

DOUTORAMENTO

Química Analítica

Development and application of enzymatic and cell-based assays to evaluate potential anticancer compounds

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Development and application of enzymatic and cell-based assays to evaluate potential anticancer compounds

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Química Analítica

Trabalho realizado sob a orientação da Professora Doutora Maria Lúcia Marques Ferreira de Sousa Saraiva e coorientação do Professor Doutor Paul Joseph Dyson

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É autorizada a reprodução integral desta tese apenas para efeitos de investigação, mediante declaração escrita do interessado, que a tal se compromete.

A handwritten signature in black ink, appearing to read 'Sarah Pereira', with a long horizontal stroke extending from the end of the name.

Assinatura do autor,

“Live as if you were to die tomorrow. Learn as if you were to live forever.” Mahatma Gandhi

“You will never know everything about anything.” Julia Child

A quem sempre esteve presente e me apoiou em todos os momentos:

Avós Elísio e Emília

Mãe e Mana

António

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Abstract

Although cancer is the principal cause of death in the world, the chemotherapy currently used still presents several adverse effects. Therefore, it is imperative to improve current cancer therapy by designing new compounds with different modes of action, namely, in new cancer biomarkers to target them as a possible treatment for this disease.

The main purpose of this thesis was to study new compounds as possible alternatives to fight cancer. According to that, the first goal was the automatic evaluation of organometallic compounds, mostly ruthenium-based compounds, against overexpressed enzymes in cancer, such as cyclooxygenase 2 (COX-2), dihydrofolate reductase (DHFR), and glutathione S-transferase P1 (GST P1). To do that, it was developed and optimized flow methodologies based on sequential injection analysis (SIA) and lab-on-valve (LOV) to measure the enzyme's activity in the presence of organometallic compounds. These results made it possible to identify the most inhibitory compounds, compared with the reference ones, for each enzyme. In these works, it was possible to highlight the potential of the SIA and μ SIA-LOV systems as simple, versatile, robust, and fast analytical tools for the automation of the determination of the IC_{50} values of compounds.

The second goal was to test new ruthenium-based compounds with different counterions in their structure and assess their cytotoxicity, cellular uptake, and extent of apoptosis effect in luminal and triple-negative breast cancer cell lines, MCF-7 and MDA-MB-231, respectively. All the compounds were also evaluated against a non-tumorigenic cell line – L929, used as a reference for the safety evaluation according to ISO 10993-1:2009 on “Biological evaluation of medical devices”. Subsequently, the interaction and binding of the most cytotoxic ruthenium-based compound with plasmid and calf thymus DNA (ctDNA) and bovine serum albumin (BSA) were characterized by gel electrophoresis, circular dichroism, and fluorescence techniques. According to all the results, one of the new ruthenium-based compounds ($[Ru(\eta^6\text{-toluene})(PPh_3)_2Cl]Cl^+$) proved to be the most cytotoxic and the most uptaken compared with the other compounds tested. The interaction with ctDNA happens through the minor groove binding mode, presenting a similar binding constant for the BSA protein.

The third and last goal of this thesis was to develop and validate a new high-throughput screening (HTS) 384-well plate assay by assessing GST P1 inhibition by applying two compound libraries ($\approx 5'830$ compounds with or without anticancer activity). Furthermore, the compounds that presented the higher inhibitory activity against GST P1 were further studied *in vitro* with MCF-7 and MDA-MB-231 breast cancer cell lines. Then, from the

results obtained, 5 compounds were identified as the most cytotoxic ones for the two breast cancer cell lines (cryptotanshinone; ZM 39923; 10058-F4; ethacrynic acid, and PRT 4165). It was also assessed which compounds are competitive and non-competitive inhibitors through a competitive enzymatic assay with GST P1. Additionally, an *in vitro* combination assay was performed, and, according to the results obtained, most of the combinations, whether in the breast cancer cell lines MCF-7 or MDA-MB-231, showed an increase in cytotoxicity of 2 and 4 times. In the case of ethacrynic acid with cryptotanshinone, in the MDA-MB-231 cell lines, an 8-fold increase in cytotoxicity was obtained. To conclude this study, a western blot experiment was achieved to evaluate the effect of 3 of the 5 most cytotoxic compounds (ethacrynic acid, ZM 39923, and PRT 4165) in a triple-negative MDA-MB-231 breast cancer cell line. It was possible to prove the expression and non-expression of GST P1 in the breast cancer cell lines MDA-MB-231 and MCF-7, respectively. Additionally, there was an approximately 2-fold reduction in this expression after 24 h of treatment of the MDA-MB-231 cell line with ethacrynic acid, ZM 39923, and PRT 4165.

In conclusion, this thesis contributed to the evaluation of several compounds with different structural characteristics using fundamentally automatic enzymatic and *in vitro* methods. Its use may contribute to the development and identification of alternatives for breast cancer treatment with more advanced *in vivo* studies, with desirably lower side effects than those known for standard chemotherapy, in a prospective clinical application.

Keywords: cancer biomarkers, automatic methodologies, enzymatic inhibition, organometallic compounds, *in vitro* assays.

Resumo

Embora o cancro seja a principal causa de morte no mundo, a quimioterapia utilizada, atualmente, ainda apresenta muitos efeitos adversos. Portanto, é imperativo melhorar a terapia atual encontrando novos compostos, com diferentes modos de ação, nomeadamente, em novos biomarcadores cancerígenos para direcioná-los como um possível tratamento para esta doença.

O principal objetivo desta tese foi estudar novos compostos como possíveis alternativas para combater o cancro. De acordo com isso, o primeiro objetivo foi a avaliação automática de compostos organometálicos, principalmente compostos à base de ruténio, contra enzimas sobre-expressas no cancro, como a cicloxigenase 2 (COX-2), a dihidrofolato redutase (DHFR) e a glutathione S-transferase P1 (GST P1). Para isso, foram desenvolvidas e otimizadas metodologias de fluxo baseadas na análise por injeção sequencial (SIA) e lab-on-valve (LOV) para medir a atividade enzimática na presença dos compostos organometálicos. Esses resultados permitiram identificar os compostos mais inibidores em comparação com os inibidores positivos de referência para cada enzima. Nestes trabalhos foi possível evidenciar o potencial dos sistemas SIA e μ SIA-LOV como ferramentas analíticas simples, versáteis, robustas e rápidas para a automatização da determinação dos valores de IC_{50} de compostos.

O segundo objetivo foi testar novos compostos à base de ruténio, com diferentes contrações na sua estrutura, e estudar a sua citotoxicidade, absorção celular e extensão do efeito da apoptose em linhas celulares do cancro da mama luminal e triplo negativo, MCF-7 e MDA-MB-231, respetivamente. Todos os compostos foram, também, avaliados numa linha celular não tumorigénica – L929, usada como referência para a avaliação da segurança de acordo com a ISO 10993-1:2009 sobre “Avaliação biológica de dispositivos médicos”. Posteriormente, procedeu-se à caracterização da interação e da ligação, do composto à base de ruténio mais citotóxico, com o DNA plasmídeo e de timo de bezerro (ctDNA) e albumina de soro bovino (BSA) por técnicas de eletroforese em gel, dicroísmo circular e fluorescência. De acordo com todos os resultados, um dos novos compostos à base de ruténio ($[Ru(\eta^6\text{-tolueno})(PPh_3)_2Cl]Cl^+$) provou ser o mais citotóxico e, o mais absorvido, em comparação com os outros compostos testados. Relativamente à interação com o ctDNA, esta acontece através do modo de ligação *minor groove*, apresentando uma constante de ligação semelhante para a proteína BSA.

O terceiro e último objetivo desta tese foi desenvolver e validar um novo ensaio em placa de 384 poços de triagem de alto rendimento (HTS) para pesquisa da inibição da GST P1

por duas bibliotecas de compostos ($\approx 5'830$ compostos com ou sem atividade anticancerígena). Além disso, os compostos que apresentaram maior atividade inibitória contra a GST P1 foram posteriormente estudados, *in vitro*, nas linhas celulares do cancro da mama MCF-7 e MDA-MB-231. Então, a partir dos resultados obtidos, 5 compostos foram identificados como os mais citotóxicos para as duas linhas celulares do cancro da mama (cryptotanshinone; ZM 39923; 10058-F4; ácido etacrínico e PRT 4165). Também se avaliou quais os compostos que são inibidores competitivos e não competitivos através de um ensaio enzimático competitivo com a GST P1. Adicionalmente, efetuou-se um ensaio de combinação *in vitro* e, de acordo com os resultados obtidos, a maioria das combinações, quer nas linhas celulares do cancro da mama MCF-7 quer MDA-MB-231 mostrou um aumento da citotoxicidade de 2 e 4 vezes. No caso do ácido etacrínico com cryptotanshinone, nas linhas celulares MDA-MB-231, foi obtido um aumento na citotoxicidade de 8 vezes. Para concluir este estudo, realizou-se um ensaio de *western blot* para avaliar o efeito de 3 dos 5 compostos mais citotóxicos (ácido etacrínico, ZM 39923 e PRT 4165) na linha celular de cancro da mama MDA-MB-231 triplo negativo. Foi possível comprovar a expressão e a não expressão da GST P1 nas linhas celulares de cancro da mama MDA-MB-231 e MCF-7, respetivamente. Adicionalmente, verificou-se a redução de aproximadamente 2 vezes desta expressão, após 24 h de tratamento da linha celular MDA-MB-231 com ácido etacrínico, ZM 39923 e PRT 4165.

Em conclusão, esta tese contribuiu para a avaliação de vários compostos, com diferentes características estruturais, usando métodos enzimáticos e *in vitro* fundamentalmente automáticos. A sua utilização pode contribuir para o desenvolvimento e identificação, com estudos *in vivo* mais avançados, de alternativas para o tratamento do cancro da mama, com efeitos colaterais desejavelmente menores, dos conhecidos pela quimioterapia padrão, numa aplicação clínica prospetiva.

Palavras-chave: biomarcadores cancerígenos, metodologias automáticas, inibição enzimática, compostos organometálicos, ensaios *in vitro*.

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Chapter II

General Introduction

Miniaturized technologies for high-throughput screening enzymatic assays and diagnostics – a review.

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Fig. 2 – Assay principles for the detection of peptide phosphorylation on peptide arrays.

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detection with a labelled phosphoamino acid chelator. Copyright 2005, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim49

Fig. 3 – Schematic of the microchip used for analysis of AChE inhibitors. The fluid reservoirs were charged with the enzyme (AChE), acetylthiocholine chloride substrate, and coumarinyl phenyl maleimide fluorophore. The inhibitor sample was changed as indicated (77). Copyright 1999 American Chemical Society52

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Tumor microenvironmental features and advanced in vitro cell culture platforms and enzyme targeting: an overview

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List of Abbreviations

μ -SIA-LOV – micro sequential injection analysis coupled lab-on-valve
 μ ARCS – micro arrayed compound screening
 μ PADs – microfluidic paper-based analytical devices
 μ TAS – micro total analysis system
2D – two-dimensional
3D – three-dimensional
5-FU – 5-fluorouracil
5-LOX – 5-lipoxygenase
7-AAD – 7-amino-actinomycin D
ABC1 – ATP-binding cassette transporter
Abs – absorbance
ABS – activity-based sensing
AChE – acetylcholinesterase
ADH – alcohol dehydrogenase
ADHP – 10-Acetyl-3,7-dihydroxyphenoxazine
AKT – activates protein kinase B
AMPK – adenosine monophosphate activated protein kinase
ARA – arachidonic acid
ASK 1 – Apoptotic signal-regulating kinase 1
ATCC – American Type Culture Collection
ATP – Adenosine triphosphate
BRACHY-T – brachyury
BSA – bovine serum albumin
BT DAN – 6-(benzo-[d]thiazol-2'-yl)-2-(N,N-dimethylamino)naphthalene
CAA – cancer-associated adipocytes
CAD – Charged Aerosol Detector
CAF – cancer-associated fibroblasts
CAT – catalase
CD – circular dichroism
CDKN1A – cyclin-dependent kinase inhibitor 1A
CDNB – 1-chloro-2,4-dinitrobenzene
CE – capillary electrophoresis
CO₂ – carbon dioxide
COD – compartment-on-demand
COX-2 – cyclooxygenase 2

COXs – cyclooxygenases
ctDNA – calf thymus deoxyribonucleic acid
CYB5R – cytochrome b5 reductase
CYP – cytochrome
DAPI – 4',6-diamidino-2-phenylindole
DAPTA - (3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane)
DFA – dihydrofolic acid
DHFR – dihydrofolate reductase
DMEM – Dulbecco's modified Eagle medium
DMSO – dimethylsulfoxide
DNA – deoxyribonucleic acid
DTT – dithiothreitol
EA – Ethacrynic acid
EB – ethidium bromide
EBS – ethidium bromide staining
ECACC – European Collection of Authenticated Cell Cultures
ECM – extracellular matrix
EDC/NHS – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide linked to N-hydroxysuccinimide
EDTA – EthyleneDiamineTetraAcetic acid
EGFR – epidermal growth factor receptor
ELISA – enzyme-linked immunosorbent assay
EMT – epithelial-mesenchymal transition
ER – estrogen receptor
ESCs – embryonic stem cells
ESI-MS – electrospray ionization mass spectrometry
F1,6BP – fructose-1,6-biphosphate
FBS – fetal bovine serum
FI – fluorescence intensity
FIA – flow injection analysis
FITC – Fluorescein-5-Isothiocyanate
FWGE – fermented whole wheat germ extract
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GLUT – glucose transporters
GM1 – monosialotetrahexosylganglioside
GM2 – monosialoganglioside
GM3 – monosialodihexosylganglioside

GSH – Glutathione
 GSK3 β – glucogen synthase kinase 3 beta
 GST P1 – glutathione S-transferase P1
 H₂O₂ – hydrogen peroxide
 HER2 – human epidermal growth factor receptor 2
 HER2⁺ – human epidermal growth factor receptor 2⁺
 HIF1 – hypoxia inducible factor 1
 HL-60 – promyelocytic cell line
 HRP – horseradish peroxidase
 Hsp70 – heat shock protein 70
 HT – high-throughput
 HTDS – high-throughput drug screening
 HTS – high-throughput screening
 IC₅₀ – half maximal inhibitory concentration
 IDO1 – indoleamine 2,3-dioxygenase
 IF – immunofluorescence
 IL-10 – interleukine 10
 IL-12 – interleukine 12
 IL-6 – interleukine 6
 IL1 β – Interleukin-1 beta
 Int – intercalation
 IUPAC – International Union of Pure and Applied Chemistry
 JNK – c-Jun N-terminal kinase
 KAT – kynurenine aminotransferase
 KP1019 – indazole trans-[tetrachlorobis(1H-indazole)ruthenate(III)]
 L929 – Adherent mouse fibroblast connective tissue cells
 LD – limit of detection
 LDH – lactate dehydrogenase
 LIMS – Laboratory Information Management System
 LKB1 – liver kinase B1
 LOV – lab-on-valve
 LQ – limit of quantification
 MajG – major groove
 MAPK – Mitogen-activated protein kinase
 MCE – microchip electrophoresis
 MCF-7 – Michigan Cancer Foundation 7 cells
 MDA-MB-231 – M.D. Anderson - Metastatic Breast 231 cells

MG – methyl green
MinG – minor groove
MKK4 – Mitogen-Activated Protein Kinase Kinase 4
MMP – matrix metalloproteinases
MMSA – microfluidic mobility shift assay
MSK1 – stress-activated protein kinase 1
MT1-MMP – membrane-type 1 matrix metalloprotease
mTOR – mammalian target of rapamycin
MTT – (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MTX – methotrexate
Na₃VO₄ – Sodium orthovanadate
NADH – Reduced nicotine adenine dinucleotide
NADPH – nicotinamide adenine dinucleotide phosphate
NaF – Sodium fluoride
NAMI-A – Imidazolium-trans-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III)
NaOH – sodium hydroxide
NBDHEX – 6-(7-nitrobenzo[c][1,2,5]-oxadiazol-4-yl)thiohexan-1-ol
NES – nestin
NFκB – antiapoptotic mediator nuclear factor κB
NFK – N-formylkynurenine
NMR – Nuclear Magnetic Resonance
NP-40 – octylphenoxypolyethoxyethanol
OCT1 – organic cation transporter 1
OCT4 – octamer-binding protein 4
P-Asch – plasma ascorbate
PBS – phosphate-buffered saline
PCNA – proliferating cell nuclear antigen
PCR – polymerase chain reaction
PDK1 – pyruvate dehydrogenase kinase isoenzyme 1
PDMS – poly (dimethylsiloxane)
PGE₂ – prostaglandin E2
PGG₂ – prostaglandin G2
PI – propidium iodide
PI3K – phosphoinositide 3-kinase
PK – pyruvate kinase
PKM2 – pyruvate kinase M2
PMMA – poly (methylmetacrylate)

POC – point of care
 PR – progesterone receptor
 PS – phosphatidylserine
 PTEN – phosphatase and tensin homolog
 PTPs – protein tyrosine phosphatases
 PVDF – polyvinylidene fluoride
 RAPTA-C – organometallic ruthenium(II) [Ru(arene)Cl₂PTA] PTA - 1,3,5-triaza-7-phosphaadamantane
 RCSC – rice callus suspension culture
 RNA – ribonucleic acid
 ROS – reactive oxygen species
 RP-HPLC – Reverse Phase-high-performance Liquid Chromatography
 RSD – relative standard deviation
 S/N – signal-to-noise ratio
 SCO₂ – cytochrome C oxidase 2
 SD – standard deviation
 SIA – sequential injection analysis
 SMM – small molecule microarray
 SOD – superoxide dismutase
 TAM – tumor-associated macrophages
 TCA – tricarboxylic acid
 TEC – Tumor endothelial cells
 TGF- β – transforming growth factor beta
 THF – tetrahydrofolate
 TIGAR – TP53-induced glycolysis and apoptosis regulator
 TME – tumor microenvironment
 TNBC – Triple-negative breast cancer
 TNF- α – tumor necrosis factor-alpha
 TOMM20 – mitochondrial transport protein
 TPO – tryptophan 2,3-dioxygenase
 TRAF2 – Tumor necrosis factor (TNF) receptor-associated factor-2
 UV – ultraviolet
 VEGF – vascular endothelial growth factor
 WB – western blot

List of Symbols

μL – microliter

μM - micromolar

μm – micrometer

% - Percentage

C – concentration

cm - centimeter

g - gravitational force equivalent

h - hour

K_m - Michaelis-Menten constant

mA – milliamperes

mg mL^{-1} – milligram per milliliter

mL min^{-1} – milliliters per minute

mm – millimetre

mM – millimolar

mol L^{-1} – moles per liter

nm – nanometers

$^{\circ}\text{C}$ – degree Celsius

s – seconds

U/mL – unit per milliliter

UN – units

V/cm – volts per centimeter

V_{max} - maximum velocity of an enzymatically catalyzed reaction

Chapter I

Aim and scope of this thesis

Cancer is the leading cause of death worldwide, reporting nearly 10 million deaths in 2020. One of the ways to detect cancer earlier and to make decisions regarding the treatment to adopt is the study of cancer biomarkers since early diagnosis and the choice of adequate treatment bring substantial benefits to the patient. Moreover, chemotherapy presents lower efficacy, and limited cancer cell selectivity, causing several side effects in patients. All these limitations, as well as the probability of the appearance of multidrug resistance to therapy, create the need to search for and study new cancer biomarkers and alternatives for the treatment of cancer.

