factors that influence the anticancer drug's partition, its location within the membrane and, ultimately, membrane fluidity. This study has contributed to identify novel biophysical mechanisms that may allow one to explain

the toxic and therapeutic effects of daunorubicin and might allow the future development of more effective anti-cancer drugs.

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[1] R.B. Weiss, The anthracyclines: will we ever find a better doxorubicin?, Semin Oncol, 19 (1992) 670-686.
[2] R. Lozano, *et al,* Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010, Lancet, 380 (2012) 2095-2128.
[3] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity, Pharmacol Rev, 56 (2004) 185-229.
[4] P.V. Escriba, Membrane-lipid therapy: a new approach in molecular medicine, Trends Mol Med, 12 (2006) 34-43.
[5] P.V. Escriba, M. Sastre, J.A. Garciasevilla, Disruption of Cellular Signaling Pathways by Daunomycin through Destabilization of Nonlamellar Membrane Structures, Proc Natl Acad Sci U S A, 92 (1995) 7595-7599.
[6] T.R. Tritton, G. Yee, The Anticancer Agent Adriamycin Can Be Actively Cyto-Toxic without Entering Cells, Science, 217 (1982) 248-250.
[7] P.V. Escriba, X. Busquets, J. Inokuchi, G. Balogh, Z. Torok, I. Horvath, J.L. Harwood, L. Vigh, Membrane lipid therapy: Modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment, Prog Lipid Res, 59 (2015) 38-53.
[8] P.V. Escriba, J.M. Gonzalez-Ros, F.M. Goni, P.K. Kinnunen, L. Vigh, L. Sanchez-Magraner, A.M. Fernandez, X. Busquets, I. Horvath, G. Barcelo-Coblijn, Membranes: a meeting point for lipids, proteins and therapies, J Cell Mol Med, 12 (2008) 829-875.
[9] P. Chandra, H.B. Noh, Y.B. Shim, Cancer cell detection based on the interaction between an anticancer drug and cell membrane components, Chem Commun (Camb), (2013).
[10] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Bio, 9 (2008) 112-124.
[11] S. Ran, P.E. Thorpe, Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy, Int J Radiat Oncol Biol Phys, 54 (2002) 1479-1484.
[12] S. Ran, A. Downes, P.E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels, Cancer Res, 62 (2002) 6132-6140.
[13] S. Riedl, B. Rinner, M. Asslaber, H. Schaider, S. Walzer, A. Novak, K. Lohner, D. Zweytick, In search of a novel target — Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy, Biochim Biophys Acta, 1808 (2011) 2638-2645.
[14] J.H. Stafford, P.E. Thorpe, Increased exposure of phosphatidylethanolamine on the surface of tumor vascular endothelium, Neoplasia, 13 (2011) 299-308.
[15] M. Shinitzky, Membrane fluidity in malignancy. Adversative and recuperative, Biochim Biophys Acta, 738 (1984) 251-261.
[16] G. Taraboletti, L. Perin, B. Bottazzi, A. Mantovani, R. Giavazzi, M. Salmona, Membrane fluidity affects tumor-cell motility, invasion and lung-colonizing potential, Int J Cancer, 44 (1989) 707-713.
[17] M. Inbar, Fluidity of membrane lipids: a single cell analysis of mouse normal lymphocytes and malignant lymphoma cells, FEBS Lett, 67 (1976) 180-185.
[18] G. Deliconstantinos, Physiological aspects of membrane lipid fluidity in malignancy, Anticancer Res, 7 (1987) 1011-1021.
[19] Y.Z. Yang, T.L. Hao, M. Qian, W.X. Dai, Y.M. Huangfu, Z.H. Zhang, Normal and leukemic lymphocyte
membrane fluidity and response to stimulation with ConA and PHA, J Tongji Med Univ, 9 (1989) 143-147.
[20] G.V. Sherbet, Membrane fluidity and cancer metastasis, Exp Cell Biol, 57 (1989) 198-205.
[21] M. Sok, M. Sentjurc, M. Schara, J. Stare, T. Rott, Cell membrane fluidity and prognosis of lung cancer, Ann Thorac Surg, 73 (2002) 1567-1571.
[22] R. Campanella, Membrane lipids modifications in human gliomas of different degree of malignancy, J Neurosurg Sci, 36 (1992) 11-25.
[23] C.M. Rodrigues, S. Sola, R.E. Castro, P.A. Laires, D. Brites, J.J. Moura, Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis, J Lipid Res, 43 (2002) 885-894.
[24] S.M. Johnson, R. Robinson, The composition and fluidity of normal and leukaemic or lymphomatous lymphocyte plasma membranes in mouse and man, Biochim Biophys Acta, 558 (1979) 282-295.
[25] G. Barcelo-Coblijn, M.L. Martin, R.F. de Almeida, M.A. Noguera-Salva, A. Marcilla-Etxenike, F. Guardiola-Serrano, A. Luth, B. Kleuser, J.E. Halver, P.V. Escriba, Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy, Proc Natl Acad Sci U S A, 108 (2011) 19569-19574.
[26] G. Agatha, R. Hafer, F. Zintl, Fatty acid composition of lymphocyte membrane phospholipids in children with acute leukemia, Cancer Lett, 173 (2001) 139-144.
[27] T. Galeotti, S. Borrello, G. Minotti, L. Masotti, Membrane-Alterations in Cancer-Cells - the Role of Oxy Radicals, Ann Ny Acad Sci, 488 (1986) 468-480.
[28] M. Lucio, J.L. Lima, S. Reis, Drug-membrane interactions: significance for medicinal chemistry, Curr Med Chem, 17 (2010) 1795-1809.
[29] C. Peetla, A. Stine, V. Labhasetwar, Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery, Mol Pharm, 6 (2009) 1264-1276.
[30] A.M. Seddon, D. Casey, R.V. Law, A. Gee, R.H. Templer, O. Ces, Drug interactions with lipid membranes, Chem Soc Rev, 38 (2009) 2509-2519.
[31] R.A. Cardone, V. Casavola, S.J. Reshkin, The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis, Nat Rev Cancer, 5 (2005) 786-795.
[32] C. Nunes, D. Lopes, M. Pinheiro, C. Pereira-Leite, S. Reis, In vitro assessment of NSAIDs-membrane interactions: significance for pharmacological actions, Pharm Res, 30 (2013) 2097-2107.
[33] L.M. Magalhaes, C. Nunes, M. Lucio, M.A. Segundo, S. Reis, J.L. Lima, High-throughput microplate assay for the determination of drug partition coefficients, Nat Protoc, 5 (2010) 1823-1830.
[34] N. Kucerka, M.A. Kiselev, P. Balgavy, Determination of bilayer thickness and lipid surface area in unilamellar dimyristoylphosphatidylcholine vesicles from small-angle neutron scattering curves: a comparison of evaluation methods, Eur Biophys J, 33 (2004) 328-334.
[35] S. Tristram-Nagle, H.I. Petrache, J.F. Nagle, Structure and interactions of fully hydrated dioleoylphosphatidylcholine bilayers, Biophys J, 75 (1998) 917-925.
[36] N. Fuller, R.P. Rand, The influence of lysolipids on the spontaneous curvature and bending elasticity of phospholipid membranes, Biophys J, 81 (2001) 243-254.
[37] H. Durchschlag, P. Zipper, Calculation of the partial volume of organic compounds and polymers, in: M.D. Lechner (Ed.) Ultracentrifugation, Steinkopff, Darmstadt, 1994, pp. 20-39.
[38] D. Illinger, G. Duportail, Y. Mely, N. Poirel-Morales, D. Gerard, J.G. Kuhry, A comparison of the fluorescence properties of TMA-DPH as a probe for plasma membrane and for endocytic membrane, Biochim Biophys Acta, 1239 (1995) 58-66.
[39] R.D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of

different fluorescence quenching analyses of membrane depth, Biochemistry, 37 (1998) 8180-8190.
[40] A. Coutinho, M. Prieto, Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrylamide: A laboratory experiment for undergraduate students, Journal of Chemical Education, 70 (1993) 425.
[41] J.R. Lakowicz, Fluorescence Anisotropy, in: Principles of Fluorescence Spectroscopy, Springer, 2006, pp. 366-375.
[42] M.N. Martinez, G.L. Amidon, A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals, J Clin Pharmacol, 42 (2002) 620-643.
[43] C. Matos, C. Moutinho, P. Lobao, Liposomes as a model for the biological membrane: studies on daunorubicin bilayer interaction, J Membr Biol, 245 (2012) 69-75.
[44] N.C. Santos, M. Prieto, M.A.R.B. Castanho, Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods, Biochimica Et Biophysica Acta-Biomembranes, 1612 (2003) 123-135.
[45] J.R. Lakowicz, Quenching of Fluorescence, in: Principles of Fluorescence Spectroscopy, Springer, 2006, pp. 278-327.
[46] L. Calzolai, E. Gaggelli, A. Maccotta, G. Valensin, Interaction of Daunomycin with Dipalmitoylphosphatidylcholine Model Membranes. A 1H NMR Study, J Magn Reson B, 112 (1996) 228-235.
[47] P.P. Constantinides, Y.Y. Wang, T.G. Burke, T.R. Tritton, Transverse location of anthracyclines in lipid bilayers. Paramagnetic quenching studies, Biophys Chem, 35 (1990) 259-264.
[48] G. Karp, The Structure of Function of the Plasma Membrane, in: Cell and Molecular Biology: Concepts and Experiments, John Wiley & Sons, Inc., 2010, pp. 133-134.
[49] M. Lucio, H. Ferreira, J.L. Lima, S. Reis, Interactions between oxicams and membrane bilayers: an explanation for their different COX selectivity, Med Chem, 2 (2006) 447-456.
[50] C. Nunes, G. Brezesinski, D. Lopes, J.L. Lima, S. Reis, M. Lucio, Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality, J Phys Chem B, 115 (2011) 12615-12623.
[51] J.M. Collins, R.N. Dominey, W.M. Grogan, Shape of the fluidity gradient in the plasma membrane of living HeLa cells, J Lipid Res, 31 (1990) 261-270.
[52] M.D. Bazzi, M.A. Youakim, G.L. Nelsestuen, Importance of phosphatidylethanolamine for association of protein kinase C and other cytoplasmic proteins with membranes, Biochemistry, 31 (1992) 1125-1134.
[53] P.V. Escriba, A. Ozaita, C. Ribas, A. Miralles, E. Fodor, T. Farkas, J.A. Garcia-Sevilla, Role of lipid polymorphism in G protein-membrane interactions: nonlamellar-prone phospholipids and peripheral protein binding to membranes, Proc Natl Acad Sci U S A, 94 (1997) 11375-11380.

Supplementary material

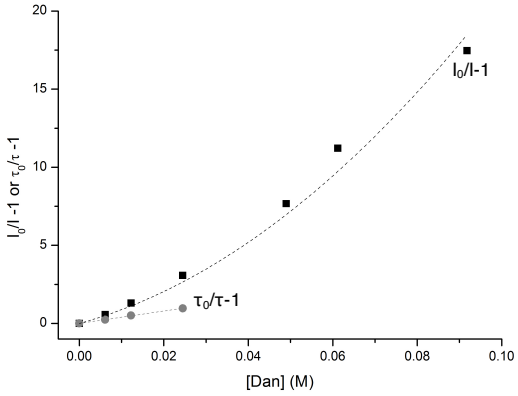


Fig. S1. Stern–Volmer plots of the probe TMA-DPH in the DMPC:DOPC:DPPS:DOPE model: square symbols represent the plot obtained by steady-state fluorescence measurements ($I_0/I-1$) and circle symbols represent the obtained by lifetime fluorescence measurements ($\tau_0/\tau-1$).

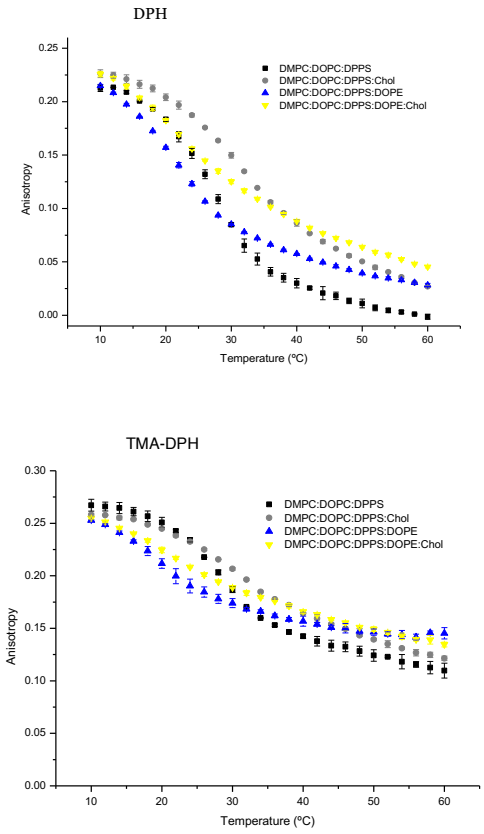
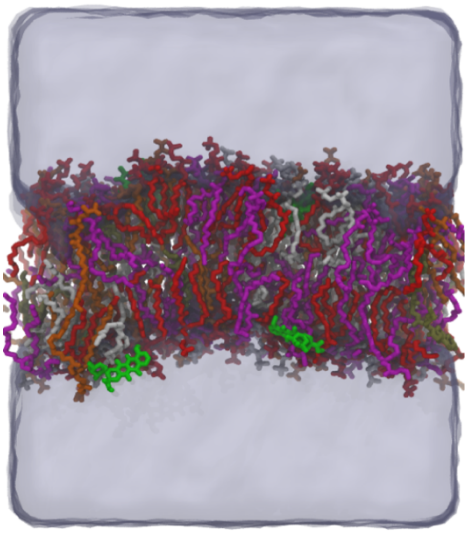


Fig. S2. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in each mimetic model, at pH 6.3. Results present the mean of at least three independent assays.

6. Doxorubicin molecular interactions with cancer membrane models: *in vitro* and dynamic simulation studies

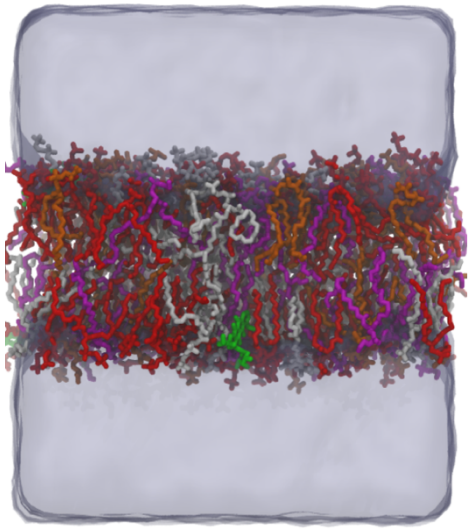
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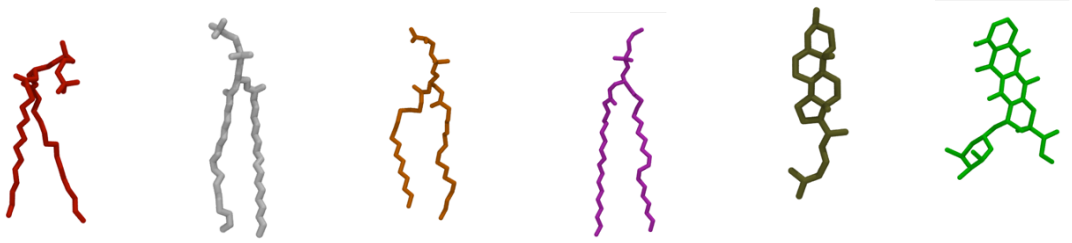
4 : 1.5 : 1.5 : 3



DMPC:DOPC:DPPS:DOPE:Chol

4 : 1 : 1 : 2.5 : 1.5





DMPC **DOPC** **DPPS** **DOPE** **Chol** **Dox**

Doxorubicin molecular interactions with cancer membrane models *in vitro* and molecular dynamic simulation studies

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Doxorubicin is extensively used for chemotherapy of diverse types of cancer. However, the mechanism through which it promotes cancer cells death still remains a matter of controversy. There is evidence that such anticancer agent can cause cytotoxic effects simply through interaction with the cell membranes.