Enzymes are considered preferred biomarkers in cancer since they give information about the diagnosis, prognosis, choice of adequate therapy, and assessment of response treatment. The amount of information available on cancer biomarkers has increased outstandingly in the latest years, and this will definitely result in earlier diagnoses and better treatments and management strategies (1).

Metabolic enzymes are considered cancer biomarkers and are extremely important for biological metabolic pathways that control the metabolites' flux according to the cell and body requirements for sustainable growth and to maintain physiological homeostasis. However, multiple metabolic pathways are changed in tumor cells to promote the proliferation of enzyme-targeting treatments against cancer by the dysregulation of metabolic enzymes (2). More than previously known pathways such as glycolysis and tricarboxylic acid cycle, there are other enzymes belonging to other metabolic pathways, such as phase II detoxification enzymes (e.g., glutathione S-transferase P1) that are also overexpressed in cancer and should be studied and be a target as a cancer biomarker (3, 4).

The study of the kinetics and inhibition of enzymes provides evidence concerning the enzymes' physiological and mechanistic properties of enzyme-catalyzed biochemical processes (5). The established methods to assess the enzymatic kinetics and inhibition are prepared by mixing solutions of a constant concentration of enzyme with different substrate concentrations. However, the inconsistency of mixing time before detection and the time spent on manually placing and moving the cuvette in and out of the detector can cause some errors such as irreproducibility and unprecise results. So, to obtain more reproducible, precise, accurate, and with the additional improvement of analysis time,

automatic systems are considered better alternatives. The automation of biochemical analyses presents several advantages: enhancement in the analytical performance, high throughput, decrease in reagents and sample consumption, and a reduction of errors associated with operator handling (6). Moreover, these techniques also improve the understanding of enzyme-inhibitor interaction and allow the screening of large libraries (7).

In order to overcome the adverse effects related to chemotherapy treatment, organometallic compounds with different metals, especially ruthenium-based compounds have been reported in the literature with fewer side effects due to their different action modes (8, 9). In several cases, ruthenium complexes were found to present high cytotoxicity against platinum-resistant cancer cell lines, making them promising candidates for more investigation (10, 11). Also notably, ruthenium compounds have established some promising activities in different breast cancer types, promoting the development of new metal-based anticancer drugs. It is noteworthy that several ruthenium compounds such as RAPTA-C (12), NAMI-A, and KP1019 (13) have successfully submitted preclinical or clinical trials for the treatment of different cancers. Since ruthenium compounds are broadly studied for their capability to induce cancer cell death, the introduction of biologically active ligands into its structure may be a promising approach for the development of new ruthenium derivatives with a broader range of anticancer activities compared to ruthenium complexes or isolated ligands (14, 15).

Since it is of the highest importance to obtain as much information about the biological, biochemical, and interactive activities and mechanisms of drug candidates, enzymatic and cell-based assays are key tools in drug discovery and analysis. Once enzymatic assays already provide a lot of information on how compounds with anticancer potential can act on enzymatic biomarkers related to cancer, the combination of these with cell-based assays is also significant due to the measurement and evaluation of the cellular responsiveness of drug compounds in overall cell function, assessing the effect of anticancer compounds on cell viability, cell proliferation, or cytotoxicity that outcomes in cell death.

Taking all these theoretical features into account, this thesis has three main objectives:

1. Development and application of automated methodologies to assess the effectiveness of several organometallic compounds, especially ruthenium-based compounds in enzymes described as overexpressed in cancer, such as cyclooxygenase 2 (COX-2), dihydrofolate reductase (DHFR) and glutathione S-transferase P1 (GST-P1).

2. Synthesis and study of new ruthenium-based compounds to evaluate their cytotoxicity in different types of breast cancer cells (luminal and triple-negative), as well as their effect at DNA and protein levels.

3. Development and application of high-throughput screening (HTS) to evaluate the enzymatic activity of GST-P1 applying compounds libraries with and without anticancer activity and subsequent evaluation of the cytotoxicity of compounds with a greater enzymatic inhibitory capacity in different types of breast cancer cells (luminal and triple-negative), and subsequent analysis of the most cytotoxic compounds in western blot.

Structure

This thesis is organized into 5 chapters.

Chapter I: Aim and scope of this thesis. This chapter explains 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 two revised manuscripts entitled “Miniaturized technologies for high-throughput drug screening enzymatic assays and diagnostics – a review” and “Tumor microenvironment features and advanced in vitro cell culture platforms and enzyme targeting: an overview” are presented. The revision contains the main theoretical concepts that are important to understand the outcomes and conclusions described in this thesis. In this context, the first review described, the miniaturized techniques currently used to evaluate the activity and inhibition of enzymes giving special attention to microplate formats, microarrays, nanoarrays, and microfluidic technologies. Also, it highlighted some of their features and skills for potential use. In addition, it also pointed out the challenges of their applications in diagnosis, analysis, and therapy, which should help improve healthcare quality globally.

The second review explained the biological part of this thesis. It mentioned the significance of the cancer microenvironment and the main cancer hallmarks. Moreover, the enzyme biomarkers targeting, the description of enzymes involved in cancer progression, and the key cell energy metabolism changes, mostly in the glycolytic pathway were revealed. Moreover, the differences between 2D and 3D cell methods were pointed out, describing the properties, purposes, advantages, and disadvantages of several 2D and 3D cell methods.

Chapter III: Theoretical background of the techniques used. The techniques and the experimental methods applied to implement the research presented in this thesis are reported. A general overview of the flow techniques, operation modes, and control is clearly explained. The enzymatic kinetic parameters were also enlightened. Moreover, the studies summarized here include the *in vitro* methods to perform the cytotoxicity assays, apoptosis analysis, and cell uptake assay. Also, gel electrophoresis, circular dichroism, and western blot techniques were also described. It also mentioned some aspects related to optimization methodologies and the statistical treatment used to assess the quality of the results.

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

- 1 – Automatic evaluation of cyclooxygenase 2 inhibition induced by metal-based anticancer compounds
- 2 – Assessment of metal-based dihydrofolate reductase inhibitors on a novel mesofluidic platform
- 3 – Automated approach for the evaluation of glutathione-S-transferase P1-1 inhibition by organometallic anticancer compounds
- 4 – Tailoring the cytotoxicity of ruthenium(II) arene compounds for enhanced activity against breast cancers
- 5 – Identification of glutathione transferase (GST P1) inhibitors via a high-throughput screening assay and implications as new treatment options for breast cancers

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.

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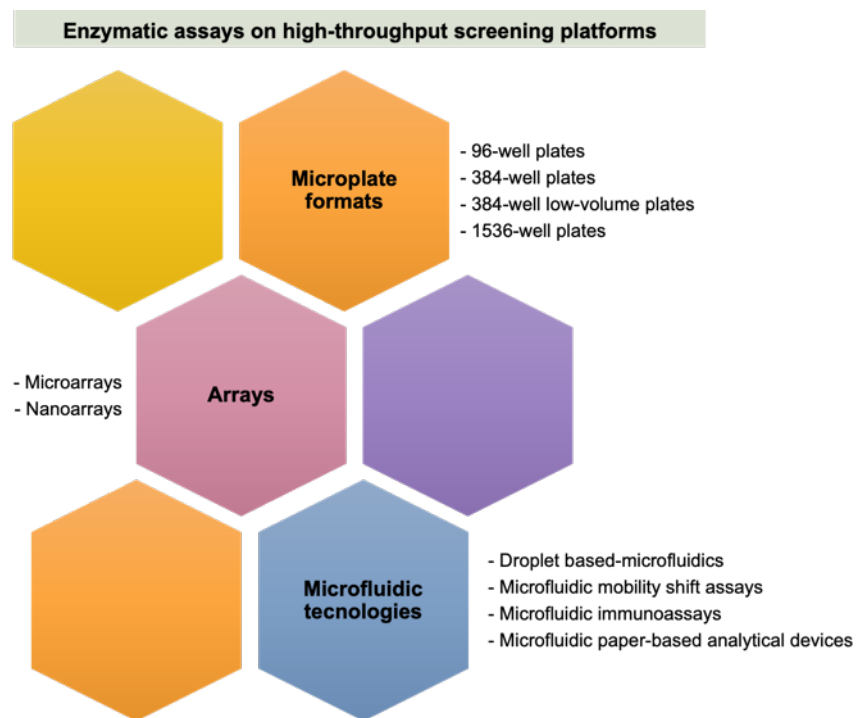
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Chapter II

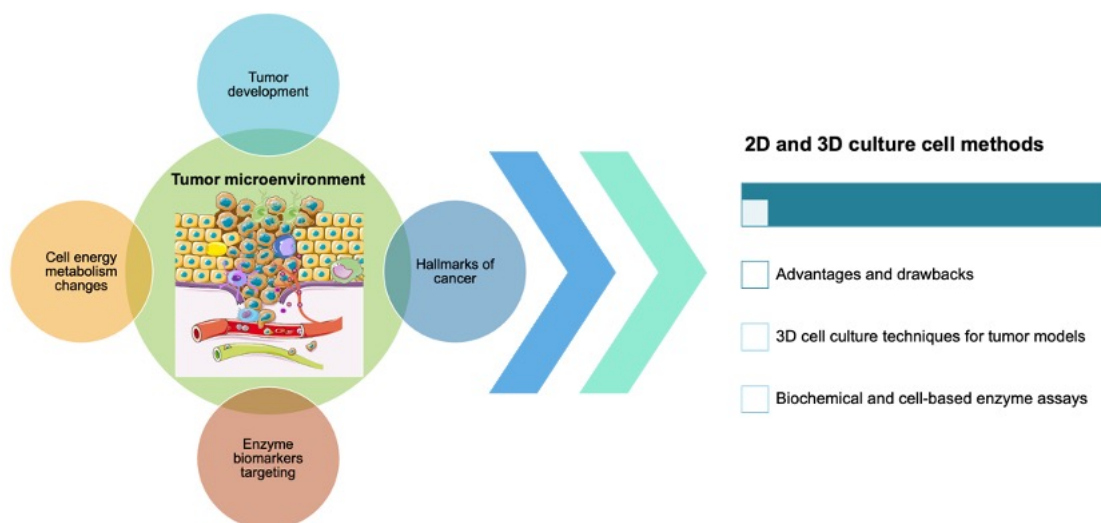
General introduction

This chapter presents two revision manuscripts entitled:

Miniaturized technologies for high-throughput drug screening enzymatic assays and diagnostics – a review



Tumor microenvironment features and advanced in vitro cell culture platforms and enzyme targeting: an overview





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Miniaturized technologies for high-throughput drug screening enzymatic assays and diagnostics – A review

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ABSTRACT

Drug discovery is a complex, multistep process, in which many challenges need to be overcome at each stage, from the discovery of a biomolecular target to the ensuring of the efficacy and safety of a compound in humans. Today's analytical methods allow tens of thousands of drug candidates to be screened for their ability to inhibit specific enzymes and the miniaturization of these approaches is highly desirable, accelerating the drug discovery process and reducing the associated costs. Herein, it is reviewed the miniaturized techniques currently used to evaluate enzymatic activity and inhibition giving special attention to microplate formats, microarrays, nanoarrays, and microfluidic technologies. It is, also, highlighted some of the characteristics and their abilities for potential uses are compared and discussed. In addition, the challenges of their applications in diagnosis, analysis, and therapy, which should help to improve the quality of healthcare globally are also pointed out.

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1. Introduction

Drug discovery is a time consuming and expensive process essentially comprising basic research (experimental and computational) in laboratories, pre-clinical studies in relevant animal models and clinical development on humans. Bringing a new drug to the market often takes more than 10 years and, towards the end of this process, it is expected that only around 10% of the drugs that enter phase I clinical trials are finally approved for commercialization by the U.S. Food and Drug Administration [1].

Increasingly, drug development is based on the discovery of new targets, usually enzymes, that are either unique or over-expressed in a disease, and these enzymes are considered druggable targets [2]. New targets are usually screened via *in silico* methods to establish what classes of compounds inhibit them, but at some point enzymatic assays are needed to validate inhibitors, to quantify their activity and confirm the inhibition mechanism [3], which usually involves determining the affinity constants of the inhibitor, for example, Michaelis-Menten constant (K_m) and the inhibitor constant (K_i) [4]. The Michaelis-Menten constants (K_m and k_{cat})

facilitate the interpretation of the IC_{50} value (the half maximal inhibitory concentration) determined for an enzyme inhibitor [5].

The environment in which the enzyme is based is highly important as it can affect its activity, e.g. by leading to denaturation. Enzyme assays are broadly classified as homogenous or heterogeneous. Homogenous assays may be considered as conventional enzyme assays, where the enzymatic reaction takes place with all the reaction components in solution [6,7]. In heterogeneous assays, the enzyme is immobilized on a solid substrate and the enzymatic reaction occurs between the solution and the surface containing the immobilized component [8–10]. Usually, in heterogeneous enzyme assays, the enzyme is immobilized either directly onto the walls of microchannels or on a solid support contained within the channel. Compared with homogenous enzyme assays, heterogeneous assays offer some advantages, including enzyme localization within devices, simplification of enzyme recycling and the possibility of continuous-flow analysis. Importantly, the enzyme must be stable and fully functional under the process conditions, therefore precise controls of the reaction conditions, i.e. temperature, solvents and pH, is essential.

In addition, physical stabilization and confinement methods have been applied to optimize enzyme immobilization and structure and to prevent denaturing [11,12]. Thus, the protein immobilization methodology is crucial [13,14]. The enzyme, matrix and

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mode of attachment are the main components of an immobilized enzyme platform [15], as summarized in Fig. 1.

Innovative testing approaches to investigate drug candidates in a fast and efficient manner are needed to accelerate drug discovery [16]. Analytical devices for this process should ideally be simple (ease of implementation and use), automated (electromechanical self-operation, involving system control) and miniaturized (small scale).

Miniaturization technologies are of increasing interest in drug screening research with microfluidic-based devices leading to decreases in analysis time, the amount of enzyme needed and the consumption of reagents. Indeed, the reduction in quantity of biological and chemical reagents and samples used per assay enable higher throughput assays due to massive parallelization and multiplex detection modes. Consequently, miniaturization promotes the development of new procedures with easier handling methods [17,18]. Considerable challenges remain, notably in the development of homogeneous assays and highly sensitive detection methods, with increased resolution and accuracy [19]. According to Ríos and Zougagh, it is possible to divide miniaturization processes into three different scales: mini-scale (several mm or μL),

micro-scale (a few mm to 50 μm and processing samples between a few μL and 10 nL) and nano-scale (below 50 μm , even 1 μm in some cases and with sample size below 10 nL or even at the pL or fL level) [20]. It is also important to consider the scale of the entire analytical process, including the detection method, etc.

Miniaturization approaches are usually based on batch and continuous systems. The former includes 96-, 384-, 1536-well microplate, microarray and nanoarray techniques, whereas flow-based miniaturized analysis, also called lab-on-a-chip or microfluidic devices and Lab-on-valve (LOV), correspond to the latter. Flow-based analysis using microfluidic devices has attracted considerable attention for chemical, biological and medical applications and should ideally integrate all components essential for microanalysis [21]. LOV systems allow continuous monitoring with automation based on precisely controlled pressure-driven programmable flow [22].

Miniaturization and automation of bioanalytical assays are now widely applied in medical diagnostics, enzyme engineering, drug discovery, functional proteomics and genomics, enabling efficient high-throughput screening (HTS) in these fields [23].

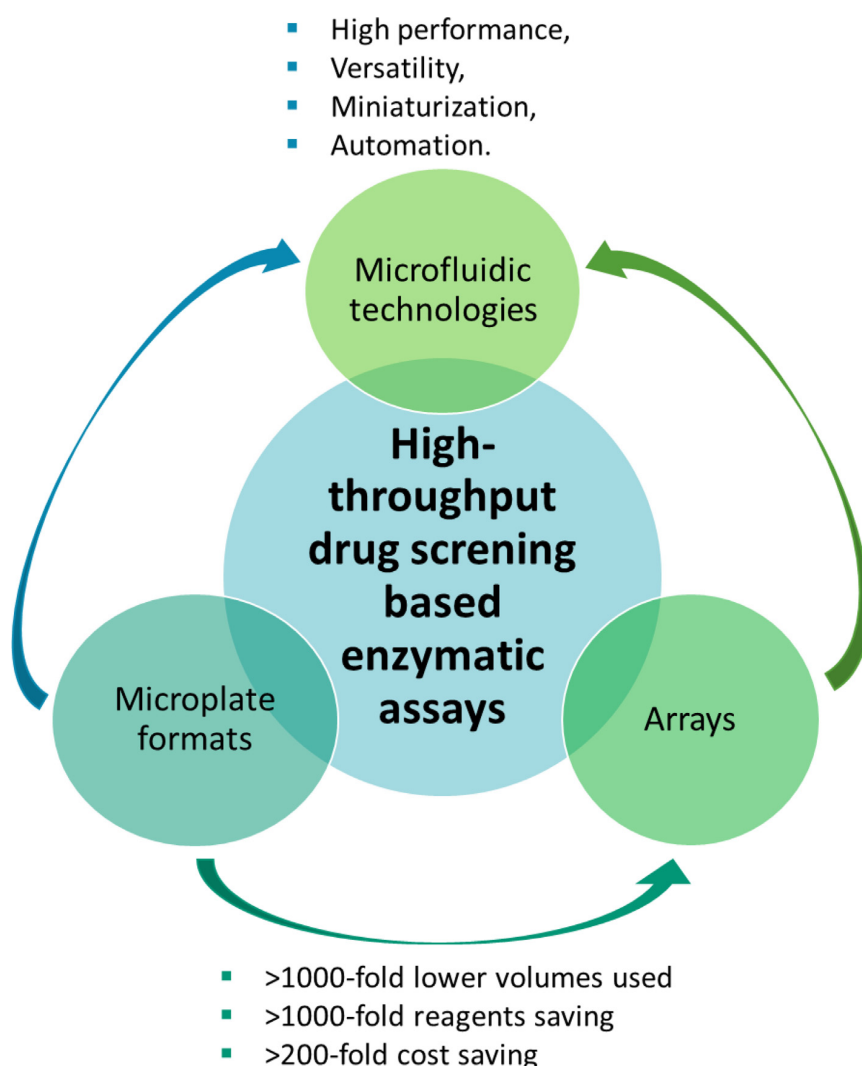


Fig. 1. – Different high throughput drug screening based enzymatic assays.

2. Enzymatic assays on high-throughput screening platforms

The development of HTS enzyme assays requires the identification of relevant enzyme and substrate forms, purification methods, kinetic parameter measurements, characterization of co-factors and buffers and the selection of a detection method [24]. HTS assays can be divided into functional and non-functional assays. Functional assays measure the activity of compounds by the modification of the function of the target protein and they tend to be more reliable than non-functional assays. Non-functional assays, on the other hand, are mainly used if a tested compound binds to a target protein [25].

The components of a HTS systems must be stable over the time course of a reaction and must not deteriorate or be impacted by dispensers or devices that are used for automation. It is also important to understand the biochemistry involved in an assay as this impacts on the quality of the assay and the interpretation of the results [24]. HTS has been enhanced by advances in technology, such as the automation of liquid handling and dispensing and the development of better tools to process huge quantities of data, and has led to a transition in all stages of drug discovery from target identification to toxicity assessment [26]. HTS programmes in the pharmaceutical industry typically operate at throughput rates in the order of 2000 to 10,000 compounds per assay per day, although ultra-high throughput rates of ca. 100,000 compounds per assays per day are also possible [27]. Hence, cost is a major factor which impacts on time and quality.

The main approaches used in HTS in drug discovery employ microtiter plates, typically used to screen drug targets against large chemical libraries [28]. In Detection methods used in enzymatic assays based on HTS platforms are listed in Table 1. In addition, Fig. 1 summarizes the main differences of high throughput drug screening based enzymatic assays.

3. Microplate formats

Microplates are flat plates, panels or dishes with multiple wells (also known as multiwell plates or microtiter plates in which, over the years, the number of wells has increased (commercially available microplates range from 96 to 1536 wells) demanded by the increase in the size of compound libraries. Microplates are available in several formats comprising different well shapes and polymer compositions [29]. The characteristics, advantages and disadvantages of the various microplate formats are listed in Table 2.

Yao and co-workers described the development and validation of six assays for the inhibition of five different cytochrome (CYP) isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A) in human liver microsomes in a fully automated 96-well microplate format and under strict kinetic conditions [30]. The metabolites of the substrates probed were also analysed by LC-MS/MS. The IC₅₀ values obtained were compatible with those reported in literature and allows the method to be used to study drug-drug interaction and drug regulatory registrations. Moreover, the use of microplates overcomes problems associated with non-specific binding and the method also described automated control of a Tecan liquid handling system.

A novel fluorescence-based 384-well microplate assay for human 5-lipoxygenase (5-LOX) using 2',7'-dichlorodihydrofluorescein diacetate was developed by Pufahl and co-workers [31]. This method enabled the characterization of several known 5-LOX enzyme inhibitors. The use of 384-well microplate format allows multiple plates to be screened in a fast and easy manner. The method was used to screen a subset library of the Pfizer compound collection, facilitating the development of a new-generation of inhibitors.

A simple HTS-compatible 96-well microplate fluorescence-based kynurenine aminotransferase (KAT) *in vitro* enzymatic assay was reported by Wong et al. [32]. This assay allowed the discovery of potential drugs for neurological diseases and tools to help unravel the role of the kynurenine pathway in brain function. The method is simple, with a quick readout and is less expensive than HPLC methods. Moreover, the assay was easily adapted to screen libraries using a 3456-well format or to study other KAT enzymes.

Kedar and co-workers developed a microplate-based assay to detect NADH-Cytochrome b5 reductase activity by fluorescence and absorbance measurements [33]. The results obtained using the microplate assay are similar to those obtained using the standard spectrophotometric method. Both sets of results agreed with the generally accepted NADH-CYB5R levels at different human stages (new-borns, adults and recessive congenital methaemoglobinemia patients).

The above mentioned assays show that it is possible to perform various types of assays in different microplate formats and have the advantage of being faster and cheaper than other methodologies (Table 2). However, it must be stressed that 96-well microplate format is easily used in routine laboratory assays since it is possible to use multichannel pipettes to perform the assays. 384- and 1536-well microplate formats are more appropriate employed in industry with automatic samplers.

4. Arrays

The emergence of micro- and nanoarrays as analytical tools and platforms to test chemical libraries has been increased by their ability to analyse reactions run at the nanolitre scale and reach densities of thousands of spot/cm², overcoming the scale and density limitations of well plates.

Comparing micro-with nanoarrays, nanoarrays can hold 10⁴ - 10⁵ more features than conventional microarrays. As a result, it is possible to perform multiplexed and rapid screening of several targets in a single experiment. Moreover, the sample and reagent volumes required are very low and only some target molecules for a specific analyte concentration can be detected. Consequently, this has led to significantly lower detection limits than that obtained using microarrays. Hence, nanoarrays require only 1/10000 of the surface area used by classic microarray devices and about 1500 nanoarray devices could be placed in the space required by a single microarray device. Lastly, nanoarrays can be used to answer important questions about biomolecular recognition, since bio-recognition is inherently a nanoscopic rather than a microscopic or macroscopic phenomenon [34].

4.1. Microarrays

Microarrays consists of biomolecules immobilized in high density and in an ordered way onto a chemically modified surface [35], while maintaining the functionality of biomolecule, usually a protein. It is important to consider the surface chemistry so the immobilized protein retains its secondary and tertiary structures and, thus, biological function [36]. Although several approaches exist, immobilization is still challenging since many protein lose their activity and function following surface attachment [37].

Most microarrays comprise either glass or silicon slides treated with an aldehyde used to immobilize the protein. Alternative materials include aluminium or gold surfaces and hydrophilic polymers [38].

Protein microarray technologies are extensively used for the identification, quantification and functional analysis of proteins [39], and offer some advantages compared with other

Table 1

Detection methods used to determine enzymatic activity on HTS systems.

Enzymes type	Detection methods	Main features	References
Protein kinases	1) Bioluminescent detection using firefly luciferase to detect the remaining ATP. 2) Non-antibody fluorescent based system to detect ADP product. 3) ADP specific antibody by fluorescence. 4) Radiolabeled ATP to measure phosphorylation of polypeptides.	1) The use of bioluminescent signal for ATP levels results in an increase in luminescence when kinase is inhibited. 2) This enzyme activity determination includes both non-antibody based system that uses coupling enzymes with a fluorescence readout. 3) In this case, they developed antibodies with >100-fold selectivity for ADP versus ATP, which enable robust detection of initial velocity rates (Z' > 0.7 at 10% substrate consumption) at ATP concentrations ranging from 0.1 μ M to 1000 μ M in a competitive fluorescence polarisation (FP) immunoassay. 4) This approach used a filter-binding assay to separate radio-labelled protein/peptide products from free radiolabel.	[140–144]
Phosphatases	1) Spectrophotometric detection to determine fluorogenic tyrosine and serine phosphatase substrates (difluoromethyl umbelliferyl phosphate).	1) Fluorogenic substrates based on fluorinated umbelliferones remains the substrate of choice for continuous assays of phosphatases.	[145]
Oxidoreductases and redox enzymes (ex. CYP450)	1) Fluorescent detection. 2) Bioluminescent detection of pro-luminescent substrates for CYP.	1) Fluorescence-based detection of pro-fluorescent substrates for specific CYP isoforms. 2) In this assay, the CYPs convert a pro-luciferin substrate to D-luciferin allowing bioluminescent detection through firefly luciferase.	[146,147]
Proteases	1) Fluorescence resonance energy transfer (FRET) detection or a pro-fluorescent substrate.	1) 5-(2-aminoethyl)aminonaphthalene-1-sulphonyl (EDANS) and 4-(4-dimethylaminophenylazo)benzoyl (DABCYL) has been used to endoproteases using FRET detection, however, compound interference and solubility are unfavourable.	[24]
Histone deacetylases (HDACs)	1) Radiometric measurements. 2) HDAC-labelled K-trap complex (coumarin label attached to a known HDAC inhibitor – k-trap). 3) Commercial fluorogenic assay.	1) These measurements stimulate the release of acetic acid from hyperacetylated tritiated histone substrate. 2) HDAC-labelled k-trap complex essentially convert the enzymatic deacetylation reaction into a binding/displacement assay type.	[24,148]
Histone methylases/demethylases	1) Spectrometric methods. 2) Time Resolved-Fluorescence resonance energy transfer (TR-FRET). 3) Fluorescent detection.	3) This method is based in Fluor-de-Lys from Biomol. 1) LC/MS and HT mass spectrometry methods to detect peptide product have been developed. 2) A TR-FRET assay using an antibody that recognizes a specific peptide (H3K4me1) but not the unmethylated product have been recently described. 3) Miniaturized fluorescent assays to detect NADH produced by formaldehyde dehydrogenase (FDH) have been developed and applied to HTS.	[149–151]
Ubiquitin proteases and ligases	1) Fluorescent detection. 2) Commercial Amplified Luminescent Proximity Homogenous Assay in 1536-well microtiter plates.	1) The cleavage of the ubiquitin chain by a deubiquitylase (DUB) can be quickly measured with a fluorescent substrate. 2) Ubiquitin ligase activity has been measured using AlphaScreen in 1536-well microtiter plates.	[152–154]

Table 2

Characteristics, advantages and disadvantages of different microplate formats.

Microplate formats	Compounds per day	Total volume per well	Standard volume per well	Advantages	Disadvantages	References
96-well plate	10.000	100–200 μ L		Main compound handling and screening format used at most pharmaceutical and biotech companies.	Transformation into higher density formats can be difficult to implement in this mode. Higher working volume which equals higher cost.	[155,156]
384-well plate	40.000	25–100 μ L	50 μ L	Biochemical and cell-based assays can be adapted without any problems.	Handling several plates in a manual mode, even using 16-channel pipettes, can be highly inaccurate.	
384-well low-volume plate	40.000	10–20 μ L		Applicability of 96/384 liquid handling and dispensing technology. Volume reduction without the concurrent infrastructure investment to adapt all processes towards 1536-well plates.		
1536-well plate	200.000	2.5–10 μ L	5 μ L	Higher density format and use few volumes of reagents	Difficulty to implement some assays in this format, e.g. assays sensitive to dimethylsulfoxide (DMSO). Requirement of special liquid delivery equipment because handling plates in a manual mode is not recommended.	

methodologies such as enzyme-linked immunosorbent assays (ELISA) and western blots, since protein microarrays use less sample and reagents and are typically 10–100-times faster than other methods [36,40].

Chemical compound microarrays developed for proteomic and genomic research provide new possibilities for miniaturization and automation assays [28]. Horiuchi and Ma identified four types of microarray devices, i.e. small molecule microarrays (SMMs), micro arrayed compound screening (μ ARCS), DiscoveryDot solution-phase chemical microarrays and chemical compound microarrays for cell-based screening. The differences between these microarray approaches and the advantages/disadvantages are presented in Table 3.

Gao and Du designed a microarray platform that includes a proteinase film. Basically, they developed, for the first time, an enzyme chip to demonstrate enzymatic activity by colour difference.

In this chromogenic assay, the influence of compounds was evaluated on elastase activity. When chemical compounds inhibit the enzyme, the substrates cannot be transformed into yellow products and blue spots are observed. When enzyme activity is maintained, the yellow products combined with blue of bromochlorophenol blue affording green spots. Subsequently, 11,680 compounds were screened and from this library and two active compounds were identified [41].

Microfluidic devices can be applied to microarrays, creating a specific and adaptable micro total analysis system (μ TAS). These systems cannot be used as alternatives to microarrays, but are fused with them to produce fully integrated modular microfluidic microarrays [42].

“Active arrays” are an alternative to microarrays that have recently been attracting attention. These devices allow the immobilization of oligonucleotides to the microarray and may be used as a set of primers for polymerase action. The immobilized primers are used to serve as templates for some enzymes such as polymerases

and transcriptases preserving the local information of the primary assay. Moreover, “active arrays” offer the possibility of performing different enzymatic assays using immobilized biomolecules on a solid support, giving more sensitivity, scalability and specificity to the analysis [43,44].

The integration of active arrays in a lab-on-chip system is, in our opinion, an excellent alternative for solving point of care problems since all the advantages presented before.

Kim et al. developed a microfluidic-based enzyme assay that combines heterogeneous reactions and microarray-based detection systems and was applied to study glucose oxidase activity. In this assay, the enzyme-substrate reaction was detected using a hydrogel microarray and the glucose concentration was continuously monitored by fluorescence. Glucose oxidase was immobilized on glass microbeads and peroxidase was entrapped in the hydrogel microarray within the microfluidic device. The enzymatic reaction was easily detected through the modification of the emission intensity ratio of resorufin inside the hydrogel microarray before and after reaction in the important physiological glucose concentration range. Moreover, this device can be used in enzyme kinetic studies and requires only low sample and reagents volumes and short analysis times [45].

Babiak and Reymond developed a high-throughput enzyme assay using silica gel plates impregnated with fluorogenic substrates. They studied different types of enzymes including lipases, glycosidases, proteases and esterases. They concluded that the assay is compatible with water-insoluble (lipases and esterases), water-soluble (glycosidases) and whole-protein substrates (proteases). Moreover, the silica gel plate assay is an alternative to well-plate high throughput screening and allows miniaturization by approximately 100-fold at basically no additional cost [46].

Peptide microarrays comprise hundreds to thousands of different peptides and they are important to determine the specificity of enzyme substrates/inhibitors, to study ligand-receptor interactions and profile antibodies [47]. Microarrays have been used

Table 3

Characteristics, requirements, advantages and disadvantages of different microarray devices.

Microarray approach	Characteristics	Requirements	Advantages	Disadvantages	References
Small molecule microarrays (SMMs)	SMM is a chemical compound microarray arrangement that covalently immobilises organic molecules on the surface of the slide.	Presence of specific reactive groups on both the microarray surface and the compound library.	Useful for probing binding partners and identifying targets.	The immobilization step has limited use with existing chemical libraries. Length and flexibility of linkers, compound binding direction, special barrier and microarray surface properties affect target binding.	[28,157,158]
Micro arrayed compound screening (μ ARCS)	μ ARCS is a chemical compound microarray that are formed by drying organic molecules dissolved in DMSO on polystyrene sheets.	The biological targets are immobilized in agarose gels and they are placed on top of the compound sheet, enabling the dissolution and diffusion of compounds into the gel and the interaction with the target.	Any chemical compound library can be deposit in a dry form on to a polystyrene sheet. 10-fold more economical than the 384-well plate assays.	The rate of re-solubilisation and diffusion of different classes of dry compounds is difficult in the dynamic range of arrayed compounds against bio-available compounds.	
DiscoveryDot: solution-phase chemical microarrays	It was created to avoid the drawbacks that SMM and μ ARCS platforms present.	This microarray uses glycerol as an anti-evaporating reagent mixed with chemicals which are arrayed on the microarray surface. microarray simultaneously.	Any chemical library can be screened making this technology the only universal chemical microarrays to date.	The microarray detectors available cannot detect fluorescent polarisation and time-resolved fluorescence energy transfer.	
Chemical compound microarray for cell-based screening	This microarray device enters a new area: cell-based high content screening.	A biodegradable poly-(D), (L)-lactide glycolide copolymer is used to arrest the organic molecules.	Can be used to perform high throughput cell-based assays.	It is challenging to find a polymer for embedding and releasing when there are millions of chemical compounds with different physical properties.	

to identify kinase substrates [48], and as an example, the principles for the detection of peptide phosphorylation on peptide arrays are shown in Fig. 2.

Phosphopeptide microarrays offer several advantages in terms of miniaturization, throughput and sensitivity for the detection of phosphatase substrates specificity [49]. Panse and co-workers

developed a phosphopeptide microarray to profile general *anti*-phosphopeptide antibodies. The peptide microarray allowed the reliable identification of peptide substrates for kinases and the detection of possible *in vivo* substrates. More than 50 different kinases were profiled using the assay, demonstrating that the phosphopeptide microarray provides a profile of selectivity,

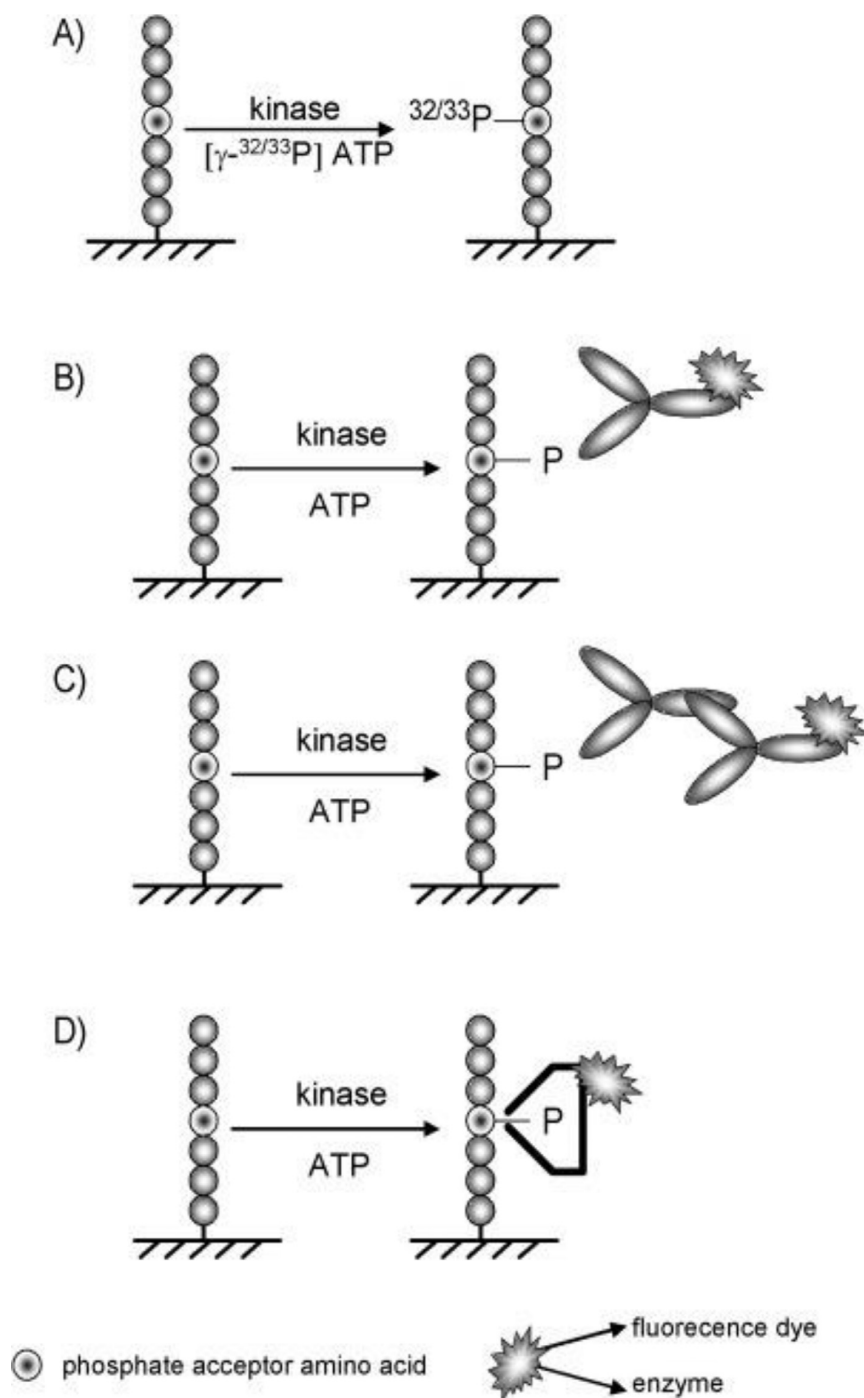


Fig. 2. — Assay principles for the detection of peptide phosphorylation on peptide arrays. A) The array is incubated with a kinase of interest in the presence of $[\gamma\text{-}^{32/33}\text{P}] \text{ATP}$, and autoradiography is used for detection. Alternatively, phosphorylation is measured with B) a labelled antiphosphoamino acid antibody or with C) an antiphosphoamino acid antibody in combination with a labelled secondary antibody. D) Phosphoamino acid detection with a labelled phosphoamino acid chelator. Copyright 2005, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

subsite specificity and cross-reactivity of *anti*-phosphopeptide antibodies [50].