Thus, the current work studies the interactions between doxorubicin and membrane mimetic models composed by different ratios of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-L-serine (DPPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol). Several biophysical parameters were assessed using liposomes as mimetic models of membranes. Thereby, the ability of doxorubicin to partition into lipid bilayers, its location within the membrane and its effect on membrane fluidity were investigated. Furthermore, in order to complement the experimental results and thus infer further insight into the mechanisms behind the interactions, molecular dynamics simulations using bilayers with the same lipid compositions were performed.

The gathered outcomes demonstrated that doxorubicin's permeation is influenced by the lipid membrane composition and causes alterations in the biophysical properties of the bilayers, e.g. membrane fluidity. Although doxorubicin only weakly interacts with cholesterol, this membrane component molecule has a significant influence on the anticancer drug behavior. In fact, significant alterations in the location and orientation of the anticancer drug molecules are observed between the mimetic systems. In the presence of cholesterol, the anticancer drug is preferentially located within the bilayer closer to lipid-water interface and an increase in the hydrogen bonds with lipids and water can be observed. Furthermore, the interaction of doxorubicin with the studied phospholipids is mainly driven by electrostatic and hydrophobic interactions that together are responsible for alterations in the membrane fluidity.

These insights demonstrated that not only membranes can affect doxorubicin accumulation in cells, but the compound itself is responsible for alterations in the properties of membranes. The changes produced by doxorubicin on the lipid structure may constitute an additional mechanism of action, which might lead to modifications in the location and, consequently, the activity of membrane signaling proteins.

doxorubicin | membrane | molecular dynamics simulation | cancer

Doxorubicin (DOX) is a cytotoxic anthracycline antibiotic widely used in chemotherapy due to its broad activity against various types of cancers such as breast cancer (*1*). Despite some controversy, various mechanisms are proposed to explain its pharmacological activity ... The main mechanism is considered to be the intercalation of the drug planar anthraquinone group between the base pairs of the DNA's double helix, which lead to a range of cytotoxic effects including inhibition of DNA replication (*2*). Albeit the DNA and nuclear components are considered their main targets, increasing evidence demonstrates that the plasma membrane is also involved in the cytotoxicity of DOX (*3*).

Biological membranes play a fundamental function in life maintenance. Actually, these structures are more than a scaffold for proteins, participating in important cellular processes that include regulation of the location and activity of many membrane proteins (*4*). In this context, modification of membrane properties by drugs can compromise the function of different proteins and/or associated signaling pathways and, in turn, affect the ability of a cell to control its metabolism and proliferation. Besides, the plasma membrane of a cell can serve either as a target or as a barrier that drugs must overcome on its path to a proposed cellular target (*5*). In fact, DOX possesses an amphipathic structure and possible membrane actions are expected to take place, which can help to explain some of the cytotoxic actions of this anticancer agent.

It is important to stress that cancer cells have been found to be distinct from healthy cells in several different aspects that range from metabolic to structural properties. In fact, a typical recognized phenotype in cancer is the increased anaerobic glycolysis that results in an extracellular acidic medium (*6*, *7*). Alteration in lipid metabolism and cell membrane are also common in cancer cells. Actually, due to a series of oncogenic events, cell growth becomes dysregulated leading to uncontrolled cell proliferation. As a consequence of their highly proliferative rate, cancer cells present a high demand for lipids to generate energy and produce signaling molecules but also to provide enough components to build new membranes in order to expand and disseminate. To satisfy these requirements, cancer cells increase the fatty acid biosynthesis once these molecules are the building blocks of biological membranes (*8*). In fact, alterations in phospholipid and fatty acid profiles have been described for different types of cancer such as breast (*9*, *10*), colorectal (*10*) and also in some chemoresistant malignant tumors (*11*). Evidence also suggests that changes in the phospholipid composition start even before the morphological tumoral

transformation (*9*). For example, it has been demonstrated that modifications in the fatty acid and phospholipid profiles occurs not only in breast cancer tissue but also in the surrounding healthy regions (*9*). It has also been observed that alterations in the lipid composition are also associated with tumor progression and growth (*12*).

Changes in cancer cells comprise not only an increase in the amounts of lipids produced but also in the type of phospholipids that constitute their membrane. Under normal conditions, phosphatidylcholine (PC) and sphingomyelin (SM) are mainly found in the outer leaflet of the plasma membrane, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are confined to the inner one (*13*). In has been described, on the other hand, that in cancer cells PS is exposed in the membranes' outer leaflet (*14-16*). Furthermore, since the distribution of PE and PS within the membrane is maintained by the same aminophospholipid translocases, it has also been demonstrated that PE can also be localized in the external leaflet of tumor endothelial cells (*17*).

Apart from lipid content, membrane fluidity of cancer cells also seems to be different from that of their normal counterparts. Membrane fluidity is a characteristic of plasma membranes that reflects the order and dynamics of phospholipid acyl chains in bilayers. It depends on the membrane's lipid and protein composition; thus, changes in lipid profiles can lead to modifications in the membrane fluidity. In this context, although no common pattern was evidenced, a number of studies have demonstrated a significant correlation between alteration of plasma membrane fluidity and cancer proliferative and invasive potential and metastatic ability (*18-20*). Evidence suggest that several tumor cells exhibit higher membrane fluidity than normal cells (*19*, *21-26*) due to low levels of cholesterol and sphingomyelin (*27*, *28*) and increased percentage of unsaturated fatty acids (*29*). Others, on the contrary, are characterized by a decrease in the membrane fluidity, such as the hepatoma tumor cell line, as a result of a lower degree of fatty acid unsaturation and increased cholesterol content (*30*).

In view of the abovementioned, it becomes clear that cancer cells are distinct from normal cells. For that reason, this works aims to better understand, from a biophysical perspective, the DOX interaction with the membrane of cancer cells. In fact, drug-membrane studies are one key to gain further insight regarding the drug's pharmacological action (*31*). Since biological cells are too complex to accurately assess their biophysical properties, liposomes were used in order to mimic some features of cancer cells (such as exposure of PS and PE in the membrane outer leaflet and acidic external pH). Thereby, the ability of DOX to partition into lipid bilayers (assessed by calculating the coefficient partition, Kp, using derivative spectrophotometry), its location within the membrane (by fluorescence quenching) and its effect on the membrane fluidity (through fluorescence anisotropy) were investigated. Molecular dynamics simulations were also performed, using the same lipid composition, in order to gain further insight regarding the interactions of DOX with the studied model membranes.

Materials and Methods

Reagents. Doxorubicin was obtained from LC Laboratories (Woburn, Massachusetts, USA). The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-L-serine (DPPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). All reagents were used without further purification. Tris maleate was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Drug solutions were prepared in Tris buffer (0.1 M, pH 6.3). The buffer was prepared using double-deionized water (conductivity less than 0.1 μS cm^{-1}).

Liposomes Preparation. The preparation of liposomes was performed according to the lipid film hydration method (*32*). The lipid film was obtained from a chloroform/methanol (3:2 v/v) solution of lipids (DMPC:DOPC:DPPS:DOPE [4:1.5:1.5:3] or DMPC:DOPC:DPPS:DOPE:Chol [4:1:1:2.5:1.5]) that was dried under a stream of nitrogen in a rotative evaporator (Buchi R-200, equipped with a thermostated bath Buchi R-490, Switzerland). Liposomes were produced by hydration of the dried lipid film using Tris buffer (pH 6.3), followed by vortexing to yield multilamellar vesicles (MLVs). The lipid suspensions were extruded eleven times (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) through policarbonate filters with a diameter pore of 100 nm (Nucleopore) at 60°C (temperature above the main phase transition temperature of the lipids) to form large unilamellar vesicles (LUVs). The size of the prepared LUVs was confirmed by dynamic light scattering analysis in a Brookhaven Instrument (Holtsville, NY). For fluorescence studies, the fluorescence probe (DPH or TMA-DPH) was co-dissolved with the lipids in the organic solvents mixture to give a probe/lipid molar ratio of 1:300. Afterwards, the labeled LUVs were identically obtained by evaporation of the organic solvents, hydration of the dried lipid film and extrusion of the MLVs.

The liposomes were prepared according to a specific lipid composition and pH in order to mimic some of the external characteristics found in cancer cell membranes, namely the presence of PS and PE and the acidic pH. As mentioned in the introduction, it has been reported that PS and PE, which are usually absent from the plasma membrane outer monolayer under normal conditions, are exposed in cancer cells (*15*, *17*).

Determination of Partition Coefficients by Derivative Spectrophotometry. The DOX partition coefficients (Kp) in the different model membranes (LUVs) was determined through derivative spectrophotometry, as described in our previous work (*32-34*).. Briefly, a set of samples containing increasing concentrations of lipid solution (in the range of 0–1250 μM) and a fixed concentration of DOX (40 μM) were prepared. References samples were identically obtained but without DOX. Samples and references were incubated

during 30 min at 37°C. The absorption spectra of all solutions were recorded at 37°C using a multidetection microplate reader (Synergy™ HT, BioTek Instruments Inc, Winooski, VT, USA). The mathematical treatment of the results obtained was performed using a developed routine, the *Kp Calculator* (*35*). The Kp values were calculated using a nonlinear regression method that fits equation 1 to the experimental data (D_T vs [L]):

$$D_T = D_W + \frac{(D_m - D_w)K_p[L]V_m}{1 + K_p[L]V_m} \quad (1)$$

where, D_T is third derivative intensity ($D=d^nAbs/d\lambda^n$) obtained from the absorbance values of the total amount of DOX (D_T), the DOX distributed on the lipid membrane phase (D_m) and the DOX distributed in the aqueous phase (D_w). [L] represents the lipid concentration (in M) and V_m is the lipid molar volume. The V_m, calculated from the weighted molecular mean and the reported volume of lipids, for each model systems studied (DMPC:DOPC:DPPS:DOPE and DMPC:DOPC:DPPS:DOPE:Chol) were 0.710 and 0.670 L mol^{-1}, respectively. The Kp values obtained are dimensionless and presented as the mean ± standard deviation calculated from at least three independent assays.

Drug Location Studies: Fluorescence Quenching. The DOX membrane location studies were performed through steady-state and time-resolved fluorescence measurements, using liposomes labeled with DPH or TMA-DPH fluorescent probes (*36*). The samples were prepared with a fixed concentration of lipid (500 μM) and increasing concentrations of DOX (0, 40 and 75 μM) and incubated at 37°C during 30 min in the dark. The fluorescence measurements were carried out at 37°C, at the excitation/emission wavelengths defined as 357/429 nm and 359/429 nm, for DPH and TMA-DPH, respectively. Fluorescence steady-state measurements were performed in a Jasco FP-6500 spectrofluorometer (Jasco, Great Dunmow, UK) equipped with a constant temperature cell holder. The fluorescence intensity values obtained were corrected for inner filter effects (quencher absorbance) at the excitation wavelength (*37*). The fluorescence time-resolved measurements were performed using a Fluorolog Tau-3 Lifetime system. The modulation frequencies were acquired between 10 and 200 MHz, with an integration time of 10 s. The fluorescence emission was detected with a 90° scattering geometry. All measurements were made using Ludox as a reference standard (τ=0.00 ns).

Membrane Fluidity Studies Evaluated by Fluorescence Anisotropy. The influence of DOX on the membrane fluidity of the developed biomimetic systems was assessed by fluorescence anisotropy studies. The samples were prepared with a fixed concentration of lipid (500 μM), labeled with DPH or TMA-DPH, and increasing concentrations of DOX (0, 40 and 75 μM), followed by incubation during 30 min at 37 °C in the dark. The steady-state fluorescence anisotropy measurements (r_s) were recorded between 10 and 60°C with intervals of 2°C. The experiments were performed in a Jasco FP-6500 spectrofluorometer with polarizers inserted (excitation/emission wavelengths were set as described for each fluorescent probe). The results obtained are presented as the mean ± standard deviation calculated from at least three independent assays.

Molecular dynamic simulations. All-atom molecular dynamics simulations of DOX molecules were performed with membrane bilayers presenting the same lipid composition used for liposomes, as mentioned above. For lipid molecules Slipids force field parameters (*38*) (*39*)were used. For water molecules we used the TIP3P (*40*) model compatible with the Slipids forcefield set. Periodic boundary conditions with minimum image convention were used in all directions. Covalent bond lengths were preserved through the linear constraint solver (LINCS) algorithm (*41*). For all systems the temperature was controlled using the Nosé-Hoover thermostat (*42*, *43*) with solvent and solute controlled independently. Pressure was controlled using the Parrinello-Rahmann barostat (*44*) with semi-isotropic control. Lennard-Jones interactions were cut off at 1.0 nm and for the electrostatic interactions the particle mesh Ewald method (PME) (*45*) was used. For all simulations physiological pressure and temperature were used (1 Bar and 37 °C). The GROMACS open source software package, version 4.6.5, was used (*46*) for all Molecular Dynamics (MD) simulations, along with all quantitative analysis. Visualisation of the trajectories was performed using the Visual Molecular Dynamics (VMD) package. (*47*). For DOX, the partial charges were derived using the RESP (*48*) procedure implemented in ANTECHAMBER (*49*). All symulated systems were run for 1.2 μs of simulated elapsed time. According to area per lipid of the membrane bilayers, first 200 ns of the simulation trajectory were considered as pre-equilibrium, and hence all the analysis was performed on the later 1 μs of the trajectories.

Statistical analysis. Statistical analysis was performed using IBM® SPSS® Statistics software (v.20.0.0.0, IBM, Armonk, NY, USA). The measurements were repeated at least three times and data was expressed as mean ± standard deviation (SD). Data was statistically analyzed through the one-way analysis of variance (ANOVA) method and differences between groups were compared by Bonferroni and Tukey post-hoc tests in which a p value lower than 0.05 ($p<0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Doxorubicin Partition Coefficients

The partition coefficient (Kp) of DOX with membranes strongly influences its pharmacokinetic and pharmacodynamic properties. In fact, the interplay of DOX with membranes is inevitable, since these structures can as a barrier that needs to be overcome on its journey to its intracellular target (such as the DNA). Furthermore, the assessment of Kp, which gives information about the distribution of the compound between the aqueous and lipid environments, can help to understand and explain drug's pharmacological activity. In this context, the DOX partition coefficient in liposomes composed of DMPC:DOPC:DPPS:DOPE and DMPC:DOPC:DPPS:DOPE:Chol was determined by derivative UV-Vis spectrophotometry.

Fig. S1 (A) (*Supporting Information*) shows, as an example, the third-derivative absorption spectra of DOX with increasing lipid concentration at pH 6.3 and 37°C, which allowed for the elimination of the background noise caused by light scattering of lipid vesicles. A shift

Table 1: Partition Coefficients (expressed as Kp and log D) of DOX in the different membrane models at 37°C and pH 6.3. [a]

MODEL	Kp	Experimental log D
DMPC:DOPC:DPPS:DOPE	2190 ± 350	3.32 ± 0.07
DMPC:DOPC:DPPS:DOPE:Chol	2731 ± 446	3.43 ± 0.07

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other model.

in the λ_{max} with increasing lipid concentrations can be observed, indicating that DOX partitions from the aqueous medium to the liposomes. The best fit of equation 1 to the third-derivative data was achieved at 546 nm and is shown in Fig. S1 (B) (*Supporting Information*). The values of Kp and log D obtained for each membrane system are listed in Table 1.