Another phosphopeptide microarray developed by Gao et al. employs protein tyrosine phosphatases (PTPs) protein substrates which were synthesized and specifically immobilized in a phosphopeptide microarray. More than 1800 different PTP/phosphopeptide enzymatic assays were simultaneously performed on the same glass slide, with high reproducibility and allowing both comparative substrate fingerprint profile between different PTPs and semi-quantitative determination of a huge number of PTP/substrate interactions. Furthermore, the method is rapid, sensitive and accurate and requires only small amounts of the enzyme and peptide substrates [51].

High-density protein microarrays for functional proteomics can be prepared by the immobilization of proteins onto slides. Such microarray-based proteomics are used for the high-throughput analysis of protein-protein, protein-small molecule and protein-nucleic acid interactions. Loch and co-workers developed a multiplexed approach in which 35 deubiquitylates (DUBs) were immobilized on a microarray platform and enzyme activity was assayed using soluble polypeptide-based substrates following immobilization [52]. The authors demonstrated the functionality of this microarray by using it to profile several small DUB inhibitors.

4.2. Nanoarrays

Nanoarrays are essentially miniaturized microarrays and corresponded to an array where the molecules are scattered in the micron or sub-micron spatial range [53].

Nanoarrays are inherently static and controlled systems. These characteristics could be advantageous in studies of some biological systems, for example cellular membranes, which are difficult to study *in vitro* due to their dynamic structure. However, the oversimplification of these arrays can give a false description of protein interactions at such dynamic interfaces. To overcome this limitation, there is an interest in developing dynamically responsive nanoarrays [54].

Nanoarrays have been used to perform biomolecular analysis and considering their characteristics, exhibit high sensitivity and selectivity and have been applied to pathogen detection at trace quantities [55].

A technique to dispense pico- and nanoliter reagent volumes based on an electrospray dispensing system was reported by Moerman and co-workers. This technique allows the deposition of several reagents in well-defined dry spots onto a solid support, into different wells, etc. [56].

Young et al. developed a laboratory-on-a-chip nanoarray incised into silicon to measure enzymatic reactions based on fluorescence (associated with NADH and rhodamine fluorescence) as well as on bioluminescence (associated with ATP). Rhodamine and NADH fluorescence could be measured up to concentrations of 1 nM and 5 μ M, respectively. *In situ* (embedded) instrumentation that allowed the measurement of NADH fluorescence and ATP-associated bioluminescence concentrations to 100 μ M and 2.4 μ M, respectively, has also been developed [57].

Dietrich and co-workers described a method for dynamic measurements of enzymatic reactions in well arrays with an individual volume capacity of about 1 nL. The nanoarray allowed the conversion of nondetectable reagents to be detected. Three different enzymes that are involved in anaerobic metabolism, i.e. enolase, pyruvate kinase (PK) and alcohol dehydrogenase (ADH) were tested. It was necessary to couple the enolase and PK assays to lactate dehydrogenase (LDH), since enzymatic activity was determined by the monitoring of the production or consumption of NAD(P)H. Two different nanoarray-based methods were used to

add the reagents, i.e. premixed and presprayed modes. In premixed mode, the authors added the commercially available enzymes or enzymes from cell-free extracts to all the reaction compounds for the assays and the reaction product is measured. On the other hand, in presprayed mode, the substrates and cofactors are sprayed into the nanoarray wells.

In the enolase assay, the premixed nanoarray led to a higher conversion rate compared to the conversion rates obtained by presprayed nanoarrays. In the case of enzyme determination in cell-free extracts, both nanoarray methods work well and give reproducible results. Moreover, the nanoarray-type enzymatic assays are cost-effective, rapid and require only low sample volumes, and are ideal alternatives to microplate and spectrophotometric assays [17].

Parajuli and co-workers developed a quantitative activity assay for horseradish peroxidase (HRP) immobilized on self-assembled polystyrene-*block*-polymethylmethacrylate (PS-*b*-PMMA). Enzymatic activity of the surface-bound HRP was compared to its activity in free solution, demonstrating that the immobilized HRP maintains a large degree of their activity and conformational integrity. Advantageously, the nanoarray does not require time-consuming fabrication or modification processes, often used to produce protein arrays, due to the diblock copolymer that promotes the construction of the enzyme nanoarray [58].

5. Microfluidic technologies

Microfluidic or lab-on-a-chip systems allow the control of fluids in a confined geometry (micro channels) with a typical length scale of one hundred nanometers to several hundred of micrometers [59]. Such systems enable high performance, versatility, speed and integration, with negligible sample and reagent consumption, miniaturization and automation [60,61]. The development of microfluidic systems requires careful optimization of the surface material.

Microfluidic techniques allow the production of micro-electromechanical-systems (MEMS) used in the electronics industry and microscale total analysis systems (μ -TASs) for biomedical applications [62]. μ -TASs exploit a diffusion phenomenon only available at a microscale, leading to low cost and portability, very low volumes of reagents and samples (ranging from microliters to picoliters), and the analysis time spent in each assay is low [63,64].

Microfluidic technologies provide new tools for multi-parallel and multiplexed assays with the distinctive ability to integrate biosensor technology with microscopy-based read-outs. Furthermore, combining them with automated imaging systems provides high-data volumes [19]. Several biochemical and biological procedures have been successfully performed in microfluidic devices, including drug screening assays [65], chemical synthesis [66], protein crystallization [67], cell counting [68], DNA sequencing [69] and enzymatic assays [70]. Microfluidic chips, i.e. miniaturized devices, with multiplexed cell culture chambers have been developed for pre-clinical drug screening, since they can be used to perform cell viability assays (e.g. to determine IC_{50} values) [71] and enzyme inhibition assays [72,73].

Initial microfluidic derivatization/separation assays were focused on non-enzymatic reactions. With the use of enzymes, selectivity is increased, and some analytically important substrates can be determined. The use of lower volumes of reagents associated with their versatility and speed, makes these microfluidic devices ideally suited to enzymatic assays [60]. It is also possible to perform several assays integrated within a single device without extending the size and complexity of the device. Additionally, several steps of an analytical procedure can be integrated and automated in the same system [4].

For enzymatic assays, microfluidic devices have already shown advantageous characteristics in the determination of enzymatic kinetic parameters [7,74], quantification of targeted substrates [75], bioactive screening and the identification of enzyme inhibitors [76,77]. The design of lab-on-a-chip devices, which includes enzymatic assays, is difficult since the voltages, microfluidic channels dimensions, enzyme concentrations and other operating parameters involved strongly affect the efficiency of the assays [78]. Electrokinetic flow is one of the several techniques used to control the flow of liquids inside a microfluidic device and exhibits certain beneficial properties and some potential challenges that are described in Table 4.

Centrifugal force is an alternative method to control the flow of liquids in microfluidic systems. Centrifugal force is generated using a centrifugal pump, e.g. a simple motor. For this reason, it is relatively safe to operate and has lower power and space requirements than electrokinetic-based systems. In contrast to electrokinetic flow, centrifugal force is not sensitive to the properties of liquids, and it functions in the presence of bubbles. Moreover, centrifugal force can be applied to a wide range of channel sizes and can therefore be used to pump liquids over a wide range of flow rates. However, in these systems it is necessary to include fundamental fluid control capabilities, i.e. spacial and temporal control over the fluids position in microfluidic channels, which tend to be controlled more precisely in electrokinetic flow systems [61].

The first microfluidic systems were based on silicon and glass [79] and were limited to bi-dimensional devices. With the addition of more functionality, e.g. pre-treatment steps, reaction steps or detection steps, other construction materials were required [80], such as poly (dimethylsiloxane) (PDMS) [81] and poly (methyl-metacrylate) (PMMA). These materials allow more complex structures to be fabricated in a facile manner and also allow the integration of different types of materials enabling different functions [80].

Baeza et al. developed low temperature fired ceramics with 3D structures that allow mechanical, electronic and fluidic modules to be integrated within a single device [82]. A microfluidic system based on this material was used to determine the conversion of o-nitrophenyl β -D-galactopyranoside in order to determine β -galactosidase activity.

A continuous flow microfluidic method was devised that allows an enzyme inhibition assay using also β -galactosidase (β -gal) and resorufin- β -galactopyranoside (a fluorogenic substrate) and lactose and galactose (competitive inhibitors) to be performed [83]. Comparing the results obtained with this microfluidic method and the conventional method (microtiter plate), the microfluidic enzyme inhibition assay takes only 2 min, whereas the plate assay requires about 15 min. Additionally, this microfluidic method was simpler to use and allowed the simultaneous measurement of enzyme activity at a range of inhibitor concentrations contrary to the plate method. This method provided initial velocity

measurements from 40 to 50-fold higher inhibitor concentrations and 50 times more determinations were performed at each inhibitor concentration compared with traditional methods.

Atalay and co-workers implemented a standard cross-channel microfluidic chip to perform a complex double-enzyme glucose assay. In this study, they used numerical simulations to study the operating parameters, i.e. flow rates, amount of enzyme and the length of the microreactor used. The transport of the reagent solutions of this enzymatic assay was performed via electrokinetic flow. It was found that the amount of enzyme used is reduced by 15-fold compared to that needed to perform the assay in a 96-well microplate format and analysis is also 30 times faster [84]. A related approach was used to quantify glucose, fructose and sucrose in food samples through a multiplexed multi-enzyme assay. In this study, optimization of the flow rate, injection sequences, concentration of enzymes and geometric parameters was undertaken to provide the maximum product signal in the shortest time [78].

Hadd et al. developed a microfluidic device for flow injection analysis and electrophoretic separation of acetylcholinesterase (AChE) inhibitors [77]. The enzyme was present in the buffer solution leading to a fluorescence background signal (Fig. 3). Integration of a separation channel for enzyme inhibitors allowed the simultaneous detection of several inhibitors since each inhibitor fraction began with a negative peak. Since the extent of inhibition corresponds to the area of the peak the inhibitor concentration and inhibition constant could be estimated.

Lab-on-a-chip devices may include a centrifugal platform called lab-on-a-disc or lab-on-a-CD. These devices do not require pumps to control the fluids, making the assays faster, with lower volumes of reagents in a compact device. Lab-on-a-CD is a promising methodology for use in point-of-care diagnosis [85].

Kawai et al. included a simple valving system in a Lab-on-a-CD with a rotatable reagent cartridge (RRC). The RRC can significantly decrease the number of valves, channels and reservoirs, since the device integrates several microvalves and reservoirs into the same cartridge, which operate with a simple mechanical force. The approach was applied to an enzymatic assay for L-lactate dehydrogenase, and achieved a quantification performance with a $R^2 > 0.999$ [86].

5.1. Droplet based-microfluidics

Interest in droplet based-microfluidics have grown in recent years due to certain advantageous features, i.e. eliminating dispersion [87–89], accelerated mixing [87], control of the chemical and physical environment [88,89] providing significant potential for high-throughput screening [89]. These systems also allow reaction volumes ranging from microliters to nano-, pico- and even femto-liters [90–92]. High quantities of droplets can be produced and analysed by a single device, improving the statistical analysis and reproducibility of an assay [93]. However, with these

Table 4
Characteristics of electrokinetic flow control.

Electrokinetic flow technique		
Features	Limitations	References
Fluid flow in the channels arises from electroosmotic flow in vessels with charged walls.	Electrokinetic flow is sensitive to physicochemical properties (pH, ionic strength, etc.) of the liquid. Challenging to pump viscous biological fluids. High voltages are required which has safety implications.	[61,159]
Easy control of the flow direction.		
Electrokinetic flow in electrophoretic mode allows separation of molecules by size and charge.		
To produce electroosmotic flow, several materials can be used such as glass, quartz and polymers.	Trapped bubbles (for example air) hinder flow.	
	High flow rates ($>1 \mu\text{L/s}$) are difficult to achieve.	

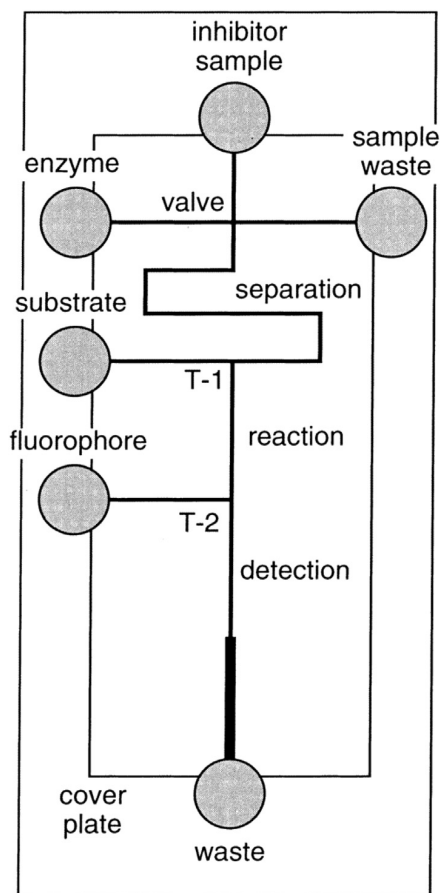


Fig. 3. – Schematic of the microchip used for analysis of AChE inhibitors. The fluid reservoirs were charged with the enzyme (AChE), acetylthiocholine chloride substrate, and coumarinyl phenyl maleimide fluorophore. The inhibitor sample was changed as indicated [77]. Copyright 1999 American Chemical Society.

microfluidic systems the manipulation of droplets after their formation, and measurements of reaction kinetics with individual droplets in real time, are difficult [93].

Droplets are prepared by immersing an aqueous sample in an immiscible carrier phase (typically an oil or gas) using hydrodynamics. It is important to control of size, shape, frequency and monodispersity of the droplets [19], which is achieved using passive (hydrodynamic) and active (electrokinetic) methods as outlined in Table 5.

Hydrodynamic methods usually use channel geometry and fluid flow characteristics to control the production of droplets [94–96]. Electrodynamics technologies have also been used for droplet formation where an electrical signal generated by integrated electrodes is used to control droplet properties [19]. Droplet-based microfluidic systems can be divided in three categories depending on the droplet actuation: mobile droplet platforms, surface droplet platforms and static droplet platforms. In mobile droplet

microfluidics, the assay is monitored continuously, which adds to the complexity of the method. However, in static droplet microfluidics, as there is no movement with the flow, monitoring can be simultaneous and at a predefined position [59]. In contrast to the other systems, in surface droplet platforms the droplets are manipulated individually in an open planar substrate [93].

Enzyme kinetics and enzyme inhibition studies have been demonstrated in several droplet-based microfluidic platforms [87,97–99]. Du et al. developed a droplet-based microfluidic system named “DropLab” that is highly versatile and can be used in multiple applications including in enzyme inhibition assays. The method has been used to evaluate the inhibition of β -galactosidase by diethylenetriaminepentaacetic acid [100].

Compartment-on-demand (COD) platforms are an alternative to flow droplet formation and decrease reagent consumption by sampling material into droplets at expressively lower throughput. Gielen et al. employed a microfluidic COD platform to evaluate the kinetics of β -glucosidase through the hydrolysis of the chromogenic substrate 4-nitrophenyl glucopyranoside. In addition, inhibition studies were performed with a known inhibitor of β -glucosidase [1-deoxynojirimycin hydrochloride] [101].

Theberge et al. demonstrated a droplet-based microfluidic assay to synthesize drug candidates as potential enzyme inhibitors. The platform has several features since it is possible to make libraries in a scalable 7 x 3 combinatorial library. Moreover, each droplet can be divided into aliquots and each aliquot can be used to screen the library against enzyme targets. The droplet-based microfluidic platform was successfully applied to study known thrombin inhibitors, however, this system can be easily integrated to many analytical and processing techniques [102].

5.2. Microfluidic mobility shift assays

Microfluidic mobility shift assays (MMSAs) combine microfluidic technology with capillary electrophoresis methods. Capillary electrophoresis (CE) (Fig. 4) has been widely used in enzymatic assays and involves the measure of the migration of compounds through a biochemical modification or a binding event, under electrophoretic conditions [103]. CE and its microfluidic counterpart microchip electrophoresis (MCE) are promising methods and their use is growing in the pharmaceutical industry for characterization of pharmaceutical compounds, drug discovery and in quality control [104].

MMSAs are performed using mobility off-chip or mobility on-chip methods (see Fig. 5). In an off-chip mobility shift assay, the enzymatic reactions take place in a 96- or 384-well plate. In this case, the reaction occurs off the chip and after reaction, as the solution is sorbed onto the chip and then the reaction mixture undergoes an electrophoretic separation in the separation flow channel in the microfluidic device. The product is usually quantified by fluorescence. In an on-chip mobility shift assay, the enzymatic reaction occurs inside the microfluidic device. Here, inhibitors are introduced together with the enzyme and the substrate in wells on the chip [103,105].

MMSAs have been applied to drug discovery, with the off-chip mobility assay mainly used in hit discovery and in the determination of IC_{50} values. The technology can be rapidly sampled to observe enzymatic linearity. MMSAs have been applied to study kinases [105–107] histone deacetylases [108,109], proteases [110], and also non-enzymatic small molecules binders of DNA and RNA [111].

Dunne et al. developed a microfluidic system to perform kinase assays using electrophoresis as part of the detection structure. The assay was developed as both an off-chip assay and an on-chip assay, since both use fluorescence detection for the electrophoresis separation of substrate and product. In their study, four different

Table 5
Methods used to produce droplets in microfluidic systems.