The DOX Kp was assessed using liposomes instead of the traditional octanol/water system, because the lipid vesicles provide an anisotropic environment that is identical to the cell membranes. Like biological membranes, liposomes are composed of phospholipids and, due to its amphiphilic nature, are able to establish not only hydrophobic, but also electrostatic interactions and hydrogen bonds with DOX. Since the partition of DOX is dependent on the degree of ionization of the compound, the pKa values and the octanol/water partition coefficient were also calculated using Marvin sketch calculator software from Chemaxon™. A value for logD, taking into account the pH value of 6.3 used in this study, was found to be -0.83: a value significantly different from those obtained using the liposome/buffer systems (Table 1). Such dissimilarity results from the fact that only hydrophobic interactions are taken into consideration between the anticancer drug and octanol in the traditional biphasic system. In fact, the pKa values (9.93) predict that around 98% of DOX molecules are positively charged at pH 6.3, confirming that DOX-membrane interaction is mainly driven by electrostatic forces. Therefore, liposomes constitute a more realistic system and provide additional information about DOX' lipophilicity obtained with the octanol/water system.

The analysis of Table 1 reveals that DOX molecules exhibit a significant membrane partition with model membranes composed of DMPC:DOPC:DPPS:DOPE and DMPC:DOPC:DPPS:DOPE:Chol.

At pH 6.3 the contribution of the positively charged DOX molecules (due to the ionization of the amine group) might be responsible for this strong partition with membranes due to the electrostatic interactions with the anionic PS and also with the negatively charged phosphate groups of the overall phospholipids. In fact, evidence demonstrate that this compound presents a strong affinity towards negative phospholipids (*50*).

On the other hand, PE display a cone shape due to its small polar head group, producing a lower surface packing density and greater lipid disorder (liquid disordered microdomains, L_d) (*51*) and, consequently, the membrane surface might become more easily accessible for the drug. This phospholipid is also known to have a non-lamellar phase propensity (negative curvature) that might favor the binding of DOX's molecules into the membrane.

The gathered values demonstrate that, not only the DOX properties such as the ionization state but also the membrane lipid composition and its biophysical features influence the drug distribution between the bilayer and aqueous medium.

Drug location studies using different fluorescent probes

Next we applied fluorescence quenching (decrease in the fluorescence intensity) of two probes to further investigate the drug-bilayer interaction. This protocol is a very sensitive and broadly used to assess the location of different molecules within liposomes and biological membranes (*52*). Fluorescence quenching efficiency of DOX in the membrane model systems we studied was determined by varying the drug concentration in liposomes labelled either with DPH or TMA-DPH. Such knowledge is only possible to obtain due to the fact that the fluorophore position in the membrane of each fluorescent probe (TMA-DPH and DHP) is well defined and documented. As such, while DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer (*53*), TMA-DPH is reported to be anchored to the polar head groups region of the phospholipids due to its charged group (*54*).

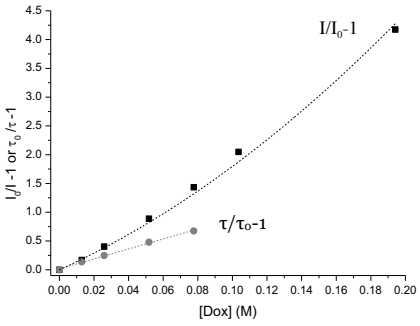


Fig. 1: Example of Stern–Volmer plots of the probe DPH in the DMPC:DOPC:DPPS:DOPE model: square symbols represent the plot obtained by steady-state fluorescence measurements ($I_0/I - 1$) and circle symbols represent the obtained by lifetime fluorescence measurements ($\tau_0/\tau - 1$).

The drug efficiency to quench the fluorophore is related with its proximity to the probe. In this sense, since the fluorophore is inserted into the membrane, only the drug that partitions into the lipid region in the vicinity of the probe will be able to act as a quencher. Therefore, in order to determine the DOX quenching efficiency, it is important to calculate its membrane concentration ($[Q]_m$) that is given by the equation 2:

$$[Q]_m = \frac{K_p[Q]_T}{K_p\alpha_m + (1 - \alpha_m)} \qquad (2)$$

where α_m is the volume fraction of membrane phase ($\alpha_m = V_m/V_T$; V_m and V_T represent the volumes of the membrane and water phase, respectively).

Table 2: Fluorescence quenching parameters (K_D, dynamic constant; K_S, static constant; K_{SV}, Stern–Volmer constant) calculated from the quenching of DPH and TMA-DPH by DOX in the different mimetic models at pH 6.3 and 37°C. [a]

Model	Probe	K_D (M^{-1})	K_S (M^{-1})	K_{SV} (M^{-1})
DMPC:DOPC:DPPS:DOPE	**DPH**	17* ± 1	11.4* ± 0.8	28.4* ± 0.5
	TMA-DPH	9.1* ± 0.6	14* ± 1	23* ± 1
DMPC:DOPC:DPPS:DOPE:Chol	**DPH**	7.8* ± 0.5	13* ± 1	20* ± 1
	TMA-DPH	5.8* ± 0.3	9.1* ± 0.5	14.9* ± 0.4

[a] All values represent the mean ± standard deviation (n=3).
* $p < 0.05$ statistically different from the other probe, for each biophysical parameter.

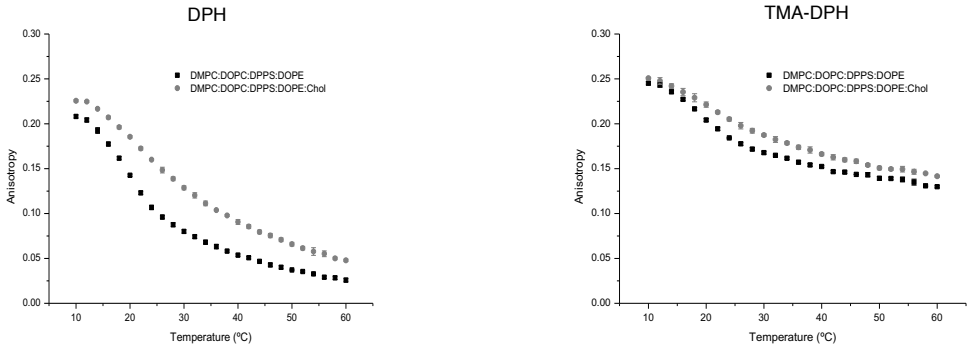


Fig. 2. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in each mimetic model. Results present the mean of at least three independent assays.

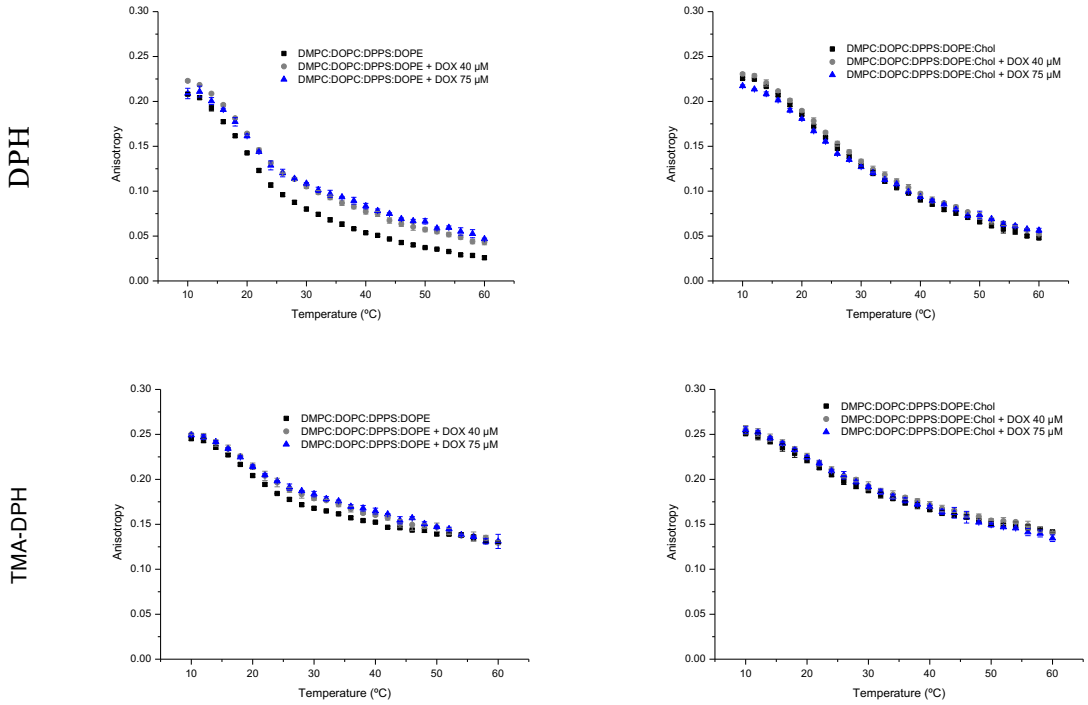


Fig. 3. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of DOX 40 μM (●) and 75 μM (▲). Results present the mean of at least three independent assays.

DOX molecules have effectively quenched both probes in both mimetic membranes. Furthermore, the wavelength corresponding to the absorption band of the probes did not change in response to the presence of DOX and thus no additional band was observed at a longer wavelength. The molecular contact between the probe (fluorophore) and the drug (quencher) can be due to different molecular interactions and can be observed by the decrease in the fluorescence intensity as represented in Fig. 1. The quenching process can result from collisions between the fluorophore and quencher, and is therefore called collisional or dynamic quenching, or can be due to complex formation, which is called static quenching (*52*). In order to distinguish the two types of quenching process, fluorescence lifetime measurements were also performed. This is possible due to the nonflourescent nature of the complexed fluorophores, where the observed fluorescence is solely due to the presence of uncomplexed fluorophores: the uncomplexed fraction is unperturbed, hence the lifetime is ~ τ_0 (τ_0 corresponds to the fluorophore lifetime in the absence of quencher). Therefore, for static quenching $\tau_0/\tau=1$ (τ_0 and τ are the fluorophore lifetime in the absence and presence of DOX, respectively) while for dynamic quenching $I_0/I=\tau_0/\tau$ (I and I_0 are the steady state fluorescence intensities with and without the quencher (DOX), respectively) (*52*).

In many cases the fluorescent probe can be quenched both by collisions and by complex formation with the same quencher. This results in an upward curvature of the Stern-Volmer plots, a positive deviation that can be seen in Fig. 1. In this sense, the dynamic portion (K_D) of the observed quenching is determined by lifetime measurements, through the following equation (equation 3) (*52*):

$$\frac{\tau_0}{\tau}=K_D[Q]_m+1 \quad (3)$$

After knowing the dynamic component, the static contribution may be calculated through linearization of the following equation (equation 4) (*52*):

$$\frac{I_0}{I}\times\frac{1}{K_D[Q]+1}=K_S[Q]_m+1 \quad (4)$$

This modified form of the Stern-Volmer equation is second order in $[Q]_m$, which accounts for the upward curvature observed when both static and dynamic quenching occur for the same fluorophore. In the Fig. 3, the Stern–Volmer plots of I_0/I-1 and τ_0/τ-1 in function of DOX membrane concentration are shown.

According to the aforementioned description of the quenching behavior, the dynamic constant (K_D), the static constant (K_S) and the Stern–Volmer constant (K_{SV}), obtained from this technique are presented in Table 2.

From the analysis of Table 2, it is possible to observe that the quenching process, in all mimetic models and probes used, results from dynamic and static interactions. This behavior translates the fact that DOX must diffuse to the fluorophore during the lifetime of the excited state but it also might form a non-fluorescent complex with each probe. Although, DOX was able to quench both probes, the K_{SV} values demonstrate that the decrease of the DPH fluorescence was more pronounced than for TMA-DPH in both membrane models. This indicates that the binding of DOX is not purely electrostatic but might also involve the insertion of drug molecules into the lipid bilayer, probably due to hydrophobic interactions between the drug's dihydroanthraquinone and the lipid fatty acid chains.

Effect of Doxorubicin on membrane fluidity

DOX molecules interact with membranes in order to reach their intracellular target and alterations in the physicochemical state of the phospholipid bilayer are thus to be expected. In this context, understanding how the anticancer influences membrane fluidity is important, since it can provide valuable information regarding their therapeutic and toxic effects. Membrane fluidity studies were performed through steady-state anisotropy (*55*), that measures lipids order and their degree of rotational motion, using fluorescent probes with a well-known membrane location (DPH and TMA-DPH). Alterations in membrane fluidity can be detected through measurement of changes in anisotropy, which reflects perturbations in the probe's rotational movement, in turn caused by changes in the stiffness of its surrounding matrix(*34*).

The effect of temperature on the DPH and TMA-DPH fluorescence anisotropy of the different model membranes without DOX is presented in Fig. 2. The results demonstrate that for all models systems developed, the anisotropy values decrease with increasing temperature in greater extent in the acyl chain region (given by the probe DPH) than in the phospholipid head region (given by the TMA-DPH probe). In fact, it is well documented that the membrane is characterized by a fluidity gradient from the aqueous interface to the bilayer interior, where the acyl chain end presents an increased disorder. It is also described that the presence of cholesterol in lipid bilayers can increase the membrane order and, therefore, decrease the fluidity, which is consistent with the obtained results.

Fig. 3 shows the influence of DOX on the DPH and TMA-DPH fluorescence anisotropy as a function of temperature for each model system studied. The obtained results reveal that the incorporation of DOX increases the anisotropy of DMPC:DOPC:DPPS:DOPE mimetic model, which, in turn, results in a decrease of membrane fluidity in both head groups and acyl chains regions. For the case of model system composed of DMPC:DOPC:DPPS:DOPE:Chol, DOX did not produce any alterations of the anisotropy values and, therefore, no changes in the membrane fluidity. Nevertheless, a strong partition of DOX with this model membrane has been observed. Therefore, the anticancer drug must adopt a disposition within the membrane that does not cause any change in the microviscosity of its surrounding environment. This might happen due to its planar structure that allows it to intercalate between the bilayer compounds in a way that the membrane fluidity does not change.