Passive methods	Active methods	References
T-junction	Electric field	[59,160]
Flow focusing	Magnetic field	
Co-flowing	Optical field	

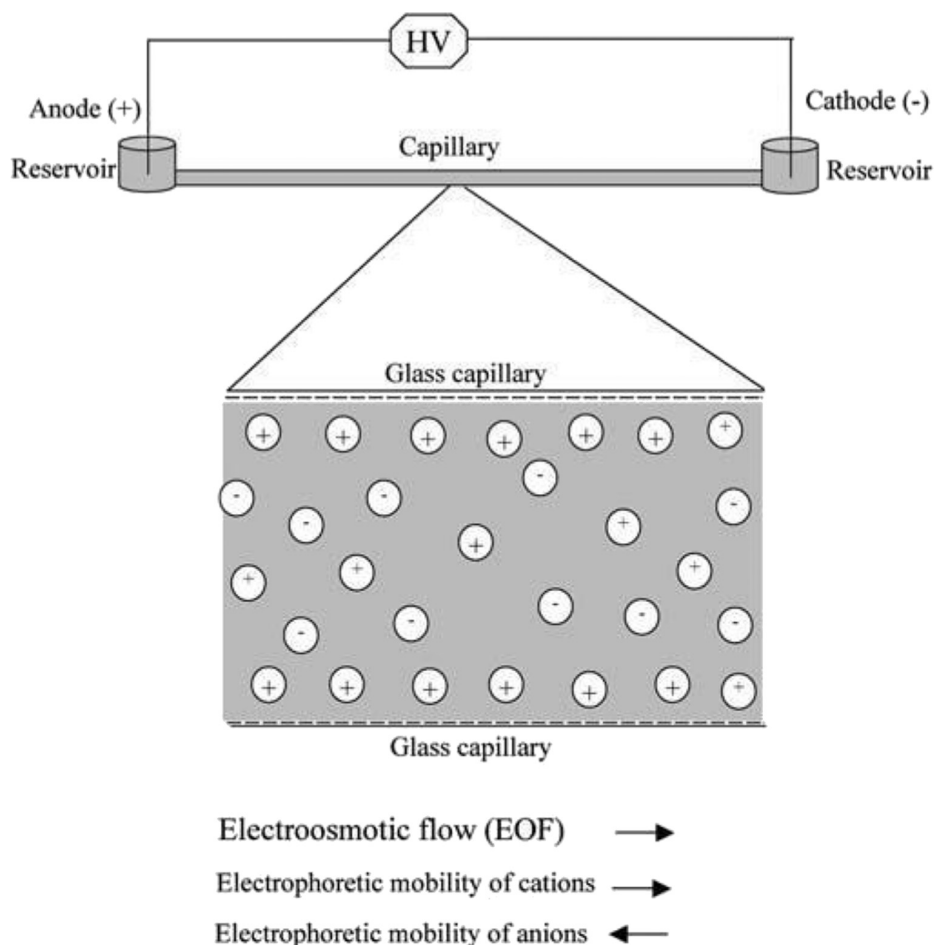


Fig. 4. – Schematic of the principles of capillary electrophoresis and electroosmotic flow [4]. Copyright Springer-Verlag 2003.

kinases were used, i.e. cyclic AMP-dependent protein kinase, mitogen- and stress-activated protein kinase 1 (MSK1), glucocorticoid synthase kinase 3 β (GSK3 β) and protein kinase b or Akt1. These kinases were incubated with 35 commercially available kinase inhibitors and a reasonable correlation between the on-chip and off-chip assays was found.

Ohla et al. developed a fast on-chip microfluidic assay to evaluate the deacetylation of acetyl-lysine residues by sirtuin enzymes. Enzymatic conversion, separation of reaction products and detection were performed within a single microfluidic chip [112]. A fast, sensitive, accurate and reproducible CE technique has been developed to analyse the enzymatic activity of human isoenzyme sirtuin 1 [113]. The CE method allows simultaneous determination of the substrate, the deacetylated peptide, nicotinamide and NAD⁺ as the enzymatic reaction product.

A miniaturized CE on-chip technique was developed for the simultaneous quantification of lactate and glucose. Using two enzymes (lactate-oxidase and glucose-oxidase) and applying an electrophoretic charge to separate the lactate from the glucose, led to the formation of two hydrogen peroxide product zones in the post-column reaction channels. The CE microfluidic device could potentially facilitate point-of-care clinical testing as it is potentially possible to measure other bioanalytes simultaneously. Moreover, the integration and miniaturization of a power supply and detector should help in the development of stand-alone microchip systems [75].

Compared to other fluorescence-based methods, MMSAs are advantageous in that the readout of the desired activity is via direct observation of the reaction product, so the detection of a secondary reagent nor a secondary enzyme is not required. Although fluorescent compounds can affect the readout it was not found to be problematic in the assays described above [103]. Microfluidic technologies can also be used to perform enzymatic assays associated to other analytical methods (immunoassays) or in different substrate materials (microfluidic paper-based analytical devices), as described below.

5.3. Microfluidic immunoassays

Immunoassays are used to detect trace amounts of antigens/antibodies in bodily fluids and are able to specifically identify a target biomarker similar to other molecules based on the high affinity between the antigen and the corresponding antibody [114,115]. Microplate-based immunoassays require long times and intensive protocols and are therefore not considered appropriate for point-of-care clinical applications [116].

The enzyme-linked immunosorbent assay (ELISA) is one of the most widely applied bioanalytical methods, employed in basic research and clinical applications, due to high specificity combined with signal amplification [117]. The microplate formats are ideally suited to high-throughput analysis [118]. However, ELISA is

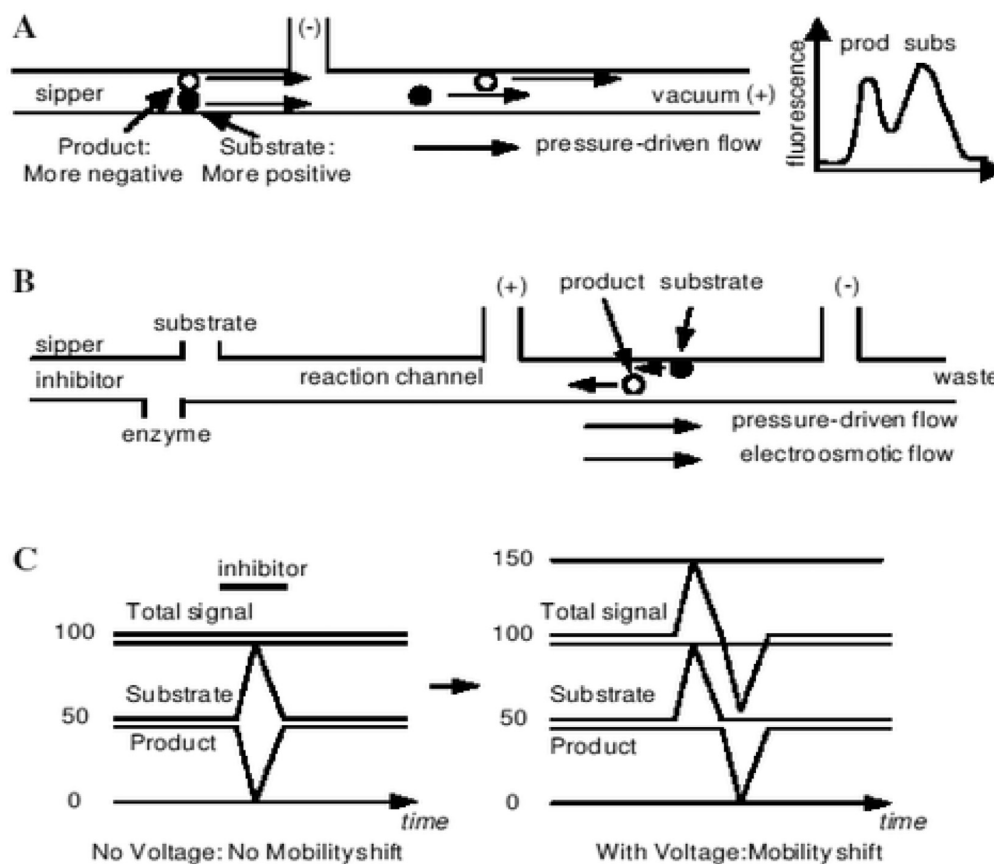


Fig. 5. — (A) Schematic of the off-chip MMSA. (B) Schematic of the on-chip MMSA. (C) In the on-chip MMSA inhibitors decrease the product in the reaction channel [105]. Copyright Mary Ann Liebert, Inc 2004.

inadequate for some particularly demanding assays (for example the detection and profiling of anti-phospholipid antibodies in antiphospholipid syndrome patients), in terms of sensitivity, assay time and/or sample size [119].

The miniaturization of this technique would reduce the incubation time and improve its sensitivity, possibly without compromising on the cost. However, the automation of immunoassays on microfluidic devices presents multiple challenges due to the higher number of fluidic processes and the different liquid reagents involved.

Hou and co-workers developed a microfluidic device to perform a sandwich ELISA (antibody-antigen-antibody) to enable HTS of enzyme inhibitors using nano-scale samples. They tested several ELISA conditions: microfluidic device surface treatment, flow effects, substrate concentrations, incubation temperature and buffer pH. Under the optimised conditions good agreement with the conventional method was obtained [120]. A microfluidic ELISA method was also developed in which the charge state of a novel enzyme substrate changes during the reaction, enabling electrophoretic separation between substrate and the product. This method results in a 54-fold lower detection limit for human TNF- α compared with commercial ELISA kits [121].

Sandwich ELISAs in microfluidic devices based on enhancing enzymatic amplification were used to study the conversion of a chromogenic substrate, o-phenylenediamine dihydrochloride by horseradish peroxidase [122]. The assays allowed prostate cancer antigen and IL-1 β to be detected with an order of magnitude improvement in the detection limit and assay speed. The method

mimics the performance and reliability of laboratory detection apparatus using low-cost optoelectronic equipment and enables the development of cheap point-of-care tests that exploit the low-cost and unique amplification capabilities of enzymatic ELISA detection with universal optical detection technologies such as smartphones and flatbed scanners. This methodology opens the way to a new generation of inexpensive and quantitative optical microfluidic point-of-care diagnostic tests.

An ELISA technique used to detect C-reactive protein was adapted to a 3D microfluidic paper-based analytical device (μ PADs). This portable device contains stored reagents that capture and detect the antibody, substrate, and buffers in isolated zones. The reagents are mixed by moving sliding-strip 3D μ PADs to different zones, allowing complex ELISAs to be performed. The assay is inexpensive, and the colorimetric readout is ideal for portable applications. However, the detection limit of the C-reactive protein (CRP) is reduced compared to a 96-well microplate format [118].

5.4. Microfluidic paper-based analytical devices (μ PADs)

Over the years, paper has been frequently used as a substrate for analytical separation, e.g. in paper chromatography and paper-supported electrophoresis. Paper substrates are attractive due to their low cost and reduced analysis volumes. Using paper as a substrate for sensors is growing, due to its exceptional mechanical (flexibility, lightness, soft texture) and structural features (absorbancy, air permeability, high surface-to-volume ratio, capillary action) as well as its natural origin (biocompatibility, easy

sterilization, chirality, biodegradability) [123]. The emergence of μ PADs has renewed interest in paper as a platform material for chemical analysis [124], and several μ PADs have been developed based on optical (colorimetric, fluorometric, surface-enhanced Raman spectroscopy, chemiluminescence, transmittance) and electrochemical (potentiometric, voltammetry and conductivity) measurements [123].

μ PADs tend to be simple, inexpensive and easy to produce and use [125]. However, some μ PADs are unable to detect biologically relevant concentrations of desired biomolecules. A few μ PADs even provide quantitative results, although these assays usually require non-ambient conditions to function correctly [126]. Paper is hydrophilic and therefore hydrophobic barriers are usually created to confine or direct fluid flow, as described in Table 6.

Only a few studies using μ PADs to perform enzyme assays. Since enzyme immobilization can lead to decreased stability in some cases, Nery and Kubota studied the impact on storage stability of several immobilization methods. In this study, the authors used glucose oxidase as a model enzyme and select an appropriate method for immobilization. Layer-by-layer methods lead to considerably higher initial activity for the same enzyme loading followed by adsorption and entrapment methods. Covalent immobilization with EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide linked to N-hydroxysuccinimide) results in an extremely low initial enzyme activity. Simple entrapment methods and the use of blocking agents reduce enzyme accessibility (highest increase of Km). The authors also noted that protective agents such as sugars, widely used to increase the shelf-life of enzymes stored in solution, do not prolong the stability of glucose oxidase on paper, and suggest that alternative blocking agents, e.g. glycine or bovine serum albumin (BSA) should be used [123].

Lewis and co-workers developed an approach to quantify enzyme analytes by measuring the time elapsed between two regions on a paper-based device that become coloured (see Fig. 6) [127]. This approach can be used to perform assays that are internally calibrated for temperature and humidity effects and sample viscosity. The method is remarkably sensitive and selective and is inexpensive and easy to use and represents a step towards quantitative POC assays without the need of auxiliary devices or electronics [127].

A systematic investigation has been conducted on gradient colour formation on μ PADs using mass spectrometry imaging techniques such as matrix-based laser desorption/ionization and desorption electrospray ionization [128]. Colour assays for glucose detection based on enzymatic reaction with glucose oxidase was chosen as a model as it has been extensively applied in μ PADs and the reaction has been well-characterized. The study showed that

attaching the chromogenic agents is the most important step in ensuring reliable analytical information from colorimetric measurements.

5.5. Reliability of miniaturized analytical technologies

Analytical reliability is an integral or cumulative concept that characterize the consistency of the analytical information in terms of quality and usefulness for costumers. The reliability comprises the results quality, the analytical processes, the sampling protocols and the suitable definition of the analytical problem [129].

Reliability is a summative of all classical analytical properties that can be divided in three categories: the capital properties responsible for quality results (accuracy and representativity), the basic properties (precision, robustness, selectivity, sensitivity and proper sampling) and finally, the productive properties (rapidity, cost-effectiveness, low risks and personnel-related factors). These last two categories are responsible for the analytical process quality that are also dependent of the capital properties [130].

Microarray devices present several weaknesses relatively to reliability. Some of them are low accuracy, precision and specificity [131] as well as the high sensitivity of experimental system to hybridization temperature changes [132] and genetic material purity and degradation rate [133]. Comparing protein microarrays with DNA microarrays, the large-scale protein microarrays is significantly more challenged because it is difficult the expression and purification of a large number of correctly folded proteins with appropriate post-translational modifications, protein instability, potential cross-reactivity and complex data handling [134].

On another perspective, microfluidic systems miniaturization and automation are known to present an increase of sensibility and specificity, higher reliability, low risks of contamination and less cost per analysis in comparison with the technologies presented in well-equipped laboratories [135].

Miniaturized mosaic immunoassay was proposed to patterning lines of immobilized antigens on the substrate surface by means of a microfluidic network. This type of miniaturized system can readily be measured by fluorescence. As it is known, fluorescence measurement is the most widely used method because is highly sensitive and there are a lot of available fluorophores. However, fluorescent dyes are costly, present limited shelf life and are influenced by chemical factors [136].

Paper-based microfluidic devices used to quantitative analysis present more accurate results when a camera phone was used for image capturing of the reacted sites and the colour intensity was analysed by the computer software in

Table 6

Techniques used to create the hydrophobic barriers and their advantages and disadvantages.

Techniques	Advantages	Disadvantages	References
Photolithography	Highly efficient and cost-effective process while producing extremely small incisions in a substrate. Control the exact size and shape of all substrate.	Photoresists are costly and expensive photolithography equipment is required.	[161,162]
Wax printing	Relatively high speed, facile process with high resolution.	High running costs and the low melting point of wax restricts use.	
Screen-printing	Higher resolution than wax printing.	Requires different printing screens for each pattern.	
Plasma treatment	Produce patterns without affecting their surface topography flexibility.	Require different masks to create different microfluidic patterns on paper.	
Flexography	Ideal for mass production.	Need to locate the two prints of polystyrene solution and require different printing plates.	
Laser treating	High resolution.	Require extra coating for liquid flow and microfluidic channels do not allow lateral fluids flow.	

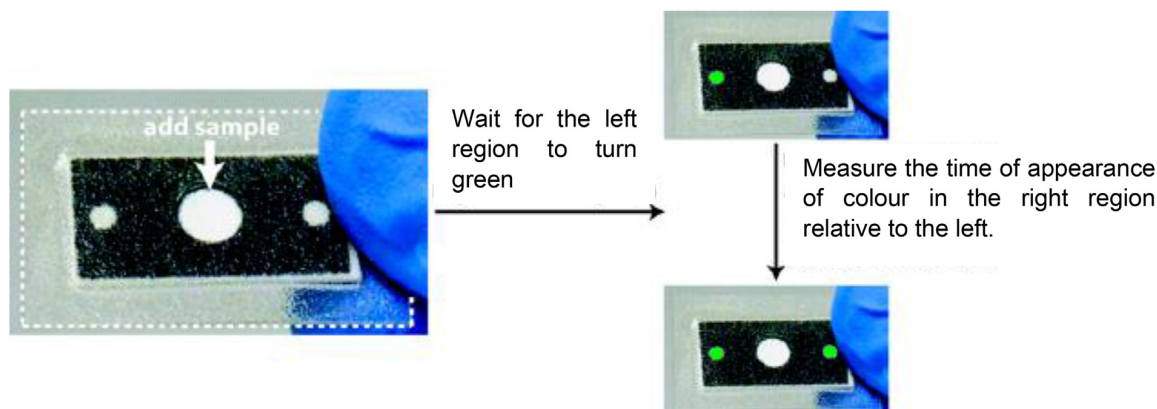


Fig. 6. – Microfluidic paper-based device that quantifies enzyme analytes by measuring the difference in time for colour to appear in the left and right regions [127]. Copyright 2013 American Chemical Society.

comparison with visual inspection [137]. If electrochemical detection is coupled to paper-based microfluidic devices, the analysis is highly sensitive and selective [138]. So, it must be pointed out the great potential of, paper-based microfluidic as a good alternative to traditional microfluidic systems for point-of-care diagnostic applications.

6. Conclusions and perspectives

Microfluidic technologies are becoming increasingly widespread in drug discovery and biomedical applications. One aim has been to develop microfluidic devices that can be engineered to enable HTS, including enzyme inhibition assays, drug-dose response measurements, protein-drug binding, etc.

Microfluidic devices present several advantages over conventionally sized systems. These devices allow the analysis and use of less volume of samples, chemicals and reagents reducing the global costs. Many operations can be performed at the same time due to their compact size, reducing the experiment time. They also offer an excellent data quality and substantial parameter control which allows process automation while preserving high performances. Microfluidic devices can process and analyse samples with minor sample handling. The microfluidic chip is elaborated so that the incorporated automation allows the user to generate multi-step reactions requiring a low level of expertise with a lot of functionality.

A future focus will be to stabilize and automatize current microfluidic devices so they can be better integrated into drug discovery platforms. As these platforms will increasingly contribute to novel drug discovery tools in combination with well-established methods. Moreover, microfluidic cell culture can be considered as the next-generation technique for biomedical and pharmaceutical applications [139].

Paper-based devices allow the easy detection of chemical and biochemical molecules without the need of expensive equipment or skilled specialists. Moreover, the broader applications associated to these devices could facilitate their implementation in the developing world to reduce health care costs by allowing home-based disease testing in these regions.

In the near future, we expect more reports of technological advancements in this unique research area. Also, we believe that further endeavours in commercialization will lead to the broader adaptation of microfluidic techniques outside the core research community and into commercial drug discovery environments.

Acknowledgments

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Tumor microenvironment features and advanced *in vitro* cell culture platforms and enzyme targeting: an overview

1. Introduction

Cancer is a diverse, complex, and the most frequent illness worldwide. Moreover, cancer is considered a systemic disease since malignant cells in their advanced stage originate human death using metastasis over vascular and lymphatic systems (1).

Tumor development is mostly constituted of four stages: tumorigenesis, proliferation, angiogenesis, and metastasis as represented in Figure 1. In brief, the influence of the tumorigenic factors causes healthy cells to enter a cancer process. In the beginning, fibroblasts and immune cells knock out the increase of tumor cells (Stage 1), hopefully clearing cancer cells. Nevertheless, since the fast-growing tumor cell number and remodeling of extracellular matrix (ECM), stromal cells will be adjusted to anomalous phenotypes and further the survival of cancer cells (Stage 2). Moreover, tumor cells will stimulate and support angiogenesis to create tumor spheroids (Stage 3). Lastly, tumor metastasis will be activated when tumor tissues can obtain a determined volume, leading to systemic injuries (Stage 4).

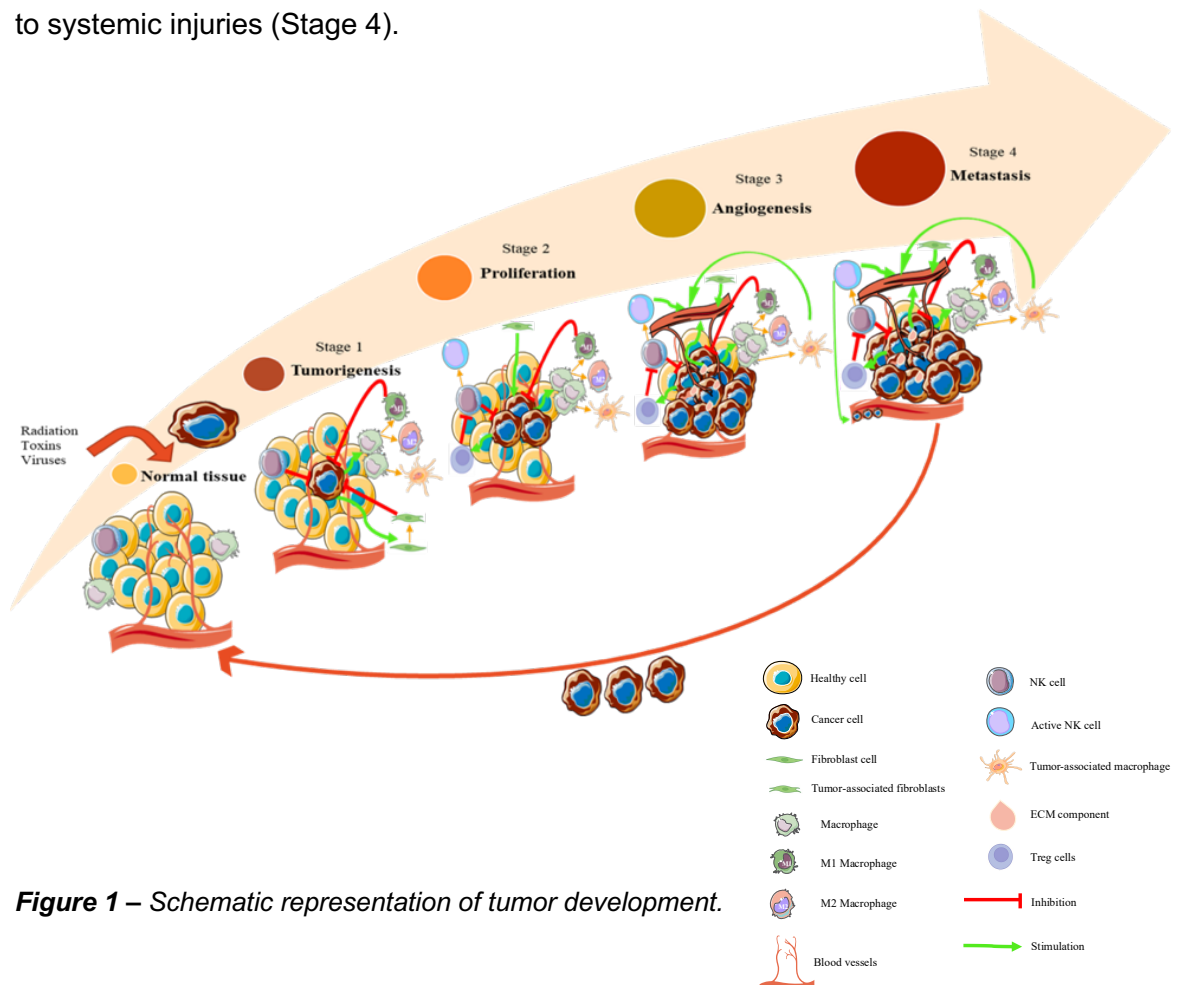


Figure 1 – Schematic representation of tumor development.

As we explained before, cancer is produced by extensive genetic and epigenetic changes that give exceptional characteristics to cancer cells that let them present independent proliferation, cell death resistance, invasiveness, immune system invasion, replicative immortality, and metastatic potential (2). Moreover, the interaction between tumor cells and the constituents of the stroma of the tumoral microenvironment has a key effect on tumor progression, leading to the greatest feature of these cells and allowing the best background for cancer development (3).

Tumorigenesis is a multistage process that gradually transforms normal cells into tumor cells. When the tumorigenesis process happens, this phenomenon can be considered a local disease mainly described by cell proliferation. However, some studies proved that genomic mutations are responsible for transforming healthy tissues into benign tumors and benign tumors into malignant tumors (4, 5). Nevertheless, the alteration of non-metastatic tumors to metastatic tumors has not been clear yet (6). It is thought that the contact of cancer cells with their neighbouring cells in the progressive stage is important to this transformation.

Cancer research has focused on cancer cells and the main variances between healthy and cancer cells (**Figure 2**).

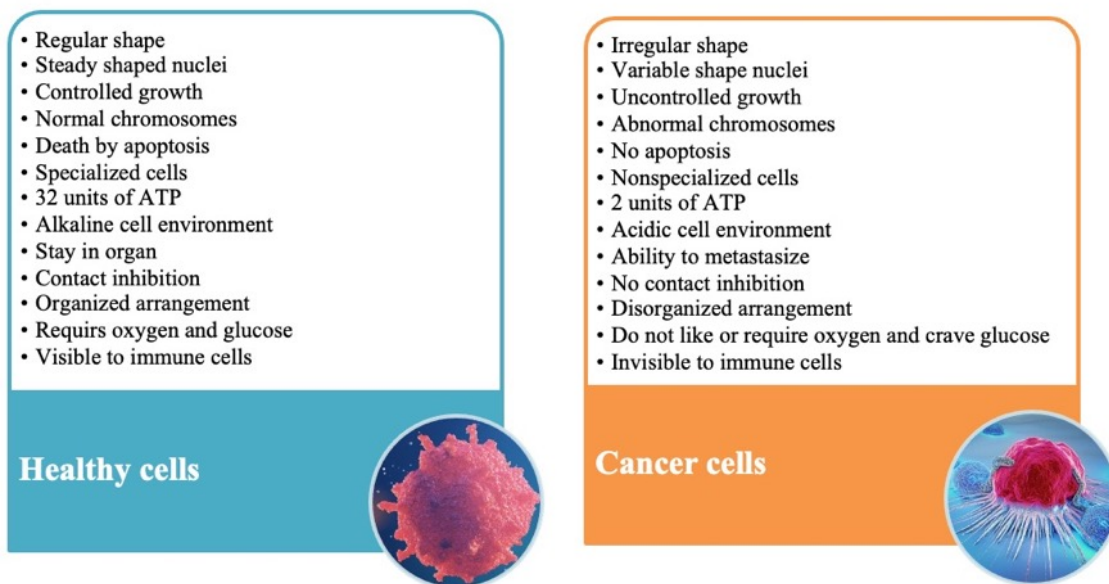


Figure 2 – Variations between healthy and cancer cells.

1.1. Tumor microenvironment

The tumor microenvironment (TME) is a complex and heterogeneous arrangement of multiple types of cell and soluble factors fixed in an extracellular matrix (ECM) which contribute directly or indirectly to the support of tumor cells (7).

The normal tissue stroma supports the organ's structure, and this constituent is crucial for preserving the tumoral microenvironment, being tissue stroma supports the organ's structure and for giving support and nutrients to cancer cells (8).

Different tumor microenvironment components provide processes associated with tumorigenesis because they are strictly correlated to cancer cells.

The tumor extension arises when a favorable microenvironment appears. In this case, the evolution into a malignant state is potentially being achieved with complete information about the cell types involved. These include endothelial and perivascular cells, immune cells including mostly macrophages, fibroblasts, and adipocytes (9).

Figure 3, there are described the tumor microenvironment components and their contribution to tumor progression.

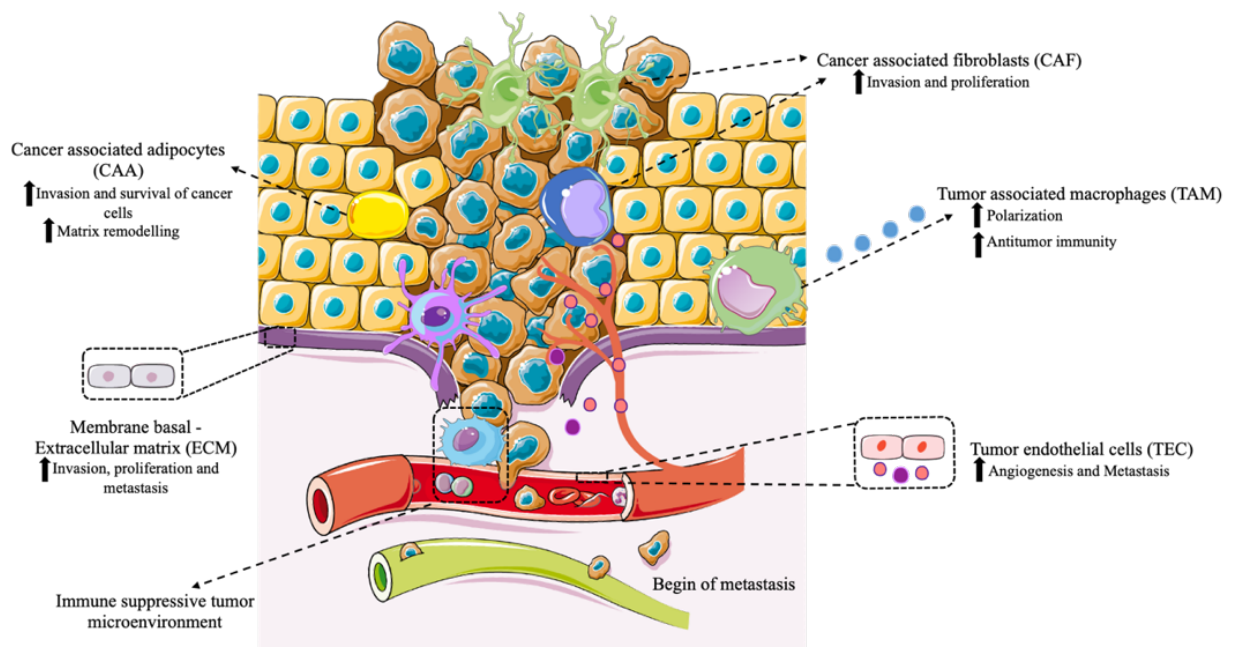


Figure 3 – Schematic representation of the components of the tumoral microenvironment and their involvement in tumor progression.

The extracellular matrix (ECM) is known as a vital constituent for promoting stromal tumors by enabling invasion, tumor progression, and metastasis through the increase of matrix remodeling and angiogenesis (10). Comparing the ECM in normal and cancer cells, the last ECM presents unregulated and disorganized biochemical, physical and architectural properties (11). A key ECM element is collagen which gives structural support and directs cell migration and chemotaxis. The significant deposition and activity of collagen and matrix metalloproteinases (MMP) respectively are characterized in cancer. Usually, the tumoral stroma presents a huge rigidity since the lysyl-oxidase activity

increase which turns by crosslinking collagen fibers, thus enabling the cancer cells' growth and proliferation (11, 12).

The immune cells transition to the tumoral microenvironment and the spread of neoplastic cells into the blood and lymph dissemination create an immunosuppressive environment. For the new vessels to grow into the tumor is crucial to the angiogenesis process. For this growth, it is crucial the existence of pro-angiogenic molecules created by endothelial cells and vascular endothelial growth factor (VEGF) which may also be generated by other neoplastic and microenvironment cells such as macrophages and fibroblasts. VEGF belongs to a huge family of angiogenic inducers and is one of the most studied vessel growth enablers. VEGF can be produced and released by several types of cells such as fibroblasts, endothelial cells, macrophages, and immune system cells; however, the main producers are tumor cells (13, 14).

Its high number makes macrophages the largest cell related to tumor immunity. Macrophages M1 are present at the beginning of the tumor process. These cells are implicated in antitumor activity and inflammatory responses through the production of pro-inflammatory cytokines such as interleukine-6 (IL-6), interleukine-12 (IL-12), and tumor necrosis factor α (TNF- α). On the other hand, macrophages' M2 phenotype and tumor-associated macrophages (TAM) are present in more advanced cancer stages presenting pro-tumorigenic activity, particularly for high production of interleukin-10 (IL-10) and transforming growth factor β (TGF- β) (15).

Cancer-associated fibroblasts (CAF) present a changed phenotype when compared with healthy fibroblast tissues and they are responsible to stimulate tumor growth, invasion, and angiogenesis (16).

Cancer-associated adipocytes (CAAs) instigate a persistent environment of inflammation, invasion, and progression of malignancy through the pro-inflammatory cytokines released. The great production of interleukin-6 (IL-6), TNF- α , and interleukin-1 β (IL-1 β) enable the straight inflammatory cells' permeation in adipose tissue, being responsible for chronic inflammation. This leads to macrophage enrolment and the excretion of substances that maintain cancer progression (17).

The discovery of tumorigenesis and tumor progression vital processes and the development of new therapies have been supported by experimental tumor modeling. However, no model can completely imitate the real system, it is important to must choose one considering its restraints and the required complexity to hold the goals of the study (18).

1.2. Hallmarks of cancer

Six hallmarks characterize cancer: sustainable proliferative signaling; avoidance of growth suppressors; resistance to cell death; enabling replicative immortality; induction of angiogenesis and activation of invasion and metastasis (19).

Moreover, in the last years, three new hallmarks were added to the previously defined: genomic instability, reprogramming of energy metabolism, and avoiding immune elimination since they arise as significant supporting features and epigenetics because they play the same significance to genetic irregularities in the beginning and development of cancer (Figure 4).

So, cancer development is only assumed by the integration of a dynamical signaling circuitry concerning cells and the tumoral microenvironment (20).

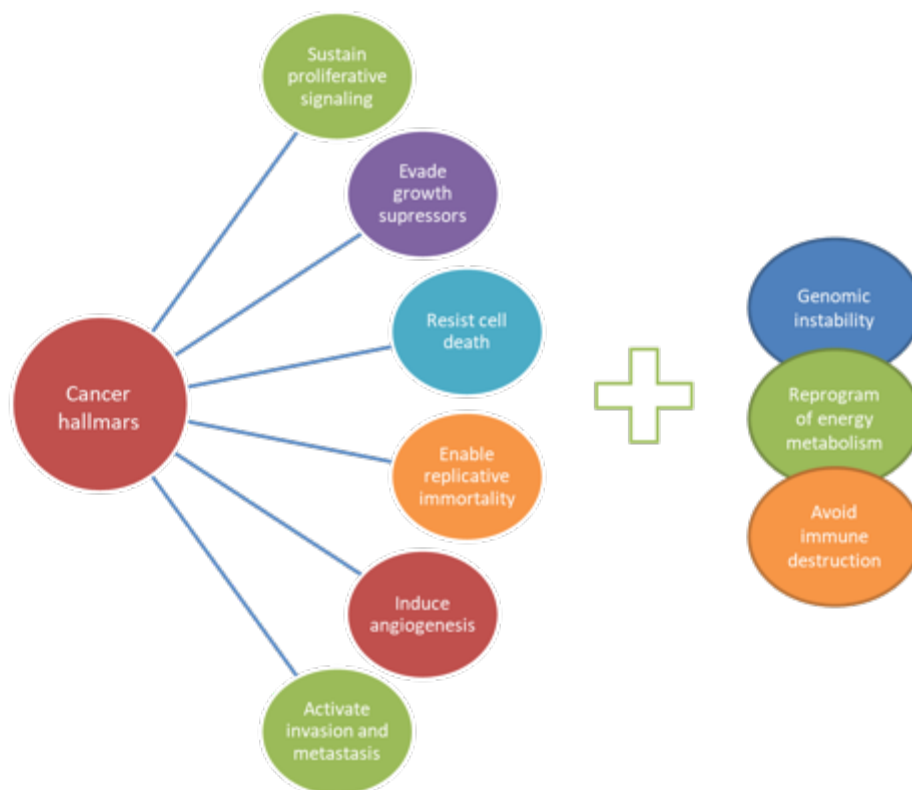


Figure 4 – Old and new cancer hallmarks.

Sustain proliferative signaling and evade growth suppressors' hallmarks are described by the number of healthy cells in a sample and/or by the conservation of proliferative capability over time since limitless proliferation is the main characteristic of all cancer cells.

Resist cell death hallmark is based on the deactivation of apoptotic pathways. Apoptosis is an organized process that leads to programmed cell death (21).

The normal cell cycle is based on an irreversible, no proliferative state and death. The uncontrolled proliferation attributed to cancer cells is regulated by the protection of the termini of chromosomes by telomeres. Telomerase is a specific DNA polymerase that enhances telomere hexanucleotide repetitions at the termini of telomeric DNA. It is almost non-existent in healthy cells, but its expression is upregulated to noteworthy levels in practically all types of cancer cells. This is the principle based on **enabling replicative immortality** hallmark (22).

The **induce angiogenesis** hallmark is based on the rapid angiogenesis by cancer cells to guarantee the tumor requirements for nutrients and oxygen (20).

The invasion-metastatic cascade is the base of **activating invasion and metastasis** hallmark. This cascade is a multi-step process that describes the spread of cancer disease. The epithelial-mesenchymal transition (EMT) is the process by which epithelial cells obtain migratory and invasive capacity (23). This transformation represents an important reprogramming of each cell biology feature, and this includes cell adherence proteins loss, cell morphology modification, raised motility, matrix-degrading proteinase expression, and apoptosis resistance. EMT can be caused by inherent reasons such as genetic mutations (24) or by extrinsic signals, such as growth factors. Nevertheless, certain tumors display deficient or incomplete EMT or invade without epithelial-mesenchymal conversion (25).

In general, most cancers exhibit different chromosomal structures and extreme rates of readjustments compared with healthy cells. The **genome instability and mutation** hallmark are characterized by microsatellite and nucleotide instability. Microsatellite instability refers to the increase or decrease of short nucleotide repeats existent in microsatellite sequences. On the other hand, nucleotide instability is described by the augmented occurrence of substitutions, deletions, and insertions in one or several nucleotides (26).