Molecular Dynamics Simulations Results

The MD trajectory analysis reveals the partitioning of DOX in the DMPC:DOPC:DPPS:DOPE and DMPC:DOPC:DPPS:DOPE:Chol membrane bilayers. The average distance between the z-coordinate of the center of mass of the DOX molecule and the nearest phosphate groups of lipids in the bilayer leaflet are calculated from the entire equilibrated trajectory as shown in Fig. 4A. This analysis shows that DOX molecule partitions towards lipid-water interface in presence of cholesterol. Further the angle of distribution

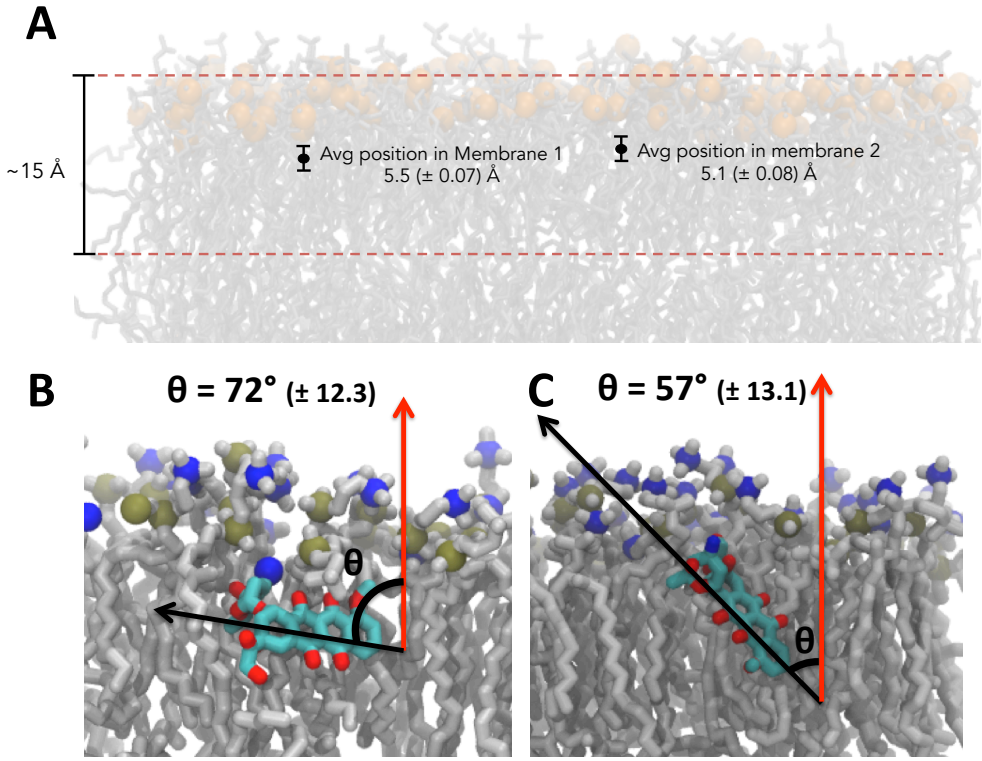


Fig. 3. Closer look at the interactions of DOX with DMPC:DPPS:DOPE:DOPC and DMPC:DPPS:DOPE:DOPC:Chol membranes. (A) Partitioning of DOX into the membrane bilayer models. The dots indicate the average distance of DOX molecule from phosphate head groups of lipids from top leaflet of the membrane bilayer. The left and right dots represent the positions of DOX in DMPC:DPPS:DOPE:DOPC and DMPC:DPPS:DOPE:DOPC:Chol respectively. (B) Angle distribution of DOX in DMPC:DPPS:DOPE:DOPC (left) and DMPC:DPPS:DOPE:DOPC:Chol (right) membranes. The red arrow represents the membrane normal.

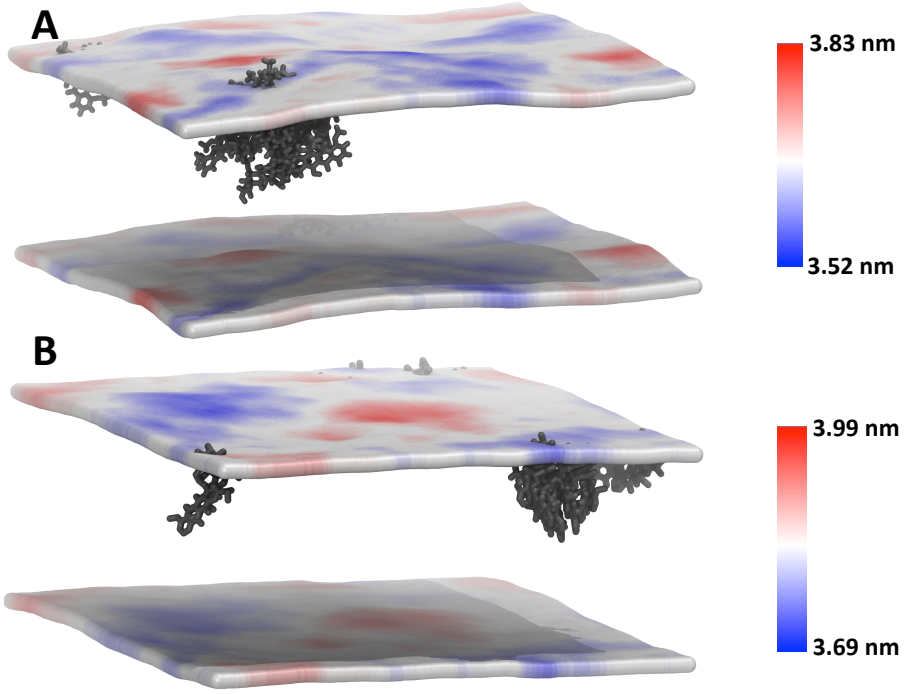


Fig. 5. Membrane thickness of (A) DMPC:DPPS:DOPE:DOPC (B) DMPC:DPPS:DOPE:DOPC:Cholesterol and average positioning of DOX conformation sampled over 1 microsecond of simulation trajectory.

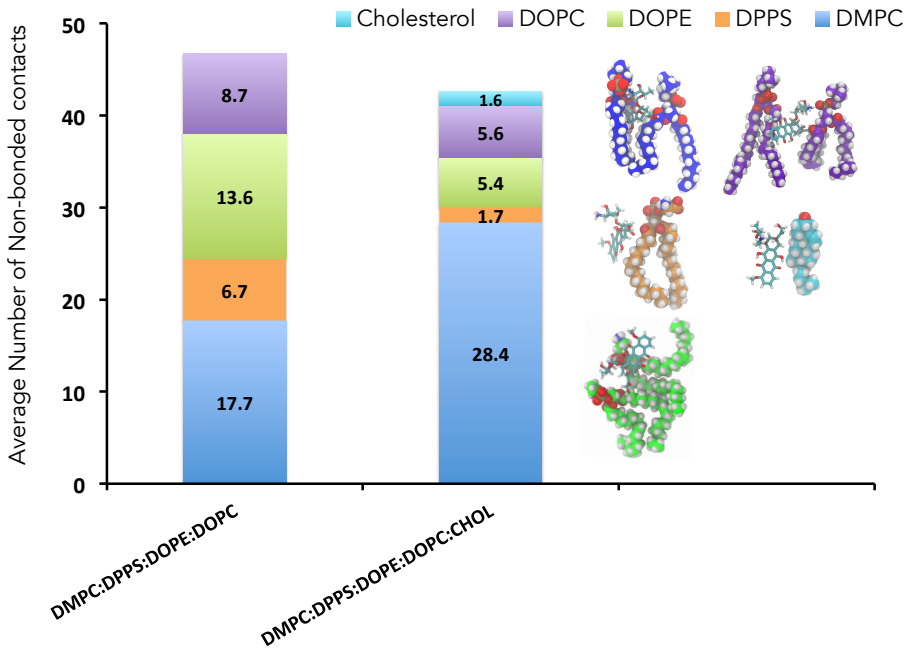


Fig. 6. Number of non-bonded contacts of DOX with DMPC, DPPS, DOPE, DOPC and Chol. These atoms are involved in electrostatic and hydrophobic interactions with DOX molecule.

of the DOX molecule with membrane normal (shown in red in Fig. 4B and C) shows that the orientation of DOX in the presence of cholesterol is significantly different. As seen in Fig. 5A, in DMPC:DOPC:DPPS:DOPE membrane bilayer, DOX anthraquinone group is perpendicular to the membrane normal, however in DMPC:DOPC:DPPS:DOPE:Chol membrane, the cholesterol increased the extent of membrane ordering, and orientation changes such that the average angle of DOX with the membrane normal is 57 degrees. Such orientation can help to explain why any significant alteration in the membrane microviscosity can be observed. Such partitioning is due to ability of DOX to form hydrogen bonds with lipid headgroups (Fig. S2, *Supporting Information*) as well as interact with lipid tails as shown in Fig. 6.

Cholesterol makes the membrane bilayer more ordered, this is evident from the increase in membrane thickness as shown in the Fig. 5B and is also in good agreement with the previous results of anisotropy. The addition of cholesterol to the mimetic model membrane causes a decrease in the number of non-bonded contacts of lipids (except for DMPC) with DOX, as can be seen in Fig. 6. On the other hand, in the presence of cholesterol, the number of hydrogen bonds that DOX forms with lipids and cholesterol increases as shown in Fig. S2 (*Supporting Information*). In Fig. S2 (*Supporting Information*) the specific groups of DOX involved in hydrogen bonding with lipids and cholesterol (A) and with water (B) are represented.

Overall, the presented results demonstrate that in the presence of cholesterol there is 1) an alteration in the orientation of the DOX molecule within the bilayer, 2) an increase in the number of hydrogen bonds formed with the membrane lipids and 3) a slight decrease in the extent of hydrophobic interactions. Nevertheless, it is interesting to observe that cholesterol does in fact show a limited degree of interaction with the DOX molecule as seen in Fig. S3 (*Supporting Information*). Fig. S3 (*Supporting Information*) shows the distance distribution of DOX with DMPC, DPPS, DOPC, DOPE and Chol. From this plot, it is clear that DOX collocates preferentially with DOPC in absence of cholesterol and with DMPC in presence of cholesterol. Hence the effect of cholesterol on interactions of DOX is, while indirect, however significant.

CONCLUSIONS

Several recent studies have shown that the membrane of cancer cells suffers several alterations in both their lipid profile and characteristics, especially in terms of fluidity, during their malignant transformation. Considering this information, the present work aims to develop different model systems to study the effects of PS and PE exposure in the external monolayer and the influence of unsaturated chains and cholesterol on DOX membrane-interactions *in vitro* and *in silico*, in order to understand how specific alterations in the lipid composition of the cancer membrane can influence in the drug's behavior. Using derivative spectrophotometry, fluorescence quenching, fluorescence anisotropy experiments combined with molecular dynamics simulations it has been demonstrated that membrane composition dictates the mode of interaction of DOX with bilayers, which is evident from differential partitioning in the membrane. In both model membranes DOX partitions into the membrane bilayer, due to its ability to interact with both lipid head groups and lipid tails. Besides, in the presence of cholesterol the DOX preferentially collocates within the bilayer closer to the lipid-water interface and an increase in the hydrogen bonds with lipids and water can be observed. Cholesterol, however, does not seen to interact directly with the drug.

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1. G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56**, 185–229 (2004).
2. O. Tacar, P. Sriamornsak, C. R. Dass, Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J. Pharm. Pharmacol.* **65**, 157–170 (2013).
3. T. R. Triton, G. Yee, The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science*. **217**, 248–250 (1982).
4. P. V. Escribá *et al.*, Membranes: a meeting point for lipids, proteins and therapies. *J. Cell. Mol. Med.* **12**, 829–875 (2008).
5. J. K. Seydel, M. Wiese, *Drug-Membrane Interactions: Analysis* (Drug Distribution, 2002).
6. S. K. Parks, J. Chiche, J. Pouyssegur, pH control mechanisms of tumor survival and growth. *Journal of Cellular Physiology*. **226**, 299–308 (2011).
7. R. A. Gatenby, R. J. Gillies, Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer*. **4**, 891–899 (2004).
8. F. P. Kuhajda, Fatty acid synthase and cancer: new application of an old pathway. *Cancer Res*. **66**, 5977–5980 (2006).
9. N. Azordegan *et al.*, Carcinogenesis alters fatty acid profile in breast tissue. *Mol. Cell. Biochem.* **374**, 223–232 (2013).
10. T. E. Merchant, J. N. Kasimos, P. W. Graaf, Phospholipid profiles of human colon cancer using 31 P magnetic resonance spectroscopy. *International journal of …* (1991).
11. P. V. Escribá, A. V. Ferrer-Montiel, J. A. Ferragut, J. M. Gonzalez-Ros, Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug-resistance phenomena. *Biochemistry*. **29**, 7275–7282 (1990).
12. L. Riboni *et al.*, Ceramide levels are inversely associated with malignant progression of human glial tumors. *Glia*. **39**, 105–113 (2002).
13. G. van Meer, D. R. Voelker, G. W. Feigenson, Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*. **9**, 112–124 (2008).
14. S. Ran, P. E. Thorpe, Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **54**, 1479–1484 (2002).
15. S. Ran, A. Downes, P. E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Res*. **62**, 6132–6140 (2002).
16. S. Riedl *et al.*, In search of a novel target - phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy. *Biochim. Biophys. Acta*. **1808**, 2638–2645 (2011).
17. J. H. Stafford, P. E. Thorpe, Increased exposure of phosphatidylethanolamine on the surface of tumor vascular endothelium. *Neoplasia*. **13**, 299–308 (2011).
18. M. Shinitzky, Membrane fluidity in malignancy Adversative and recuperative. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. **738**, 251–261 (1984).
19. G. Taraboletti *et al.*, Membrane fluidity affects tumor-cell motility, invasion and lung-colonizing potential. *International Journal of Cancer*. **44**, 707–713 (1989).
20. R. Zeisig, T. Koklic, B. Wiesner, I. Fichtner, M. Sentjurc, Increase in fluidity in the membrane of MT3 breast cancer cells correlates with enhanced cell adhesion in vitro and increased lung metastasis in NOD/SCID mice. *Arch. Biochem. Biophys.* **459**, 98–106 (2007).
21. M. Inbar, Fluidity of membrane lipids: a single cell analysis of mouse normal lymphocytes and malignant lymphoma cells. *FEBS Lett.* **67**, 180–185 (1976).
22. Y. Z. Yang *et al.*, Normal and leukemic lymphocyte membrane fluidity and response to stimulation with ConA and PHA. *J. Tongji Med. Univ.* **9**, 143–147 (1989).
23. G. V. Sherbet, Membrane fluidity and cancer metastasis. *Exp. Cell Biol.* **57**, 198–205 (1989).
24. M. Sok, M. Sentjurc, M. Schara, J. Stare, T. Rott, Cell membrane fluidity and prognosis of lung cancer. *Ann. Thorac. Surg.* **73**, 1567–1571 (2002).
25. R. Campanella, Membrane lipids modifications in human gliomas of different degree of malignancy. *J Neurosurg Sci*. **36**, 11–25 (1992).
26. C. M. P. Rodrigues *et al.*, Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis. *J. Lipid Res.* **43**, 885–894 (2002).
27. S. M. Johnson, R. Robinson, The composition and fluidity of normal and leukaemic or lymphomatous lymphocyte plasma membranes in mouse and man. *Biochim. Biophys. Acta*. **558**, 282–295 (1979).
28. G. Barceló-Coblijn *et al.*, Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 19569–19574 (2011).
29. G. Agatha, R. Häfer, F. Zintl, Fatty acid composition of lymphocyte membrane phospholipids in children with acute leukemia. *Cancer Lett.* **173**, 139–144 (2001).
30. T. Galeotti, S. Borrello, G. Minotti, L. Masotti, Membrane alterations in cancer cells: the role of oxy radicals. *Ann. N. Y. Acad. Sci.* **488**, 468–480 (1986).
31. M. Lúcio, J. L. F. C. Lima, S. Reis, Drug-membrane interactions: significance for medicinal chemistry. *Curr. Med. Chem.* **17**, 1795–1809 (2010).
32. C. Nunes, D. Lopes, M. Pinheiro, C. Pereira-Leite, S. Reis, In vitro assessment of NSAIDs-membrane interactions: significance for pharmacological actions. *Pharm. Res.* **30**, 2097–2107 (2013).
33. C. Pereira-Leite, C. Nunes, J. L. F. C. Lima, S. Reis, M. Lúcio, Interaction of celecoxib with membranes: the role of membrane biophysics on its therapeutic and toxic effects. *J. Phys Chem. B*. **116**, 13608–13617 (2012).
34. C. Nunes *et al.*, Lipid-drug interaction: biophysical effects of tolmetin on membrane mimetic systems of different dimensionality. *J. Phys Chem. B*. **115**, 12615–12623 (2011).
35. L. M. Magalhães *et al.*, High-throughput microplate assay for the determination of drug partition coefficients. *Nat Protoc*. **5**, 1823–1830 (2010).
36. J. Brittes, M. Lúcio, C. Nunes, J. L. F. C. Lima, S. Reis, Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects. *Chem. Phys. Lipids*. **163**, 747–754 (2010).
37. A. Coutinho, M. Prieto, Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrylamide: A laboratory experiment for undergraduate students. *Journal of Chemical Education*. **70**, 425 (1993).
38. J. P. M. Jämbeck, A. P. Lyubartsev, An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J Chem Theory Comput*. **8**, 2938–2948 (2012).
39. J. P. M. Jämbeck, A. P. Lyubartsev, An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J Chem Theory Comput*. **8**, 2938–2948 (2012).
40. W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics*. **79**, 926–935 (1983).
41. B. Hess, H. Bekker, H. Berendsen, LINCS: a linear constraint solver for molecular simulations. *Journal of computational …* (1997).
42. W. G. Hoover, Canonical dynamics: Equilibrium phase-space distributions. *Phys. Rev. A*. **31**, 1695–1697 (1985).
43. S. Nosé, A unified formulation of the constant temperature molecular dynamics methods. *The Journal of Chemical Physics*. **81**, 511–519 (1984).
44. M. Parrinello, A. Rahman, Polymorphic transitions in single crystals: A new molecular dynamics method. *Journal of Applied Physics*. **52**, 7182–7190 (1981).
45. U. Essmann *et al.*, A smooth particle mesh Ewald method. *The Journal of Chemical Physics*. **103**, 8577–8593 (1995).
46. S. Pronk *et al.*, GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics*. **29**, 845–854 (2013).
47. W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics. *Journal of Molecular Graphics*. **14**, 33–38 (1996).
48. C. I. Bayly, P. Cieplak, W. Cornell, P. A. Kollman, A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys Chem.* **97**, 10269–10280 (2002).

49. J. Wang, W. Wang, P. A. Kollman, D. A. Case, Automatic atom type and bond type perception in molecular mechanical calculations. *Journal of Molecular Graphics and Modelling*. **25**, 247–260 (2006).
50. F. A. de Wolf *et al.*, Comparable interaction of doxorubicin with various acidic phospholipids results in changes of lipid order and dynamics. *Biochim. Biophys. Acta*. **1096**, 67–80 (1990).
51. P. V. Escribá *et al.*, Membrane lipid therapy: Modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment. *Prog. Lipid Res.* **59**, 38–53 (2015).
52. J. R. Lakowicz, *Quenching of Fluorescence, in Principles of Fluorescence Spectroscopy.* (Springer, 2006).
53. R. D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth. *Biochemistry*. **37**, 8180–8190 (1998).
54. D. Illinger *et al.*, A comparison of the fluorescence properties of TMA-DPH as a probe for plasma membrane and for endocytic membrane. *Biochim. Biophys. Acta*. **1239**, 58–66 (1995).
55. J. R. Lakowicz, (Springer, 2006), pp. 366–375.

Supporting Information

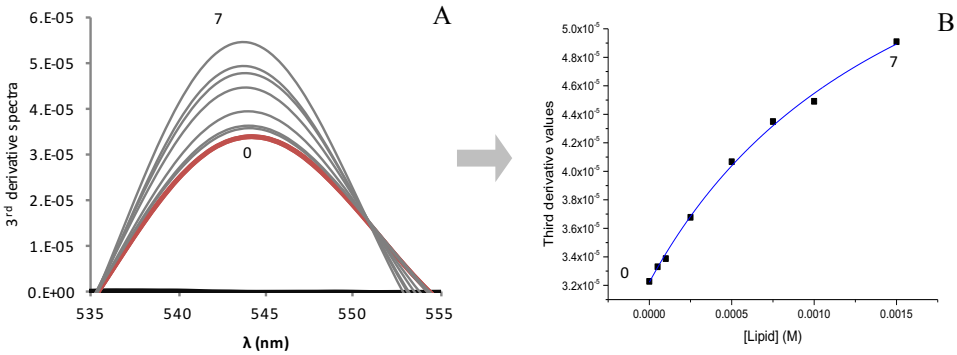


Fig. S1. Third-derivative absorption spectra (A) of DOX (40μM) alone (red line, 0), incubated in DMPC:DOPC:DPPS:DOPE:Chol model membrane with increasing lipid concentration (7, represents the maximum lipid concentration) at 37°C and pH 6.3. Plot (B) represents the best fitting curve to the experimental third-derivative spectrophotometric data (DT vs. [L]) using a nonlinear regression method at a wavelength of 546 nm.

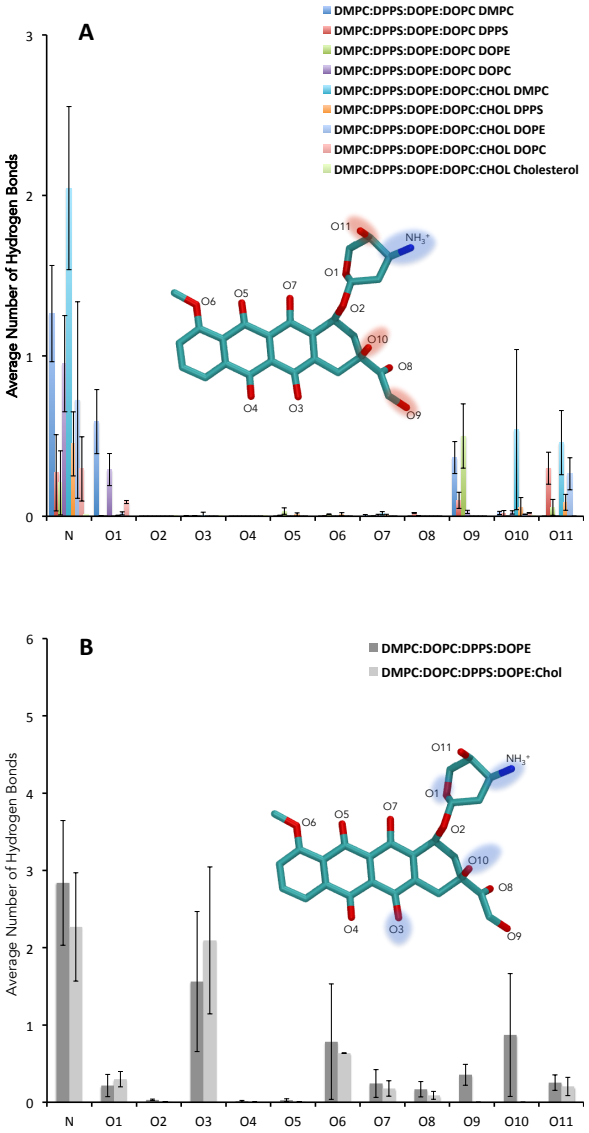


Fig. S2. Molecular interactions of DOX with individual components of the membrane bilayers (A) Number of hydrogen bonds of DOX with lipids. (B) Number of hydrogen bonds of DOX with water molecules, highlighted atoms of DOX show part of the molecule involved in hydrogen bonds.

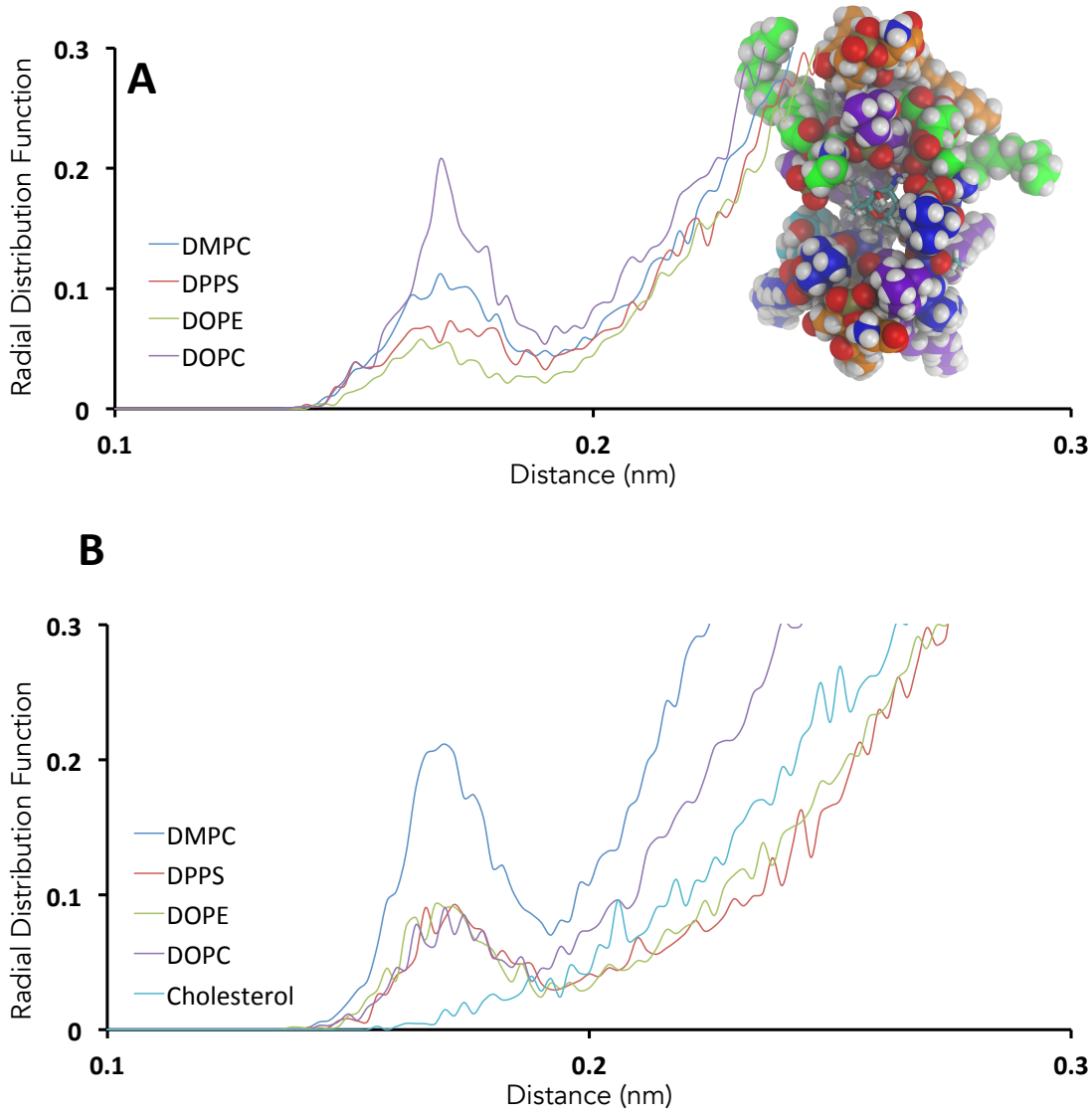


Fig. S3. Radial distribution function of DOX. Distance distribution of DMPC, DPPS, DOPE, DOPC and cholesterol from DOX molecule. The plot shows the distance distribution of center of mass of DOX molecule with DMPC, DPPS, DOPE, DOPC and cholesterol over 1 microsecond trajectory.

7. Synchrotron SAXS and WAXS investigation of the interactions of anthracyclines with lipid membranes

Synchrotron SAXS and WAXS investigation of the interactions of anthracyclines with lipid membranes

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In preparation

The anthracyclines drug-membrane interactions play a crucial role in the molecules' bioavailability and drug-induced toxicity process. In fact, the cell boundary is involved in several biological activities that lead to cell survival, differentiation and death. Thus, structural changes in the lipid phase induced by these drugs can disturb the membrane function and indirectly modulate membrane proteins activity. Structural information of DMPC:DOPC:DPPS:DOPE [4:1.5:1.5:3] and DMPC:DOPC:DPPS:DOPE:Chol [4:1:1:2.5:1.5] bilayers was accessed by small angle and wide angle X-ray measurements (SAXS and WAXS) that are powerful techniques to investigate the anthracycline drugs' influence on the long range bilayer order and the hydrocarbon chain packing, respectively.
The present study provides evidence of significant alterations in the structural properties of phospholipid bilayers that mimic the cancer membrane composition, in particular the presence of phosphatidylethanolamine and phosphatidylserine in the outer leaflet, that can be correlated with both daunorubicin and doxorubicin mechanism of action.

SAXS | WAXS | anthracyclines | drug-membrane interactions

Anthracyclines are considered one of the most effective group of anticancer drugs ever developed [1, 2]. These compounds display a lipophilic flat anthraquinone moiety coupled to an amino sugar and differ only by their side chain: the side chain of doxorubicin (DOX) ends with a primary alcohol, whereas that of daunorubicin (DAN) ends with a more lipophilic methyl. This small structural difference has relevant consequences on their spectrum of activity and pharmacokinetics [1]. Thus, while DAN shows activity in acute lymphoblastic or myeloblastic leukemia's [3], DOX is used in the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas [1].

The most prevalent opinion is that their cytotoxic activity is mainly due to a direct interplay with nucleic acids, leading to DNA damage and inhibition of DNA synthesis. However, this still represents a matter of considerable debate, since there is also strong evidence suggesting that interactions with cell membranes plays an important role in their activity [4]. For example, in 1982 Tritton *et al*. have demonstrated that anthracyclines, without entering the cell, could exert their cytotoxic effect only through their interplay with the membrane [5]. Even considering that the main mechanism is the interaction with nucleic acids, in order to reach the target, anthracyclines must pass through a variety of other structures. First, the drugs must overcome the selective barrier to entry into the cell that is the plasma membrane and, finally, the nuclear membrane. For this reason, interaction with lipid membranes is an unavoidable step in the anticancer drugs activity, independently of the main mechanism of action.

Drug-membrane interactions produce alterations in the physical properties of the cell membrane, as a consequence of the behavior of the drug within the membrane. How the drug orients in the membrane, which components of the lipid membrane the drug interacts with and what interactions within the membrane the drug disrupts, are all factors that alter the biophysical characteristics of the membrane [6]. This can, in turn, lead to modifications in cell signaling and function of membrane proteins, such as transporters and ion channels [7]. It has been demonstrated, for example, that DAN causes alteration of both G-proteins and protein kinase C-associated signaling pathways, through destabilizing the non-lamellar membrane structures that are involved in their location and activity [8, 9]. Since these proteins participate in fundamental functions of the cell, including proliferation and differentiation, their modulation could be responsible for the antitumor activity of some drugs [8].

Even though the emergence of cancer cell may have a genetic link, significant data shows that cells, during malignant transformation, suffer alterations in their membrane's lipid profile and biophysical properties [6, 10]. In fact, under normal conditions, phosphatidylcholine (PC) and sphingomyelin (SM) are mainly found in the outer leaflet of the plasma membrane, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are confined to the inner one [11]. In contrast with this scenario, it has been observed that PS is exposed in the outer leaflet of cancer cells' membranes [12-14]. Since the distribution of PE and PS within the membrane is maintained by the same aminophospholipid translocases, it has been demonstrated that PE along with PS can also be localized in the external leaflet of tumor endothelial cells [15].

In addition to lipid content, membrane fluidity of cancer cells seems to be different from their normal counterparts. In fact, although no common pattern was evidenced, a number of studies have demonstrated a significant correlation between plasma membrane fluidity and cancer malignancy [16, 17]. It appears that some tumor cells exhibit higher membrane fluidity than normal cells [17-24] due to low levels of cholesterol and sphingomyelin [25, 26] and increased percentage of unsaturated fatty acids [27]. Other cancer cells present a decreased membrane fluidity, such as the hepatoma tumor cell line, as a result of a lower degree of fatty acid unsaturation and increased cholesterol content [28].