The changes in **energy metabolism** are so common in cancer cells that it has been integrated as one of the new cancer hallmarks. Active proliferating cancer cells need to control their metabolism to supply continuous energy to the growth of cells. In this case, the increase of glycolysis rate while there is a limitation of oxidative phosphorylation, cells get in a state described as anaerobic glycolysis, similarly recognized as the Warburg effect (27).

There is a complex process that is extremely delimited by several cell types and is vastly studied and described in the tumor context named immunity. The success of tumor progression is an important feature of immune cell scape. So, to allow the growth and metastasis of cancer cells it is important to **avoid immune destruction**, this being the last new hallmark described by (28).

The monitoring of a given hallmark and selecting a suitable *in vitro* assay is a challenging task since this is difficult to analyze only one biomarker (generally it is performing the concomitant study of several hallmarks). In these cases, the combination of some assays is considered. These assays yield further information, however, simultaneously this multiplexing analysis needs to be compatible with parallel or sequential measurements.

1.3. Enzyme biomarkers targeting and cell energy metabolism changes

As we described earlier, the development of cancer cells passes through several stages, including angiogenesis, escape of tumor cells from the primary tumor, cell migration and invasion of adjacent tissues and blood vessels, and metastasis. A fundamental step of all these processes is ECM degradation and modulation. For the tumor cells' invasion and metastasis, these cells must yield hydrolytic enzymes that degrade proteins of ECM to allow the movement of tumor cells into blood and lymph vessels (29-31). The production of hydrolytic enzymes also takes place when there is the reformulation and migration of new tumor blood vessels by the ECM.

There are several types of enzymes involved in these steps. Enzymes are responsible for catalyzing several ranges of chemical reactions crucial for cellular metabolism, signaling, and defense. The transcription and translation of important genes and proteins can be regulated by cells. Otherwise, it can also straightforwardly regulate the activity of the protein by post-translational modifications, the availability of cofactors intracellularly, and substrates or feedback mechanisms (32-34).

Some enzyme families that are associated with ECM degradation are enzymes located in the extracellular and cell surface from the matrix metalloproteinase and serine hydrolase and cysteine cathepsin protease (intracellular enzymes) families. Some of these hydrolytic and proteolytic enzymes have huge potential for the diagnosis and prognosis of human cancers as tumor biomarkers (29-31, 35, 36).

Changes in energy metabolism are considered hallmarks of cancer, as we explained before, and the target of the glycolytic pathway enzymes gives an idea of how to treat the disease. In the last years, extensive investigations have been done to identify and validate enzymes that contribute to this metabolic pathway as potential targets.

The enzyme biomarkers could be divided into two main groups: enzymes involved (Figure 5) or not involved in the glycolytic pathway.

The glycolytic pathway is considered the primary phase where the energy required for cancer cells is produced. For that, the involvement of several enzymes is required. The consumption of two molecules of ATP through glucose to fructose-1,6-bisphosphate (F1,6BP) conversion is next catalyzed by several enzymes such as hexokinase, phosphoglucose isomerase, and phosphofructokinase. To produce energy, the conversion of

F1,6BP to pyruvate step by step, produces four molecules of ATP and two molecules of NADH. The end-product of glycolysis is lactate or CO₂ when glucose is completely oxidated via respiration in mitochondria. In cancer cells, the glycolysis and glucose uptake rates are extremely increased, so there is a higher production of ATP (energy). (Figure 5-A).

The Warburg effect is mentioned as the most significant modification in metabolic events during tumor transformation (37). Even when oxygen is in normal concentrations, the production of ATP is from glycolysis and not through oxidative phosphorylation. So, in these transformed cells, some energy arises from aerobic glycolysis, by the conversion of the most external glucose to lactate instead of metabolizing it through oxidative phosphorylation inside the mitochondria. This requests that tumor cells implement an abnormal glucose high-rate uptake for their need for higher energy, biosynthesis, and redox needs (37, 38). Looking for Figure 5-B and to describe the underlying mechanisms, phosphoinositide 3-kinase (PI3K) activates protein kinase B (AKT), which promotes glycolysis through the direct glycolytic enzymes regulation and by the activation of the mammalian target of rapamycin (mTOR). The liver kinase B1 (LKB1) tumor suppressor, which encodes a serine-threonine kinase, due to the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), faced the change in glycolytic phenotype through the mTOR inhibition. mTOR changes the glycolytic metabolism in several ways, however, it influences the glycolytic phenotype by augmenting the hypoxia-inducible factor 1 (HIF1), which involves a hypoxia-adaptive transcriptional program. HIF1 is responsible for the raised glucose transporters (GLUT) expression, glycolytic enzymes, and pyruvate dehydrogenase kinase isoenzyme 1 (PDK1), which hinder the access of pyruvate into the tricarboxylic acid (TCA) cycle. MYC is a genes and proto-oncogenes regulator that codes for transcription factors, that conjoin with HIF in triggering various genes that convert glycolytic proteins and rise mitochondrial metabolism. The tumor suppressor p53 faces the glycolytic phenotype by quelling glycolysis over TP53-induced glycolysis and apoptosis regulator (TIGAR), enhancing the metabolism of mitochondria via cytochrome C oxidase 2 (SCO2) and auxiliary the expression of phosphatase and tensin homolog (PTEN). Organic cation transporter 1 (OCT1) works in a divergent manner to stimulate the gene transcription that leads to glycolysis and oxidative phosphorylation repression. The change in the pyruvate kinase M2 (PKM2) isoform influences glycolysis not only by decelerating the reaction of pyruvate kinase but also by the deviation of substrates into biosynthetic substitutes and by reducing the generation of nicotinamide adenine dinucleotide phosphate (NADPH-) pathways (Figure 5-B).

B

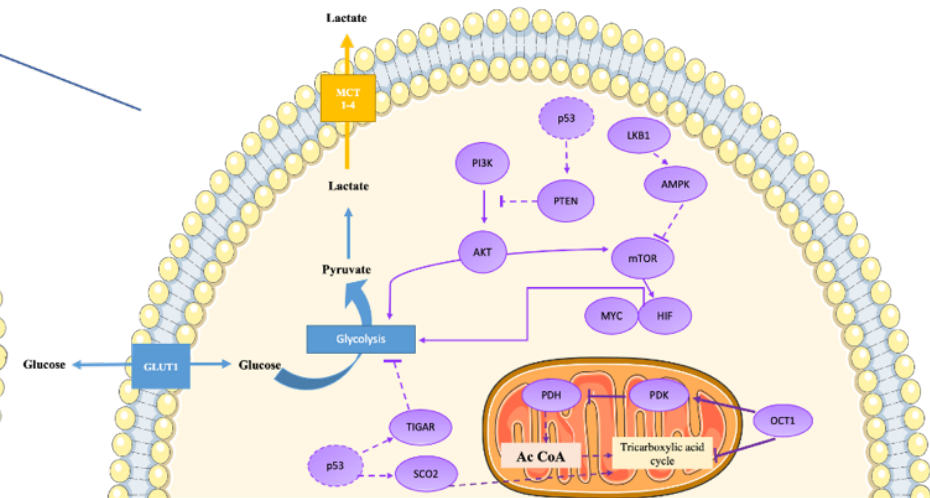
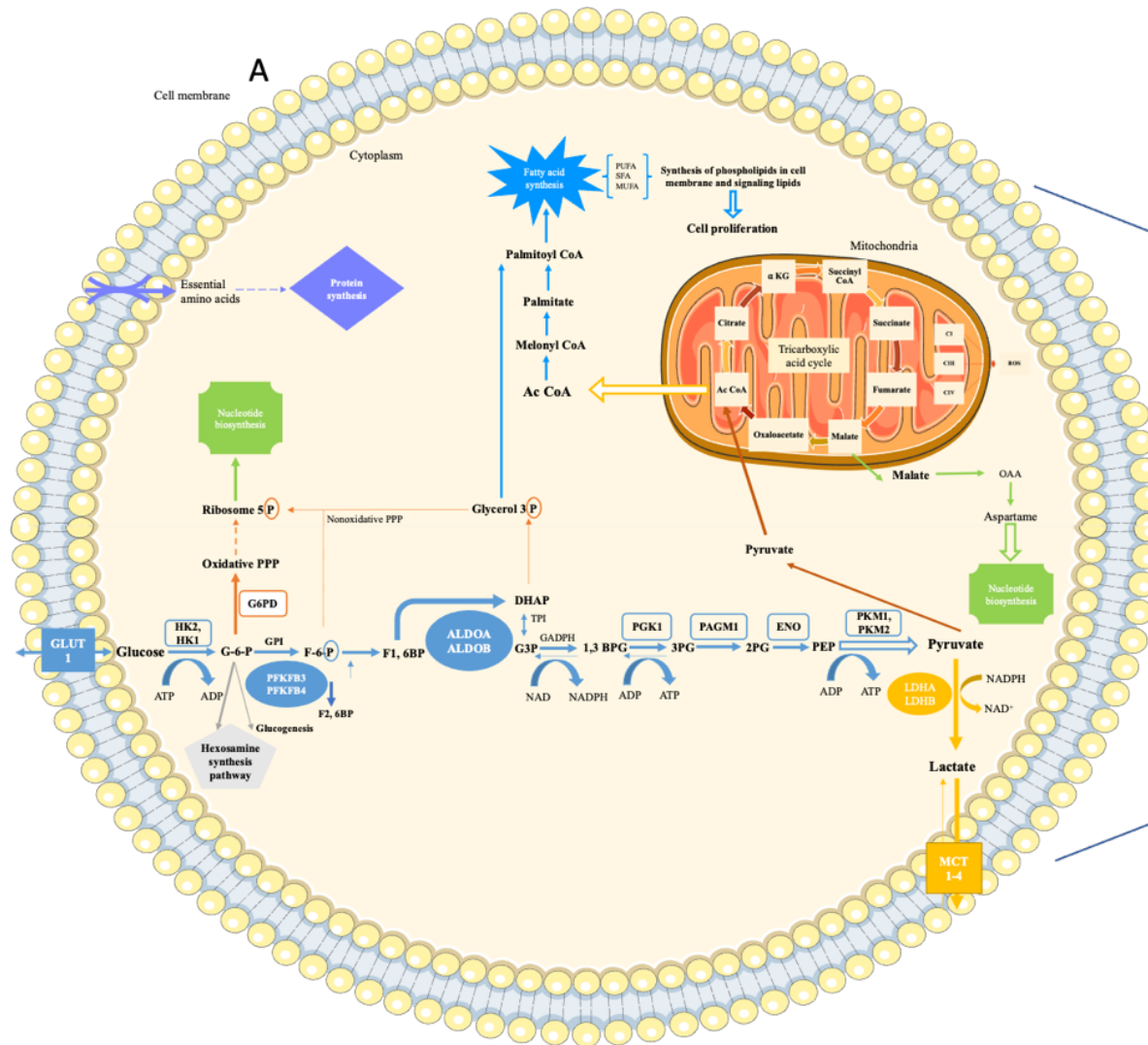


Figure 5 – Schematic representation of modified enzymatic activity in cancer cells. **A)** Glycolysis and glucose metabolism become faster in cancer cells than in normal cells owing to the higher expression of enzyme isoforms and transporters that support the flux of glucose to assemble the anabolic requirements of cancer cells. **B)** Molecular mechanisms that promote the Warburg effect. The dashed lines indicate the loss of the p53 function. **α KG** - α -ketoglutarate; **1,3BPG** - 1,3-bisphosphoglycerate; **2 PG** - 2-phosphoglycerate; **3 PG** - 3-phosphoglycerate; **PFKFB** - 6-phosphofructo 2-kinase/fructose-2,6-bisphosphatase; **Ac CoA** - acetyl-CoA; **AKT** - protein kinase B; **ALDO** - aldolase; **AMPK** - 5' adenosine monophosphate-activated protein kinase; **CI-IV** - Complex I, III and IV of the mitochondrial electron transport chain; **ENO** - enolase; **F1,6BP** - fructose-1,6-bisphosphate; **F2, 6BP** - fructose-2,6-bisphosphate; **F6P** - fructose-6-phosphate; **GLUT** - glucose transporter; **G6P** - glucose-6-phosphate; **GPI** - glucose-6-phosphate isomerase; **G3P** - glyceraldehyde-3-phosphate; **GADPH** - glyceraldehyde-3-phosphate dehydrogenase; **Glycerol-3P** - glycerol-3-phosphate; **HIF** - hypoxia-inducible factor 1; **HK** - hexokinase; **LDH** - lactate dehydrogenase; **LKB1** - serine-threonine kinase product; **mTOR** - mammalian target of rapamycin; **MUFA** - monounsaturated fatty acid; **MYC** - regulator of genes and proto-oncogenes that code for transcription factors; **OAA** - oxaloacetate; **OCT1** - organic cation transporter 1; **p53** - cytoplasmic protein; **PDH** - pyruvate dehydrogenase; **PDK** - pyruvate dehydrogenase kinase; **PPP** - pentose phosphate pathway; **PEP** - phosphoenolpyruvate; **PI3K** - phosphoinositide 3-kinase; **PFK1** - phosphofructokinase 1; **PGK1** - phosphoglycerate kinase 1; **PGAM1** - phosphoglycerate mutase 1; **PUFA** - polyunsaturated fatty acid; **PK** - pyruvate kinase; **PKM2** - pyruvate kinase M2; **PTEN** - phosphatase and tensin homolog; **ROS** - reactive oxygen species; **SCO2** - cytochrome C oxidase 2; **SFA** - saturated fatty acids; **TCA** - tricarboxylic acid cycle; **TIGAR** - TP53-induced glycolysis and apoptosis regulator; **TPI** - triosephosphate isomerase.

Looking at the importance of the glycolytic pathway in cancer progression, targeting this pathway has been studied as a desired therapeutic approach (39, 40).

One of the cancer environmental factors studied is oxidative damage to cellular macromolecules. This damage mostly influences cancer development due to the stimulation of DNA damage, disrupting intracellular signal transduction pathways.

Tumor spheroids also present this change in energy metabolism (Figure 6). The increase of lactate in the microenvironment of tumors leads to acidosis that promotes tumor invasion by the adjacent normal cell destruction and the degradation of the ECM by acid induction and promotion of apoptosis (41).

There are some reports in the literature that support this. Bergman et al. described the increase in the production of GLUT-1 and lactate that changes the expression of the multidrug resistance-associated protein 1 and P-gp, leading to drug resistance (42). Moreover, Khaitan et al. observed that the consumption of glucose and the production of lactate were two- to three-fold higher in Cellosaurus cell line (BMG-1) glioma spheroids than in BMG-1 monolayer cell culture (43).

From another perspective, Longati et al. found that the expression of messenger RNA (mRNA) of GLUT-1 and lactate dehydrogenase on pancreatic cancer cells (PANC-1) cultured in 2D and 3D models was around 3.5 and 7.5, respectively (44). Likewise, another report also revealed that the expression of GLUT-1 in MCF-7 breast cancer spheroids was 0.5-fold upper than in 2D cell cultures (45).

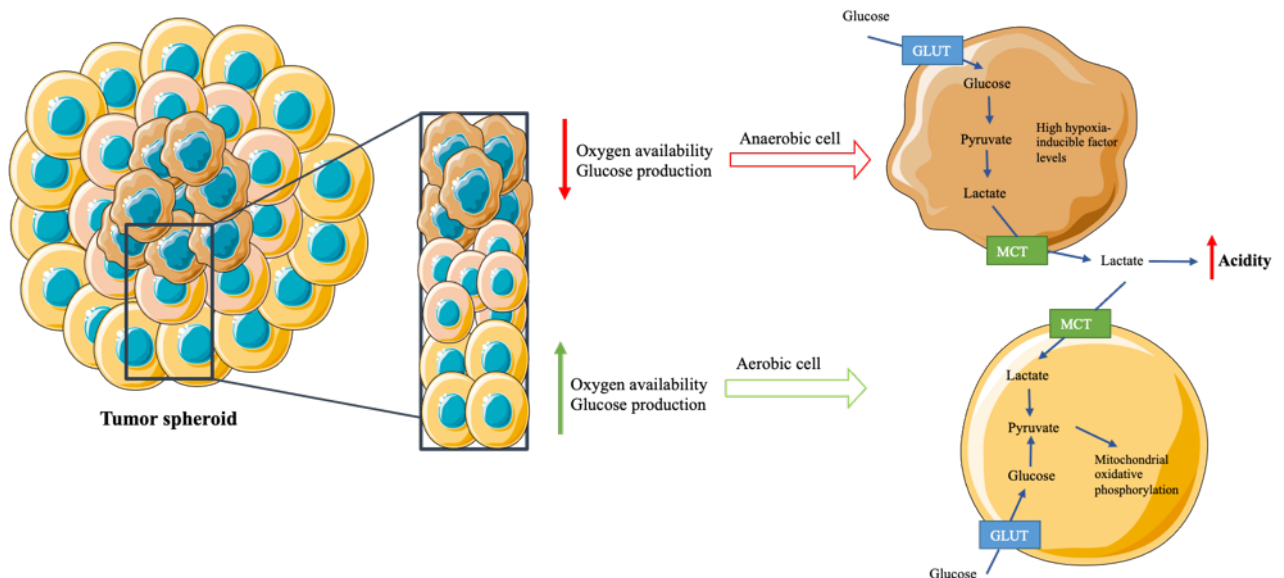


Figure 6 – Differences between oxygen distribution in tumor spheroids leading to the modifications in energy metabolism in cancer cells.

To identify the susceptibility to DNA damage and cancer risk, the expression levels, and the presence of genetic polymorphisms of some enzymes, such as catalase (CAT), glutathione-S-transferase (GST), and superoxide dismutase (SOD) have been studied. CAT is responsible for the catalysis of hydrogen peroxide (H_2O_2) to water and oxygen, being the major contributor to removing high fluxes of H_2O_2 in tumor cells. However, according to Doskey et al. study, on average, cancer cells are 2-fold lower rates of removing the extracellular H_2O_2 than normal cells, and the activity of CAT in tumor cell lines from different tissues source presents a broad difference in the tumor cells' capability to eliminate the extracellular H_2O_2 . This work also proves the involvement of H_2O_2 in the mechanism of plasma ascorbate ($P-AsCH^+$) toxicity to cancer cells and CAT is essential to remove this. Besides, *in vivo* CAT activity measurements in tumors may evaluate which cancers will respond to pharmacological ascorbate therapy, this leads to the possibility to find new therapies increasing the treatment efficacy for those tumors with higher CAT activity (46).