In view of the abovementioned, it becomes clear that cancer cell membranes are altered in many different aspects. From the pharmacological point of view, it is important to understand the molecular mechanisms through which drugs interplay with cell membranes. In fact, the different characteristics of membranes can, themselves, influence a drugs' pharmacological activity, being fundamental for their pharmacokinetics, efficacy and toxicity [29-31]. On the other hand, the interaction of drugs with membranes can modulate their biophysical properties, affect their function and integrity and, ultimately, modify protein activity [6, 32, 33]. Drug-membrane studies arise, therefore, as fundamental assays to unveil the biophysical mechanisms behind the therapeutic and toxic effects of daunorubicin. For that reason, the main goal of this work is to systematically characterize the influence of daunorubicin on the biophysical properties of membranes and further

correlate these effects with some of their pharmacological actions. The use of membrane model systems, such as liposomes, allows one to more easily perform these studies. Actually, liposomes mimic biomembranes constitution and dimensionality and, at the same time, minimize their molecular complexity. In this context, different mimetic models were designed in order to simulate some of the abovementioned features encountered in cancer cells as lipid content is concerned (presence of PS and PE). Cholesterol has also been included since it represents an important component of biological membranes, being involved not only in the control of membrane fluidity but also in its structure [6]. The external pH conditions encountered by daunorubicin in the tumor microenvironment (acidic pH) were also taken into account. In fact, several studies reported that this acidic extracellular pH, caused by the high glucose consumption rates [34], is a typical intrinsic feature of tumor phenotype and benefits cancer cells once it promotes invasiveness.

In this work, small- and wide-angle X-ray scattering (SAXS and WAXS), which measure the membrane thickness and the crystalline packing of lipids in the membrane plane, were performed to assess the influence of DOX and DAN on the structural organization of the membrane models at the molecular level.

Materials and Methods

Materials and reagents. Daunorubicin (DAN) was obtained from Biovision (Milpitas, California) and doxorubicin (DOX) from LC Laboratories (Woburn, Massachusetts, USA). The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), sphingomyelin (SM) and cholesterol (Chol) were supplied by Avanti Polar Lipids, Inc. (Alabama, USA). All compounds were used without further purification. Drug solutions were prepared with HEPES buffer (10 mM, pH 7.4). The buffer was prepared with double deionized water (conductivity inferior to 10 μScm−1) and ionic strength adjusted to mimic physiological conditions with NaCl (I=10 M).

Multilamellar vesicles (MLVs) preparation. The several molar fractions of doxorubicin (DOX) and daunorubicin (DAN) (0, 10 and 20 mol%) were mixed with DMPC:DOPC:DPPS:DOPE [4:1.5:1.5:3] or DMPC:DOPC:DPPS:DOPE:Chol [4:1:1:2.5:1.5] in a chloroform/methanol mixture (3:1 v/v). Lipid films were formed by drying the samples under nitrogen stream and left overnight under reduced pressure to remove all traces of the organic solvents. After that, the lipid films were hydrated with Tris buffer (pH 6.3) and vortexed, at 60°C (well above the main phase transition temperature of the lipid mixtures). The lipid dispersions were transferred into glass capillaries of 1.5 mm diameter and the flame-sealed capillaries were stored at 4°C until the X-ray measurements.

X-ray scattering measurements. The assessment of the long-range membrane order and the hydrocarbon chain packing by X-ray scattering measurements were performed as described elsewhere [35, 36]. Small-angle and wide-angle X-ray scattering (SAXS and WAXS) experiments were done at the Austrian SAXS beamline in the electron storage ring Elettra (Trieste, Italy) [37]. A monochromatic radiation with a wavelength of 1054 nm was used and the SAXS and WAXS detectors Pilatus (Dectris, Baden Switzerland) were calibrated with silver behenate powder (*d*-spacing =58.376 Å) [38] and with p-bromo benzoic acid with *d*-spacing taken from Ohkura *et al*. [39], respectively. Measurements were performed at several temperatures (10°C, 37°C and 50°C) using a custom-made sample cell with a water bath Unistat (Huber, Offenburg, Germany) for cooling. Each diffraction pattern is presented as normalize scattering intensity in arbitrary units (a.u.) in function of the reciprocal spacing in nm^{-1}, s ($s = (2 \sin \theta) / \lambda$, where θ is the diffraction angle and λ is the X-ray wavelength). The obtained X-ray diffraction patterns was then subjected to detailed analysis using a Peak Fitting tool of OriginPro 8.5.1 Software (OriginLab Corporation), where each Bragg peak was fitted with Lorentzians. The positions of maximum intensities (s) were determined and used to calculate the repeat distances – the lipid bilayer plus the water layer distances, d ($d = 1/s$), while the widths at half maximum (w) were used to calculate the correlation lengths between the lipid bilayers, ξ ($\xi = 2\pi/w$).

Results and Discussion

The structural effect of DAN and DOX on the order and packing of DMPC:DOPC:DPPS:DOPE [4:1.5:1.5:3] and DMPC:DOPC:DPPS:DOPE:Chol [4:1:1:2.5:1.5] bilayers at pH 6.3 was assessed by employing X-ray scattering techniques (SAXS and WAXS). SAXS measurements allow one to obtain information regarding the distances between the thickness of the bilayer plus the water layer and, thus, on the long range bilayer organization, while WAXS measurements permit to calculate the distances between the phospholipids head groups, proving information about the short range order of the system [40]. The experiments were performed at 10°C, 37° and 50°C in order to study the drugs effect on the different lipid phases, because the order and packing of lipids are temperature-dependent [41].

Anthracyclines influence on the structure of DMPC:DOPC:DPPS:DOPE bilayers

The small angle X-ray diffraction patterns of DMPC:DOPC:DPPS:DOPE in Tris buffer (pH 6.3) at all temperatures are presented in Figure 1, and in Tris buffered solutions containing 10 and 20mol% of anthracyclines (DAN or DOX) at a physiological relevant temperature (37ºC) are presented in Figure 2. The repeat distance, *d*, deduced from the SAXS and WAXS patterns of DMPC:DOPC:DPPS:DOPE as well as the respective correlation between the bilayers (ξ) of the first-order Bragg peaks for all temperatures studied are depicted in Table 1.

The lipid mixture DMPC:DOPC:DPPS:DOPE is characterized by a SAXS diffraction pattern with a broad peak between the first and second-order Bragg peaks, which is more evident at 10ºC. The first and second-order peaks are in the ratio of 1:2 that is a clear indication of a lamellar structure. At the same time the presence of a broad peak can be an indication of non-correlated bilayers formation, such as the hexagonal inverted phases (H_{II}), probably due to the presence of phosphatidylethanolamines. Taken together the diffraction patterns demonstrate that probably the model membrane presents both lamellar and non-lamellar structures. However, more studies using simple model membranes are required to fully understand the structural behavior of these lipid mixtures.

The addition of anthracyclines on this model system led to some changes in the small angle X-ray diffraction patterns. In fact, the observed first order Bragg peaks are in a ratio of d-spacing that is a clear indication of a lamellar structure for both anticancer drugs studied. In this context, the presence of DAN and DOX causes a membrane stabilization, by the elimination of the non-lamellar phases. However, regarding the short-range

Table 1: Long and short-range distances (d) and correlation lengths (ξ) of DMPC:DOPC:DPPS:DOPE in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C, 37°C and 45°C and pH 6.3.

[Drug]	T	Long distances (Å)						Short distances (Å)	
(mol %)	(°C)	d_1	d_2	d_3	ξ_1	ξ_2	ξ_3	d	ξ
0	10	74.8 ± 0.5	79.7 ± 0.5	109.9 ± 0.5	2596 ± 10	2452 ± 10	456 ± 10	4.27 ± 0.05	273 ± 10
	37	66.1 ± 0.5	---	---	3523 ± 10	---	---	4.55 ± 0.05	145 ± 10
	50	65.5 ± 0.5	---	---	2668 ± 10	---	---	4.56 ± 0.05	165 ± 10
DAN 10	10	66.7 ± 0.5	77.4 ± 0.5	---	5694 ± 10	8667 ± 10	---	4.25 ± 0.05	275 ± 10
	37	61.3 ± 0.5	63.7 ± 0.5	---	3740 ± 10	4055 ± 10	---	4.54 ± 0.05	160 ± 10
	50	59.7 ± 0.5	62.1 ± 0.5	---	3861 ± 10	3225 ± 10	---	4.55 ± 0.05	145 ± 10
DAN 20	10	65.6 ± 0.5	77.3 ± 0.5	---	5605 ± 10	8667 ± 10	---	4.21 ± 0.05	420 ± 10
	37	59.1 ± 0.5	63.0 ± 0.5	---	4174 ± 10	3530 ± 10	---	4.56 ± 0.05	174 ± 10
	50	57.7 ± 0.5	62.9 ± 0.5	---	4368 ± 10	2302 ± 10	---	4.58 ± 0.05	174 ± 10
DOX 10	10	70.2 ± 0.5	81.2 ± 0.5	---	2917 ± 10	5025 ± 10	---	4.22 ± 0.05	460 ± 10
	37	64.1 ± 0.5	74.6 ± 0.5	---	2624 ± 10	4590 ± 10	---	4.56 ± 0.05	146 ± 10
	50	63.4 ± 0.5	72.5 ± 0.5	---	2213 ± 10	5068 ± 10	---	4.59 ± 0.05	160 ± 10
DOX 20	10	68.6 ± 0.5	82.2 ± 0.5	---	3643 ± 10	4677 ± 10	---	4.22 ± 0.05	400 ± 10
	37	64.4 ± 0.5	75.2 ± 0.5	---	2722 ± 10	7570 ± 10	---	4.55 ± 0.05	150 ± 10
	50	63.3 ± 0.5	72.3 ± 0.5	---	2293 ± 10	7096 ± 10	---	4.56 ± 0.05	162 ± 10

order of the system, no alterations were observed with the addition of both anticancer drugs.

Furthermore, it can be seen that in the presence of daunorubicin there is a lipid phase separation characterized by a smaller bilayer thickness plus the water layer when compared with the membrane without the drug. In addition, each lipid phase has a well-defined membrane thickness plus the water distance, as can be observed for example in Fig. 2, when compared with the higher distance range in the bulk model membrane. On the other hand, DOX also leads to a lipid phase separation that is more pronounced when compared with DAN.

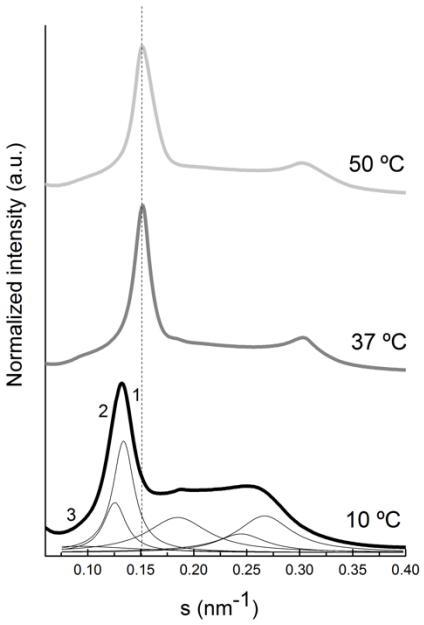


Fig. 1. X-ray diffraction patterns (SAXS) at 10, 37 and 50°C for DMPC:DOPC:DPPS:DOPE at pH 6.3.

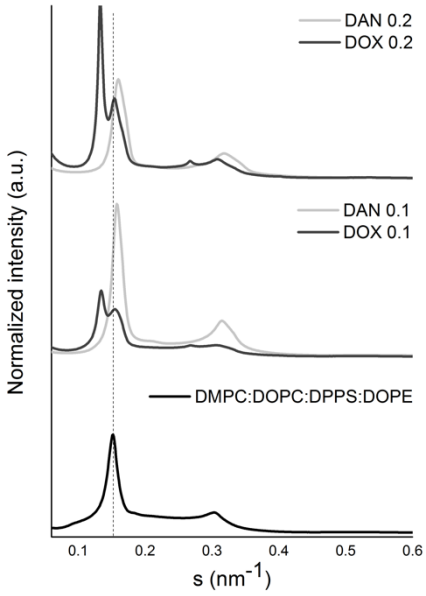


Fig. 2. X-ray diffraction patterns (SAXS) of DMPC:DOPC:DPPS:DOPE in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 37°C and pH 6.3.

Anthracyclines influence on the structure of DMPC:DOPC:DPPS:DOPE:Chol bilayers

The small angle X-ray diffraction patterns of DMPC:DOPC:DPPS:DOPE in Tris buffer (pH 6.3) at all temperatures is presented in Figure 3, and in Tris buffered solutions containing 10 and 20mol% of anthracyclines (DAN or DOX) at a 10ºC is presented in Figure 4. Table 2 displays the long- and short-range repeat distances (*d*) and the respective correlation length (*ξ*) of each first-order Bragg peak at all the investigated temperatures.

At 10ºC the appearance of broader Bragg peaks in this model system leads to the idea that the presence of

Table 2: Long-range and short-range distances (d) and correlation lengths (ξ) of DMPC:DOPC:DPPS:DOPE:Chol in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C, 37°C and 50°C and pH 6.3.

[Drug]	T	Long distances (Å)						Short distances (Å)	
(mol%)	(°C)	d1	d 2	d 3	ξ1	ξ2	ξ3	d	ξ
0	10	69.9 ± 0.5	74.2 ± 0.5	108.0 ± 0.5	4330 ± 10	1028 ± 10	904 ± 10	4.36 ± 0.05	156 ± 10
	37	70.0 ± 0.5	---	---	2081 ± 10	---	---	4.64 ± 0.05	120 ± 10
	50	67.8 ± 0.5	---	---	1973 ± 10	---	---	4.60 ± 0.05	170 ± 10
DAN 10	10	64.4 ± 0.5	80.0 ± 0.5	---	7630 ± 10	9254 ± 10	---	4.38 ± 0.05	135 ± 10
	37	---	---	---	---	---	---	---	---
	50	---	---	---	---	---	---	---	---
DAN 20	10	63.5 ± 0.5	79.9 ± 0.5	---	5320 ± 10	7681 ± 10	---	4.37 ± 0.05	170 ± 10
	37	61.8 ± 0.5	77.0 ± 0.5	---	3337 ± 10	6746 ± 10	---	4.60 ± 0.05	179 ± 10
	50	59.6 ± 0.5	64.1 ± 0.5	---	6196 ± 10	2223 ± 10	---	---	----
DOX 10	10	68.1 ± 0.5	83.0 ± 0.5	---	3418 ± 10	5457 ± 10	---	4.33 ± 0.05	247 ± 10
	37	64.7 ± 0.5	78.0 ± 0.5	---	2808 ± 10	4782 ± 10	---	4.60 ± 0.05	138 ± 10
	50	63.1 ± 0.5	74.9 ± 0.5	---	2624 ± 10	4303 ± 10	---	---	---
DOX 20	10	67.2 ± 0.5	82.6 ± 0.5	---	4415 ± 10	7315 ± 10	---	4.33 ± 0.05	206 ± 10
	37	65.1 ± 0.5	77.3 ± 0.5	---	3967 ± 10	5861 ± 10	---	4.60 ± 0.05	145 ± 10
	50	65.2 ± 0.5	75.1 ± 0.5	---	3361 ± 10	6308 ± 10	---	---	---

cholesterol induces the formation of domains with distinct membrane thicknesses, as evidenced by the deconvolution in Fig. 3. However, with temperature increase the lipids become more homogeneous distributed along the membrane and, probably, such domains disappear.

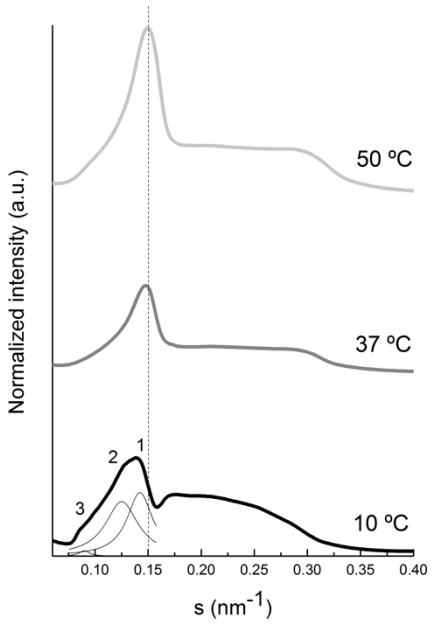


Fig. 3. **X-ray diffraction patterns (SAXS) at 10, 37 and 50°C for DMPC:DOPC:DPPS:DOPE:Chol at pH 6.3.**

Furthermore, at higher temperatures the increase in the bilayer plus the water layer distance caused by cholesterol can be attributed to its condensing effect, where the phospholipids acyl chains become organized and fully extended. Regarding the anticancer drugs' effect, it is possible to conclude that once again the compounds induce a lipid redistribution within the bilayer. There is membrane region (higher distances) comprising a similar bilayer thickness and w ater layer in the presence of both DAN and DOX. However, concerning the second lipid phase region, DAN promoted a higher decrease in the thickness of the bilayer plus the water layer when compared with DOX.

The 10%mol daunorubicin sample at 37 and 50ºC was compromised and, thus, the experiments at both temperatures with this sample need to be repeated.

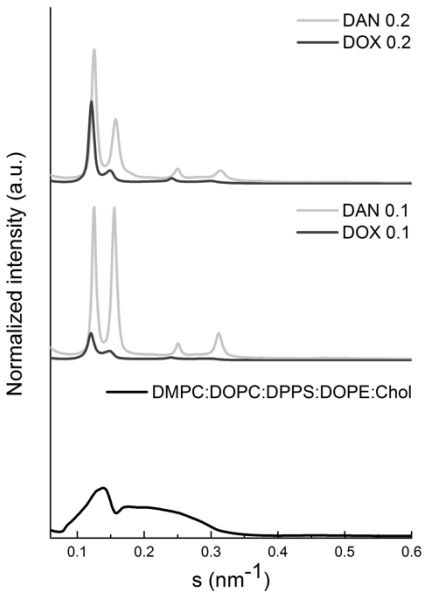


Fig. 4. X-ray diffraction patterns (SAXS) of DMPC:DOPC:DPPS:DOPE:Chol in the absence and presence of 10 and 20 mol% of daunorubicin (DAN) or doxorubicin (DOX) at 10°C and pH 6.3.

Conclusions

Over the past decades, several evidences have been demonstrating the involvement of cell membranes in the cytotoxic mechanism of action of anthracyclines, which modulate membrane properties and functions. The results from SAXS and WAXS clearly indicate that DAN and DOX interact with membranes by incorporation into the lipid bilayers and produce significant alteration in the membrane' structural organization. In fact, anthracyclines caused a pronounced stabilization of the bilayer by the elimination of non-lamellar phases. In addition, these compounds also induced a clear lipid phase separation comprising well-defined thicknesses, contrary to the bulk membranes.

Nevertheless, it is crucial to perform more studies using model systems with simpler lipid composition in order to depict and understand the structural organization of these mixtures but also the behavior of these anthracyclines.

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1. Weiss, R.B., *The anthracyclines: will we ever find a better doxorubicin?* Semin Oncol, 1992. **19**(6): p. 670-86.
2. Lozano, R., et al., *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010*. Lancet, 2012. **380**(9859): p. 2095-128.
3. Minotti, G., et al., *Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity*. Pharmacol Rev, 2004. **56**(2): p. 185-229.
4. Bourgaux, C. and P. Couvreur, *Interactions of anticancer drugs with biomembranes: what can we learn from model membranes?* J Control Release, 2014. **190**: p. 127-38.
5. Tritton, T.R. and G. Yee, *The Anticancer Agent Adriamycin Can Be Actively Cyto-Toxic without Entering Cells*. Science, 1982. **217**(4556): p. 248-250.
6. Escriba, P.V., et al., *Membranes: a meeting point for lipids, proteins and therapies*. J Cell Mol Med, 2008. **12**(3): p. 829-75.
7. Escriba, P.V., et al., *Role of lipid polymorphism in G protein-membrane interactions: nonlamellar-prone phospholipids and peripheral protein binding to membranes*. Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11375-80.
8. Escriba, P.V., M. Sastre, and J.A. Garciasevilla, *Disruption of Cellular Signaling Pathways by Daunomycin through Destabilization of Nonlamellar Membrane Structures*. Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(16): p. 7595-7599.
9. Giorgione, J., et al., *Role of phospholipids containing docosahexaenoyl chains in modulating the activity of protein kinase C*. Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9767-70.
10. Chandra, P., H.B. Noh, and Y.B. Shim, *Cancer cell detection based on the interaction between an anticancer drug and cell membrane components*. Chem Commun (Camb), 2013.
11. van Meer, G., D.R. Voelker, and G.W. Feigenson, *Membrane lipids: where they are and how they behave*. Nature Reviews Molecular Cell Biology, 2008. **9**(2): p. 112-124.
12. Ran, S. and P.E. Thorpe, *Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy*. Int J Radiat Oncol Biol Phys, 2002. **54**(5): p. 1479-84.
13. Ran, S., A. Downes, and P.E. Thorpe, *Increased exposure of anionic phospholipids on the surface of tumor blood vessels*. Cancer Res, 2002. **62**(21): p. 6132-40.
14. Riedl, S., et al., *In search of a novel target — Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy*. Biochimica et Biophysica Acta, 2011. **1808**(11-15): p. 2638-2645.
15. Stafford, J.H. and P.E. Thorpe, *Increased exposure of phosphatidylethanolamine on the surface of tumor vascular endothelium*. Neoplasia, 2011. **13**(4): p. 299-308.
16. Shinitzky, M., *Membrane fluidity in malignancy. Adversative and recuperative*. Biochim Biophys Acta, 1984. **738**(4): p. 251-61.
17. Taraboletti, G., et al., *Membrane fluidity affects tumor-cell motility, invasion and lung-colonizing potential*. Int J Cancer, 1989. **44**(4): p. 707-13.
18. Inbar, M., *Fluidity of membrane lipids: a single cell analysis of mouse normal lymphocytes and malignant lymphoma cells*. FEBS Lett, 1976. **67**(2): p. 180-5.
19. Deliconstantinos, G., *Physiological aspects of membrane lipid fluidity in malignancy*. Anticancer Res, 1987. **7**(5B): p. 1011-21.
20. Yang, Y.Z., et al., *Normal and leukemic lymphocyte membrane fluidity and response to stimulation with ConA and PHA*. J Tongji Med Univ, 1989. **9**(3): p. 143-7.
21. Sherbet, G.V., *Membrane fluidity and cancer metastasis*. Exp Cell Biol, 1989. **57**(4): p. 198-205.
22. Sok, M., et al., *Cell membrane fluidity and prognosis of lung cancer*. Ann Thorac Surg, 2002. **73**(5): p. 1567-71.
23. Campanella, R., *Membrane lipids modifications in human gliomas of different degree of malignancy*. J Neurosurg Sci, 1992. **36**(1): p. 11-25.
24. Rodrigues, C.M., et al., *Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis*. J Lipid Res, 2002. **43**(6): p. 885-94.
25. Johnson, S.M. and R. Robinson, *The composition and fluidity of normal and leukaemic or lymphomatous lymphocyte plasma membranes in mouse and man*. Biochim Biophys Acta, 1979. **558**(3): p. 282-95.
26. Barcelo-Coblijn, G., et al., *Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy*. Proc Natl Acad Sci U S A, 2011. **108**(49): p. 19569-74.
27. Agatha, G., R. Hafer, and F. Zintl, *Fatty acid composition of lymphocyte membrane phospholipids in children with acute leukemia*. Cancer Lett, 2001. **173**(2): p. 139-44.
28. Galeotti, T., et al., *Membrane-Alterations in Cancer-Cells - the Role of Oxy Radicals*. Annals of the New York Academy of Sciences, 1986. **488**: p. 468-480.
29. Lucio, M., J.L. Lima, and S. Reis, *Drug-membrane interactions: significance for medicinal chemistry*. Curr Med Chem, 2010. **17**(17): p. 1795-809.
30. Peetla, C., A. Stine, and V. Labhasetwar, *Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery*. Mol Pharm, 2009. **6**(5): p. 1264-76.
31. Seddon, A.M., et al., *Drug interactions with lipid membranes*. Chem Soc Rev, 2009. **38**(9): p. 2509-19.
32. Escriba, P.V., *Membrane-lipid therapy: a new approach in molecular medicine*. Trends in Molecular Medicine, 2006. **12**(1): p. 34-43.
33. Escriba, P.V., et al., *Membrane lipid therapy: Modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment*. Progress in Lipid Research, 2015. **59**: p. 38-53.
34. Cardone, R.A., V. Casavola, and S.J. Reshkin, *The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis*. Nat Rev Cancer, 2005. **5**(10): p. 786-95.
35. Nunes, C., et al., *Effects of non-steroidal anti-inflammatory drugs on the structure of lipid bilayers: therapeutical aspects*. Soft Matter, 2011. **7**(6): p. 3002-3010.
36. Neves, A.R., et al., *Resveratrol interaction with lipid bilayers: a synchrotron X-ray scattering study*. Langmuir, 2016.
37. Amenitsch, H., et al., *First performance assessment of the small-angle X-ray scattering beamline at ELETTRA*. J Synchrotron Radiat, 1998. **5**(Pt 3): p. 506-8.
38. Blanton, T.N., et al., *JCPDS—International Centre for Diffraction Data round robin study of silver behenate. A possible low-angle X-ray diffraction calibration standard*. Powder Diffraction, 1995. **10**(2): p. 91-95.
39. Ohkura, K., S. Kashino, and M. Haisa, *The Crystal and Molecular Structure of p-Bromobenzoic Acid*. Bulletin of the Chemical Society of Japan, 1972. **45**(8): p. 2651-2652.
40. Pili, B., et al., *Interaction of an anticancer drug, gemcitabine, with phospholipid bilayers*. Journal of Thermal Analysis and Calorimetry, 2009. **98**(1): p. 19.
41. Pereira-Leite, C., et al., *Biophysical characterization of the drug–membrane interactions: The case of propranolol and acebutolol*. European Journal of Pharmaceutics and Biopharmaceutics, 2013. **84**(1): p. 183-19.

Chapter VI

Concluding remarks

1. Final conclusions

In this chapter the questions that guided this research, expressed in the introduction, will be taken up and a summary of the obtained results from the membrane biophysical studies will be outlined. The outcomes clearly indicate that both daunorubicin and doxorubicin are able to be incorporated into lipid bilayers, penetrating into the acyl membrane region but also positioning their positively charged amino group near the head group region of the membrane. In addition, the gathered data points to an anticancer drug's behavior that is responsible for significant alterations in the membrane organization, to which extent depends not only on the chemical structure of the drug but also on the bilayer lipid composition and physical state. In fact, the small structural difference between daunorubicin and doxorubicin (presents an hydroxyl instead of a methyl group in the side chain) has a significant impact in the drug-membrane interactions.

Comparing the anthracyclines behavior in models membranes composed of DMPC:SM it is possible to observe that doxorubicin has a higher partition than daunorubicin in these bilayers. Furthermore, while the presence of liquid-ordered phase, induced by cholesterol in DMPC:SM, significantly reduced the daunorubicin membrane partition, it did not impair the doxorubicin penetration within the bilayer. The formation of such liquid-ordered domains is important for different signal transduction pathways, through the regulation of membrane-associating receptors, proteins and enzymes. In fact, lipid rafts have been associated to organizing centers for the assembly of apoptotic and signaling molecules involved in cell death. Among the proteins thought to be associated with these liquid-ordered domains is P-glycoprotein P (P-gp) that effluxes a variety of hydrophobic, neutral and positively charged drugs from the cell. This protein is a component of the normal cell defense system against xenobiotics, but it also overexpressed in cancer cells. Anthracyclines are among the subtracts effluxed by P-gp, which is responsible for the reduced drug concentration inside the cell and, for that reason, this protein plays a key role in drug resistance. According to the gathered results it is possible to conclude that the resistance mechanism that lowers the efficacy of these anticancer drugs, results from an increased density in the membrane regions (liquid-ordered domains) where the efflux proteins are present.

Furthermore, although both drugs present a broad distribution along the membrane, due to their ability to interact electrostatically with the lipids head groups and also with the acyl chains trough hydrophobic interactions, doxorubicin assumes a more superficial location in bilayers comprising DMPC:SM:Chol than daunorubicin, as observed by the dynamic simulations and location studies. In addition, in the presence of cholesterol, doxorubicin preferentially locates within the bilayer closer to the lipid-water interface. Cholesterol, however, was found to not interact directly with the drug; the effect of the presence of cholesterol was solely due to the ordering effect cholesterol has on membrane structure. Anthracyclines alterations on the long-range bilayer order and in the hydrocarbon chain packing were revealed by X-ray diffraction measurements and highlight the perturbing but distinct effect of these compounds in the studied membranes.

The fluid phase of membranes is considered as the most biologically relevant, since great part of the cellular membranes composition are fluid bilayers that have the necessary permeability to allow the exchange of substances between the extracellular and intracellular media. Hence, the interactions of drugs with membranes at the fluid phase play an important role in activity of the drugs. In the fluid phase of the membrane composed of DMPC:SM, both drugs decrease the membrane fluidity. Nevertheless, in the presence of cholesterol (liquid-ordered domains) the drugs' ability to alter the membrane fluidity is reduced. Once again, this fact can be also be correlated with the mechanism of resistance, a clinical problem that leads to therapy failure.

In this work, model membranes containing PE and PS that are known to be expressed in the outer leaflet of cancer cells were also used to better understand the behavior of anthracyclines. Results show that daunorubicin and doxorubicin also present a high partition with these mimetic bilayers, which can be correlated with their cytotoxicity. Nevertheless, the compounds effect in the membrane fluidity was also different between then and dependent on the lipid composition. In fact, the increase in the acyl chains order were more pronounced by daunorubicin and by doxorubicin. At the same time, the presence of cholesterol in these membranes, besides reducing the fluidity and increasing the thickness of the bilayer, it also modulated the anticancer drugs action in the membrane structural properties. In fact, cholesterol without directly interacting with doxorubicin, reduced the compounds' ability to influence the membrane microviscosity.

In conclusion, the biophysical interaction studies of anthracyclines with model membranes provide a rational approach to a better understanding of the overall mechanism of action that are behind the pharmacological and therapeutic activities of these compounds.

2. Limitations of the experimental studies

One of the major limitations pointed to the biophysical studies of drug-membrane interactions is generally the lack of all the constituents generally present in the membranes such as proteins and/or the lipid composition chosen. However, the biophysical studies should be interpreted in the most cases as retrospectives studies. Thus, in the presence of a certain phenomenon not fully explained by the conventional point of view of a drug's mechanism of action (i.e. drug that has a specific target), the biophysical studies may help to get a higher knowledge about complementary and membrane dependent mechanism of action, using for that, simple but reliable membrane models. It is important to report that the simplification and, therefore, a complete control of so complex structures is critical to understand the interactions at the molecular level. In addition, the concentrations used are in most cases much higher in comparison with the achieved therapeutic plasmatic concentration of anthracyclines. This is related with the sensibility of the techniques used.

3. Future perspectives

At the end of this thesis, it becomes evident that the gathered results raise new questions and hypothesis that point the way for future research. Among the several studies that can be performed to deepen the knowledge regarding anthracyclines-membrane interactions it would be useful to:

- Test membrane models with different lipid composition and, eventually, include proteins (proteoliposomes), enzymes or important membrane receptors with the goal of getting close to biomembranes. In fact, the assessment of receptors-anthracyclines interaction or of the effect of anthracyclines-membrane interaction in the receptor activity may prove to be important for the designing of more effective and selective drugs.
- Apply the assays used in this work to the investigation of other anthracyclines, which could serve to establish relations between the parameters evaluated and the pharmacological or side effects presented by the drugs.
- Studies of drug location by other techniques that do not depend on the quenching ability of a third fluorescent molecule (probe). The location and conformation of the studied drugs appears to be crucial in the establishment of their therapeutic action, as well as in their pharmacological features. More information about the exact location of the drugs in the membrane could be obtained by nuclear magnetic resonance (NMR) or neutron diffraction. In fact, performing neutron diffraction studies would be very important once it would allow assessing without doubt the thickness of the water layer between bilayers and,

thereby, clarifying the assumptions made in the SAXS and WAXS experiments. Besides, techniques such as Grazing incidence X-ray diffraction (GIXD) and X-ray reflectivity may be applied to the monolayers in order to study, respectively, the changes of the phospholipids molecular order induced by the drugs or other lipids and to study the influence in the structure of the monolayer.

- These biophysical studies may be interpreted as the preliminary studies to the further design and development of an innovative of a nanocarrier lipid based (e.g. liposomes and lipid nanoparticles) for targeted delivery of anthracyclines. Indeed, the present thesis seeks to deepen the scientific knowledge about the biophysics underlying drug-membrane interactions, in order to choose lipid based nanocarriers systems with a rational composition selection based in the biophysical properties observed for the drug- membrane interactions. The chosen membrane models allow us to predict the type of interactions of anthracyclines with drug delivery systems such as liposomes, which could be critical for an efficient drug delivery lipid-based systems.

Bibliography

1. Triton TR, Yee G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science (New York, NY). 1982;217(4556):248-50.
2. Escribá PV, Sastre M, García-Sevilla JA. Disruption of cellular signaling pathways by daunomycin through destabilization of nonlamellar membrane structures. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(16):7595-9.
3. Seydel JK, Wiese M. Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling: Wiley-VCH Verlag GmbH & Co.; 2002.
4. Jesorka A, Orwar O. Liposomes: technologies and analytical applications. Annual review of analytical chemistry. 2008;1:801-32.
5. Escriba PV, Gonzalez-Ros JM, Goni FM, Kinnunen PK, Vigh L, Sanchez-Magraner L, et al. Membranes: a meeting point for lipids, proteins and therapies. Journal of cellular and molecular medicine. 2008;12(3):829-75.
6. Mills TT, Huang J, Feigenson GW, Nagle JF. Effects of cholesterol and unsaturated DOPC lipid on chain packing of saturated gel-phase DPPC bilayers. Gen Physiol Biophys. 2009;28(2):126-39.
7. Llado V, Lopez DJ, Ibarguren M, Alonso M, Soriano JB, Escriba PV, et al. Regulation of the cancer cell membrane lipid composition by NaCHOleate: effects on cell signaling and therapeutical relevance in glioma. Biochim Biophys Acta. 2014;1838(6):1619-27.
8. Saiz L, Bandyopadhyay S, Klein ML. Towards an understanding of complex biological membranes from atomistic molecular dynamics simulations. Bioscience Rep. 2002;22(2):151-73.
9. Rappolt M. Bilayer thickness estimations with "poor" diffraction data. J Appl Phys. 2010;107(8).
10. Mouritsen OG. Life as a matter of fat - the emerging science of Lipidomics. Dragoman D, Dragoman M, Elitzur AC, Silverman MP, Tuszynski J, Zeh HD, editors: Springer; 2005.
11. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. Science (New York, NY). 2010;327(5961):46-50.
12. Sodt AJ, Sandar ML, Gawrisch K, Pastor RW, Lyman E. The molecular structure of the liquid-ordered phase of lipid bilayers. J Am Chem Soc. 2014;136(2):725-32.
13. Seddon AM, Casey D, Law RV, Gee A, Templer RH, Ces O. Drug interactions with lipid membranes. Chem Soc Rev. 2009;38(9):2509-19.
14. Seydel JK, Wiese M. Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling. Mannhold R, Kubinyi H, Folkers G, editors: Wiley-VCH; 2002.
15. Brasseur R, Deleu M, Mingeot-Leclercq MP, Francius G, Dufrene YF. Probing peptide-membrane interactions using AFM. Surf Interface Anal. 2008;40(3-4):151-6.
16. Pott T, Dufourcq J, Dufourc EJ. Fluid or gel phase lipid bilayers to study peptide membrane interactions? Eur Biophys J Biophy. 1996;25(1):55-9.
17. Gaspar D, Lucio M, Wagner K, Brezesinski G, Rocha S, Lima JLFC, et al. A biophysical approach to phospholipase A(2) activity and inhibition by anti-inflammatory drugs. Biophys Chem. 2010;152(1-3):109-17.
18. Wenk MR. The emerging field of lipidomics. Nat Rev Drug Discov. 2005;4(7):594-610.
19. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Bio. 2008;9(2):112-24.
20. Singer SJ, Nicolson GL. Fluid Mosaic Model of Structure of Cell-Membranes. Science (New York, NY). 1972;175(4023):720-&.
21. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. Nature reviews Molecular cell biology. 2008;9(2):112-24.
22. Nelson DL, Cox MM. Lehninger Principles of Biochemistry: W.H.Freeman & Co; 2004.

23. Skwarek M. Recent controversy surrounding lipid rafts. Archivum immunologiae et therapiae experimentalis. 2004;52(6):427-31.
24. Fendler JH. Membrane mimetic chemistry: Wiley-Interscience; 1982.
25. Martin DK. Nanobiotechnology of Biomimetic Membranes. Ferrari M, editor: Springer; 2007.
26. Chan YHM, Boxer SG. Model membrane systems and their applications. Curr Opin Chem Biol. 2007;11(6):581-7.
27. Scrimin P, Tecilla P, Moss RA, Bracken K. Control of permeation of lanthanide ions across phosphate-functionalized liposomal membranes. J Am Chem Soc. 1998;120(6):1179-85.
28. Matos C, Lima JLFC, Reis S, Lopes A, Bastos M. Interaction of antiinflammatory drugs with EPC liposomes: calorimetric study in a broad concentration range. Biophys J. 2004;86:946-54.
29. Lasic DD. Liposomes - from Physics to Applications. New York: Elsevier; 1993.
30. Mui B, Hope MJ. Formation of Large UnilamellarVesicles by Extrusion. In: Gregoriadis G, editor. Liposome Preparation and Related Techniques. Liposome Technology: Informa Healthcare; 2007.
31. Peetla C, Stine A, Labhasetwar V. Biophysical Interactions with Model Lipid Membranes: Applications in Drug Discovery and Drug Delivery. Mol Pharmaceut. 2009;6(5):1264-76.
32. Langmuir I. The constitution and fundamental properties of solids and liquids. II. Liquids. J Am Chem Soc. 1917;39:1848-906.
33. Buehler LK. Cell Membranes. New York: Garland Science Taylor & Francis Group; 2015.
34. Esteves F, Moutinho C, Matos C. Correlation between octanol/water and liposome/water distribution coefficients and drug absorption of a set of pharmacologically active compounds. J Liposome Res. 2013;23(2):83-93.
35. Marcelino J, Lima JL, Reis S, Matos C. Assessing the effects of surfactants on the physical properties of liposome membranes. Chemistry and physics of lipids. 2007;146(2):94-103.
36. Ferreira H, Lucio M, Lima JL, Matos C, Reis S. Interaction of clonixin with EPC liposomes used as membrane models. Journal of pharmaceutical sciences. 2005;94(6):1277-87.
37. Gombar VK, Enslein K. Assessment of n-octanol/water partition coefficient: when is the assessment reliable? Journal of chemical information and computer sciences. 1996;36(6):1127-34.
38. Martinez MN, Amidon GL. A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals. Journal of clinical pharmacology. 2002;42(6):620-43.
39. Santos NC, Prieto M, Castanho MARB. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. Bba-Biomembranes. 2003;1612(2):123-35.
40. Sethi B, Soni M, Kumar S, Gupta GD, Mishra S, Singh R. Lipophilicity Measurement Through Newer Techniques. Journal of Pharmacy Research. 2010;3(2):345-51.
41. Betageri GV, Rogers JA. The Liposome as a Distribution Model in Qsar Studies. Int J Pharm. 1988;46(1-2):95-102.
42. DD L. Liposomes--From Physics to applications. New York: Elsevier; 1993.
43. Castro B, Gameiro P, Lima JLFC, Matos C, Reis S. A fast and reliable spectroscopic method for the determination of membrane-water partition coefficients of organic compounds. Lipids. 2001;36:89-96.
44. Ferreira H, Lúcio M, Siquet C, Reis S. Utilização de modelos membranares na avaliação da actividade de fármacos. QUÍMICA. 2005;99:39-51.
45. Kitamura K, Imayoshi N, Goto T, Shiro H, Mano T, Nakai Y. Second Derivative Spectrophotometric Determination of Partition Coefficients of Chlorpromazine and Promazine Between Lecithin Bilayer Vesicles and Water. Analytica Chimica Acta. 1995;304:101-6.

46. Santos NC, Prieto M, Castanho MARB. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. Biochimica et Biophysica Acta. 2003;1612:123-35.
47. Lelkes PI, Miller IR. Perturbations of Membrane-Structure by Optical Probes .1. Location and Structural Sensitivity of Merocyanine-540 Bound to Phospholipid-Membranes. J Membrane Biol. 1980;52(1):1-15.
48. Ojeda CB, Rojas FS. Recent developments in derivative ultraviolet/visible absorption spectrophotometry (review). Analytica Chimica Acta. 2004;518 1-24.
49. Omran AA, Kitamura K, Takegami S, El-Sayed AAY, Abdel-Mottaleb A. Determination of partition coefficients of diazepam and flurazepam between phosphatidylcholine bilayer vesicles and water by second derivative spectrophotometric method. Journal of Pharmaceutical and Biomedical Analysis. 2001;25(2):319-24.
50. Ferreira H, Lúcio M, Castro B, Gameiro P, Lima JLFC, Reis S. Partition and location of nimesulide in EPC liposomes: a spectrophotometric and fluorescence study
. Analytica and Bioanalytical Chemistry. 2003;377:293-8.
51. Ferreira H, Lúcio M, Lima JLFC, Cordeiro-da-Silva A, Tavares J, Reis S. Effect of anti-inflammatory drugs on splenocyte membrane fluidity. Analytical Biochemistry. 2005;339(1):144-9.
52. Mansilha CIGR. Estudos de partição e localização de fármacos em lipossomas por técnicas espectroscópicas [Tese de Doutoramento]. Porto: Universidade do Porto; 2002.
53. Loura LMS, de Almeida RFM, Coutinho A, Prieto M. Interaction of peptides with binary phospholipid membranes: application of fluorescence methodologies. Chemistry and physics of lipids. 2003;122(1-2):77-96.
54. Slavic J. Fluorescent probes in cellular and molecular biology. Michigan CRC Press: Ann Arbor; 1994.
55. Kaiser RD, London E. Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth. Biochemistry. 1998;37(22):8180-90.
56. Repáková J, Capková P, Holopainen JM, Vattulainen I. Distribution, Orientation, and Dynamics of DPH Probes in DPPC Bilayer. Journal of Physical Chemistry B. 2004;108:13438-48.
57. Kaiser RD, London E. Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analysis of membrane depth. Biochemistry. 1998;37:8180-90.
58. Vazquez JL, Montero MT, Merino S, Domenech O, Berlanga M, Vinas M, et al. Location and nature of the surface membrane binding site of ciprofloxacin: A fluorescence study. Langmuir. 2001;17(4):1009-14.
59. Lakowicz JR. Principles of Fluorescence Spectroscopy. 3rd ed. ed. New York: Springer; 2006.
60. Coutinho A, Prieto M. Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrylamide. Journal of Chemical Education. 1993;70:425.
61. Lucio M, Ferreira H, Lima JLFC, Matos C, de Castro B, Reis S. Influence of some anti-inflammatory drugs in membrane fluidity studied by fluorescence anisotropy measurements. Phys Chem Chem Phys. 2004;6(7):1493-8.
62. Valeur B. Molecular Fluorescence Principles and Applications: Wiley-VHC; 2002.
63. Kriechbaum M, Laggner P. States of phase transitions in biological structures. Prog Surf Sci. 1996;51(3):233-61.
64. Müller-Goymann CC. Physicochemical characterization of colloidal drug delivery systems such as reverse micelles, vesicles, liquid crystals and nanoparticles for topical administration. European Journal of Pharmaceutics and Biopharmaceutics. 2004;58:343-56.
65. Larsson M. X-Ray diffraction studies of lung surfactant membrane sctructures. In: Nag K, editor. Structure and Dynamics of Membranous Interfaces: Wiley; 2008.
66. Mills TT, Tristram-Nagle S, Heberle FA, Morales NF, Zhao J, Wu J, et al. Liquid-liquid domains in bilayers detected by wide angle x-ray scattering. Biophys J. 2008;95(2):682-90.

67. Gunstone FD, Harwood JL, Padley FB. The Lipid Handbook. 2nd edition ed. New York: Chapman & Hall; 1994.
68. Butt H-J, Graf K, Kappl M. Thin films on surfaces of liquids. Physics and Chemistry of Interfaces: Wiley-VCH; 2003.
69. Feng SS, Gong K, Chew J. Molecular interactions between a lipid and an antineoplastic drug paclitaxel (taxol) within the lipid monolayer at the air/water interface. Langmuir. 2002;18(10):4061-70.
70. Schmidt TF, Caseli L, Nobre TM, Zaniquelli MED, Oliveira ON. Interaction of horseradish peroxidase with Langmuir monolayers of phospholipids. Colloid Surface A. 2008;321(1-3):206-10.
71. Wang ZN, Yang SH. Effects of Fullerenes on Phospholipid Membranes: A Langmuir Monolayer Study. Chemphyschem. 2009;10(13):2284-9.
72. Stine KJ. Brewster Angle Microscopy. Supramolecular Chemistry: John Wiley & Sons, Ltd; 2012.
73. Hönig D, Möbius D. Reflectometry at the Brewster angle and Brewster angle microscopy at the air-water interface. Thin Solid Films. 1992;210:64-8.
74. Roldan-Carmona C, Giner-Casares JJ, Perez-Morales M, Martin-Romero MT, Camacho L. Revisiting the Brewster Angle Microscopy: the relevance of the polar headgroup. Adv Colloid Interface Sci. 2012;173:12-22.
75. Alonso C, Alig T, Yoon J, Bringezu F, Warriner H, Zasadzinski JA. More than a monolayer: relating lung surfactant structure and mechanics to composition. Biophys J. 2004;87(6):4188-202.
76. Mendelsohn R, Flach CR. Infrared Reflection – Absorption Spectrometry of Monolayer Films at the Air – Water Interface. Handbook of Vibrational Spectroscopy: John Wiley & Sons, Ltd; 2006.
77. Blaudez D, Turlet J-M, Dufourcq J, Bard D, Buffeteau T, Desbat B. Investigations at the air/water interface using polarization modulation IR spectroscopy. Journal of the Chemical Society, Faraday Transactions. 1996;92(4):525-30.
78. Mendelsohn R, Mao GR, Flach CR. Infrared reflection-absorption spectroscopy: Principles and applications to lipid-protein interaction in Langmuir films. Bba-Biomembranes. 2010;1798(4):788-800.
79. Mendelsohn R, Flach CR. Infrared reflection-absorption spectroscopy of lipids, peptides, and proteins in aqueous monolayers. Curr Top Membr. 2002;52:57-88.