GST enzymes are phase II detoxification enzymes, that reduce the glutathione (GSH) level by the conjugation of several reactive electrophiles, being essential in cellular defense by the reduction of these levels. Some genetic polymorphisms, e.g., GSTP1 and

GSTT1 are related to not only the increased risk of different cancers, such as breast, lung, liver, and stomach cancers but also to the chemoresistance of several anticancer drugs such as 5-fluorouracil (5-FU) and cisplatin-resistant gastric cancer cells or adriamycin-treated breast cancer cells (47, 48). According to cellular metabolism, GSTP1 is also responsible to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), increasing its activity in breast cancer cells (49). Moellering et al. and Hildebrandt et al. established that the conversion of 3-phosphoglycerate to 1,3-bisphosphoglycerate mediated by GAPDH is limited by the glycolytic reaction rate in cancer cells by the intensification of the aerobic glycolysis. Moreover, these reports also confirmed that the activation of GAPDH by GSTP1 is an important metabolic focus so, it is possible to identify promised therapeutics for breast cancer patients through GSTP1 inhibitors (50, 51).

SOD enzymes are responsible for the superoxide radicals' conversion to oxygen and peroxides, protecting cells against the toxic products produced during aerobic respiration. SOD1, one of the three forms of SODs, is the major intracellular SOD form, being near 80% of the total SOD protein. Looking from a cancer perspective, SODs present a crucial role in some aspects of human cancer, however, the three SODs have different roles depending on the localization and distribution in cells and tissues and their biological functions. SOD1 could be easily closed related to cancer since carcinogenesis cause redox homeostasis and oxidative stress. From this point of view, the loss of SOD1 increases the reactive oxygen species (ROS) level and is so inherently believed that cause oxidative DNA damage and stimulate carcinogenesis. From another perspective, cancer cells are recognized to present elevated ROS levels and turn progressively dependent on the activation of antioxidants such as SOD1 to avoid extreme cellular damage and tumor progression apoptosis (52). Looking at SOD2, this enzyme form has been associated to be a tumor suppressor by the decreasing SOD2 expression in tumors. Generally, the SOD2 levels present different levels according to the tumor stage (53). Normally, SOD2 is lower in the first stages of tumors which is reliable with the reduction of antioxidants associated with the increase of ROS and oxidative genomic DNA damage and thus carcinogenesis but in late-stage tumors, specifically metastatic tumors, the SOD2 level is usually higher. Nevertheless, the complexity of the situation could be increased since the SOD2 level can also be determined by specific oncogenic drivers and the complete state of the whole redox system. According to Papa et al., there were evaluated some breast cancer cells to identify the levels of SOD1 and SOD2 enzymes. The results suggest that the combination of the decrease in SOD2 and the increase in SOD1 may identify SOD1 as the key dismutase activity in breast cancer. To prove that they measured the activity of total dismutase using a board of breast cancer cells. They

discovered that MCF7, BT474, and MDA-MB-231 breast cancer cells showed an increase of 5-, 6.7-, and 3.2-fold, respectively, in the activity of dismutase, when compared with MCF-10A cells. After that, they also determined the SOD1 and SOD2 relative contribution to the total dismutase activity in these cells. To measure the dismutase activity, they used potassium cyanide to inhibit SOD1 activity. They established that the SOD1 inhibition hugely reduced the total dismutase activity in MCF7, BT474, and MDA-MB-231, to 10%, 34%, and 27%, respectively (54).

Looking for cancer cell proliferation, the enzymes that play the key role are the glycolytic enzymes. In this group, we can include lactate dehydrogenase (LDH), cyclin-dependent kinases, caspases, and enzymes redox-detox regulated by p53. Generally, to produce energy quickly, cancer cells use non-glycolytic enzymes which unsurprisingly cause extreme lactate production by LDH (55, 56). Consequently, the production of NADH and NAD via glycolytic fluxes provides simultaneously anabolic substrates that stimulate the progression of cancer (57) which seems to be the first checkpoint target of both fermented whole wheat germ extract (FWGE) and rice callus suspension culture (RCSC) to produce an anti-proliferative effect. FWGE looks to be responsible for the inhibition of glucose uptake by cancer cells and exhibited cytotoxic and cytostatic effects in several cancer cells (58), while RCSC is responsible to inhibit LDH, so it activates oxidative phosphorylation rising the ROS production and the following apoptosis and also exhibited a stronger anti-proliferative effect than paclitaxel and etoposide in renal and colon cancer (59, 60).

Moreover, caspases, that belong to the cysteine-aspartic acid protease family, are also triggered by these extracts, and when activated, they are responsible for the initiation and execution of apoptosis (61). Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A) and the transcription factor Jun (c-Jun) play important roles in the regulation of cyclin-dependent kinases and then in the cell cycle preservation and development (62). Additionally, the p53 expression, a negative regulator of cell growth, is also controlled by them, which applies its effect by the regulation of the redox and detox enzymes (63).

Other enzymes are already reported as overexpressed enzymes in cancer, and they also affect the metabolism of cells, such as Cyclooxygenase-2 (COX-2) and Dihydrofolate reductase (DHFR).

Cyclooxygenases (COXs) are desirable enzymes to convert metabolically arachidonic acid into prostaglandins, including PGE₂, the main inflammation, and angiogenesis intermediary. There are two isoforms of COXs: COX-1 and COX-2. Both are present as essential, membrane-bound proteins, sited mainly on the endoplasmic reticulum luminal side and nuclear envelope (64). Although they present a huge sequence identity level (ca. 60%), they present slight differences in the active site and specificity of ligand and each

one presents different functions in the human body (65). COX-2 is usually undetectable in healthy tissues and can be quickly upregulated in reaction to several pro-inflammatory agents comprising tumor promoters specially involved in inflammation, pain, fever, or tumor formation. Recently, the overexpression of COX-2 has been described in various human cancers such as breast (66, 67), lung (67, 68), skin (69), colon (70), cervical (71), and so on. The COX-2 constitutive expression and the consequent biosynthesis of PGE₂ interfere in the principal functions in the beginning and advancement of cancer progression. PGE₂ facilitates these effects over several signaling pathways such as VEGF is important to increase angiogenesis, cell proliferation, and the potential of metastatic and invasive (72); improving the protooncogene BCL-2 and EGFR expression by the activation of the MAPK and the phosphoinositide 3-kinase (PI3K)/AKT pathway, respectively (73); enhancing the antiapoptotic mediator nuclear factor κ B (NF κ B) transcriptional activity; increasing the metastasis and the invasion through the stimulation of matrix metalloproteases (MMP-2 and MMP-9) (73); and immunosuppression by the suppression of the IL-12 production (74).

The reduction of dihydrofolate to tetrahydrofolate using NADPH is catalyzed by the dihydrofolate reductase (DHFR) enzyme. NADPH is considered a key cofactor involved in the biosynthesis of purines, thymidylate, and various amino acids. Also, DHFR is related to the synthesis of raw material for cell proliferation (75). The folate pathway is considered an important target in cancer. Antifolate compounds can strongly bind to DHFR and inhibit DNA synthesis and cell proliferation, so these compounds are considered potential anticancer drugs. An example of an antifolate compound is methotrexate. In 2009, Martin et al. proved that MTX can induce oxidative DNA damage leading to the treatment of hereditary non-polyposis colon cancer (76). Nevertheless, cancer cells can develop MTX resistance through some mechanisms such as changes in drug transporters, DHFR overexpression, and/or promotor polymorphism.

All these mechanisms explained before for each enzyme described could be challenging to develop and study in cell-based enzyme assays, however, some studies have been performed to understand that.

2. *In vitro* assays

In vitro cell cultures allow the advanced knowledge of mechanisms that cause the behavior of the cells *in vivo* such as cell biology, differentiation, migration, growth; tissue morphology; disease mechanisms, and drug action (77). Furthermore, these mechanisms are possibly used in preclinical research of several drugs in cancer research and gene function (78).

2.1. Cellular characteristics

To perform *in vitro* model systems, it is possible to use different cell types. Nowadays, primary cells isolated straightforwardly from donors' material or using recognized cultures located in cell banks is one of the cell types that can be used and is considered the golden standard of *in vitro* model, being greatly recommended for their implementation into laboratory practice (79, 80). **Primary cultures** typically comprise populations of diverse cell types existent in the source tissue. These cultures present two main disadvantages: isolation difficulty and short lifespan. Moreover, tumor microenvironment preservation following the use of initial passages is also critical in these cell cultures. Generally, the initial cell passaging carries modifications in gene expression, proliferation rate, and drug response that improves certain sub-clones, changing the high translational importance of this culture model. So, the initial steps of primary culture management are extremely important if valuable translational results must be attained (81). Nevertheless, the main advantage is that they thoroughly mimic the *in vivo* genetic tumor characteristics (82). Comparing primary cells with **immortalized cells**, the first cells present noteworthy advantages in drug discovery relatively target identification, hit identification, and lead optimization. Most significantly, the protein physiology expressed in primary cells has stronger matches to cells *in vivo* than immortalized cells. Many primary cells in culture present another better characteristic when compared with immortalized cells that are as they do not proliferate, the extended consequences of compounds screened can be distinguished from effects of cell division. The formation of immortalized cell line must have three key procedures: stromal cell selection; cell immortalization; and the cell line description based on the morphological and biomolecular features (83). Immortalization can be promoted with or without genetic modifications (84, 85).

Table 1 presents the main differences between primary and immortalized cell lines.

Table 1 – Main differences between primary and immortalized cell lines.

Parameters	Primary cell line	Immortalized cell lines
Lifespan	Limited	Infinite
Specificity	Retains	Loses
Source	Tissue	Vial with high mutations and clonal selections
Characterization	Ready to use	Required authentication
Donor	Varied	Single

From another point of view, the importance of cellular crosstalk phenomena is currently taken on as increased interest in using **co-cultures systems**.

Co-culture is a culture methodology where more than two different cell types are in one culture dish or well with some degree of contact between them. There are several motivations to use these systems: studying the natural interactions between populations, enhancing culturing success for some cells, or trying the establishment of synthetic interactions between different cell populations. This technique has been mostly used to perform three different assays: embryonic stem cells on feeder cell culture, consideration of immune cell interactions, and assessment of mesenchymal-stromal interactions.

The use of co-cultures for drug research is extremely significant since they give a more representative human *in vivo*-like tissue model than animal models. Also, it is an important strategy to apply in high-throughput analysis and extensively monitorisation of the effect of drugs on cell-cell interactions (86).

In literature, some studies prove the advantages of co-culture compared with monoculture, specifically in breast cancer cells, in some enzyme's expressions, such as aromatase and cytokine (87).

2.2. *In vitro* models

Over the past years, pharmaceutical industries and specifically the cancer-related scientific community have been spending their time and money on the development of new anticancer drugs, which must be mandatorily assessed using several *in vitro* and *in vivo* assays. Moreover, they are vital for the drug development progression in preclinical screening and help in the evolution of the best-performing compounds for human clinical trials (88). The truth is there is a rise in drug development data and the advances in technology should have approved a more reproducible and cost-effective improvement of successful therapies (89), however, only 5% of the novel anticancer molecules magnificently profit clinical approval since the 95% fail because of toxicity and poor efficacy of the compounds (90-92). So, the effective, safe, and economic development of feasible anticancer drugs is still the main challenge. One of the explanations for this happen is the low connexion of the preclinical *in vitro* and *in vivo* data with the results from the clinical trials (93). The reason behind this is the absence of preclinical models with clinical relevance skilled to mimic tumor physiopathology and review the complexity of the tumor concerning the tumoral microenvironment as well as the tumor cells' interaction with the neighboring medium (94, 95).

Thus, three-dimensional (3D) cell models have attracted more and more attention from the scientific community since they present several advantages, that will be described in detail below, bridging the gap between the two-dimensional (2D) cell culture systems and *in vivo* studies (96, 97). Moreover, 3D models are suggested to be used instead of using

animals in biomedical research, especially for toxicity assays, respecting the 3R principles (replacement, reduction, and refinement) which are well-defined by ethical and regulatory laws (97, 98).

2.2.1. 2D and 3D cell culture methods

To mimic what happens in our body and to evaluate the cytotoxicity of drugs and other compounds, *in vitro* models are established using cell lines or dissociated primary cells that can be cultured in two different dimensional forms based on cell morphology: two-dimensional (2D) and three-dimensional (3D) culture systems. The differences between these two-dimensional forms are presented in Table 2.

Two-dimensional (2D) *in vitro* cell culture models have been used to analyze cellular responses to stimulations from biophysical and biochemical signals. Most of the biological experiments on tumors are established on 2D cell models. 2D cell culture consists of the adherence to a rigid surface to provide mechanical sustenance for the cells mimetizing the ECM composition, promoting cell adhesion, and supporting desired cell behavior such as proliferation or differentiation (99, 100). Using these models, the cell grows and proliferates homogeneously due to the number of nutrients and growth factors available in the medium. 2D cell methods present some advantages related to simple and low-cost conservation of the cell culture and the implementation of functional assays.

Nevertheless, most of the 2D cell methods present some drawbacks that can impact cell morphology and shape (101), internal cell structural organization, secretion and cell signaling (102, 103), survival (104), proliferation and differentiation (105), loss of diverse phenotype (106, 107), and disease mechanisms (108).

Table 2 – Differences of 2D and 3D models.

Culture type	2D model	3D model	Ref.
Cells characteristics	- Monoculture – the study of individual cell types. - Alteration of morphology cell and division mode. - Lost of different phenotypes. - Lost of cells polarity since changes in interaction with the external environment.	- Resemblances between morphology and behavior of cells growing. - Maintain division mode. - Different phenotypes and polarity. - Cell proliferation is higher in the peripheral part of the 3D structure.	(106, 107, 109-113)
Cells interactions	- Lost of cell-cell and cell-extracellular environment interactions.	- Suitable interactions of cell-cell and cell-extracellular environment to get a simulation of the structure of the tissue. - Stimuli received from the local environment as <i>in vivo</i>.	(114-118)
Molecular mechanisms	- Modifications in gene expression and mRNA splicing. - Variations on cells biochemistry and topology.	- Genes expression and mRNA splicing. - Similar to cell growth <i>in vivo</i> related to cellular topology, gene expression, signaling, and metabolism.	(119-126)
Culture time formation	- Between minutes to a couple of hours.	- Since a few hours to a few days.	(127-129)
Culture usage and analysis	- High performance and reproducibility. - Long-term culture. - Easy to understand. - Culture simplicity.	- Less performance and reproducibility. - Difficult to understand. - Difficulty in carrying out the culture.	(130)
<i>In vivo</i> mimic	- Do not mimic the natural structure of tissues or tumors.	- Mimic the physical and biochemical features of tissues and tumors.	(131)
Access to main components	- Unlimited access to oxygen, nutrients, metabolites, and signaling molecules. - More cells are in the same stage of the cell cycle.	- Limited access to oxygen, nutrients, metabolites, and signaling molecules. - The main cells often continue inactive because they have less oxygen and growth factors from the medium.	(97, 132-135)

Drug sensitivity	- Cells often have less resistance to drugs what seems that the drugs are a successful treatment. - Lower metabolization of drugs. - Cells proliferation at an abnormally fast pace.	- Cells are often more resistant to drugs treatment. - Better drug metabolism. - Precise representation of the drug's effect. - Proliferation rates are representative and depending on the type of cells studied or the technique applied could be high or low.	(135-137)
Gene and protein expression level	- Often extremely different compared to <i>in vivo</i> models.	- Levels that resemble the ones found on <i>in vivo</i> cells.	(134, 135, 138)
Apoptosis	- Easily induce cells apoptosis from drugs.	- High resistance rates for drug-induced apoptosis.	(134)
Maintenance culture costs	- Low cost. - Tests and media are commercially available.	- More expensive. - More time-consuming. - Tests are commercially available in fewer quantities.	(139, 140)

Another disadvantage of 2D cell culture is the unlimited access to oxygen, nutrients, metabolites, and signal molecules presented in the cell medium, however, for *in vivo* cancer cells, the accessibility of these products is more inconstant due to the design of the tumor (97). Looking at all these drawbacks associated with 2D cell models, it is necessary to discover new unconventional models that mimetizing in a better way the natural environment of normal and tumor cells such as 3D culture systems.

3D tumor models attracted increasing attention from the scientific community. Tumor cells are spread in three dimensions and progressively from spheroids supported by scaffolds or hydrogels and contain nutritional gradient and phenotypic diversity (141). 3D cell models have been broadly used for the analysis of several tumor types such as breast and prostate cancer (142). The advantages of 3D models offset the drawbacks of 2D since they improved to mimic the *in vivo* microenvironment, enable the cell-cell and cell-ECM interactions, reproduce the mechanical conditions of human tissues, and present similarities in gene expression, cellular topology, signaling, and metabolism (123-125, 132). The higher quantity of cell-cell interactions recognized in spheroids can regulate cancer cell behavior mainly their cell signaling, proliferation, survival, and drug sensitivity. On the other hand, in 3D models, the cells do not exhibit polarity, live in a lower rigidity microenvironment, and restrict the distribution and migration (143, 144). Moreover, with 3D tumor models, tumor migration, invasion, and drug resistance results proved to be more reliable compared with 2D models (139, 145).

Some studies in the literature evaluated the consequences of 2D and 3D cell methods on proliferation, differentiation, gene expression cell levels, apoptosis prevention, motion and migration, ECM matrix mechanical, chemical, and topographical properties, and mechanical responses.

Pineda and co-workers proved that octamer-binding protein 4 (OCT4) in mouse embryonic stem cells (ESCs) was reduced in 2D and 3D cell cultures, displaying a pluripotency loss, while nestin (NES) and brachyury (BRACHY-T) markers presented in ectoderm and mesoderm, respectively, exhibited an increase. The ESCs cultured on glass slides (2D) coated with collagen type I presented a fast rate of differentiation than in 3D cultures (embryonic bodies and collagen type I gels). The embryonic body culture proved to have the best ability to support cardiovascular differentiation. Moreover, neural differentiation was only maintained in embryonic bodies (3D) that had been constant over time (146). Also, Chitcholtan et al. demonstrated that some tumor cell features are not suitably modeled in the cell monolayer. They used four endometrial cancer cell lines that were illustrative of the diverse pathological morphologies of these routine tumors in the clinic. According to the results, cancer cells lead to the formation of spherical structures in the 3D reconstituted basement membrane (3D rBM) and the morphological aspect was

cell line dependent. The results also showed that there was a reduced proliferation related to a decrease in the expression of proliferating cell nuclear antigen (PCNA) in 3D rBM than in 2D cell monolayers. The uptake of glucose, the VEGF secretion, and prostaglandin E₂ (PGE₂) was selectively different in 3D rBM, so the 3D *in vitro* model showed that can influence the endometrial cancer cell proliferation and metabolism compared to 2D cell monolayer, however, they assume the importance of study targeted anticancer agents in this 3D *in vitro* model (147).

Recently, two different cell lines (5637 and T24 bladder carcinoma cells) were tested in 2D and 3D cell models to compare their proliferation. The dead cell number in the 2D cell culture was higher than that obtained in the 3D platform. Furthermore, they evaluate on days 2 and 3, the cell growth on both methodologies and they realized that the cell growth was greater in the 3D platform than in the 2D cell culture in both days for 5637 cell line (in day 2: 4.89 ± 3.77 [2D] vs. 25.84 ± 3.33 [3D], $p < 0.05$), (in day 3: 13.39 ± 4.04 [2D] vs. 45.19 ± 3 [3D], $p < 0.01$); and just in day 3 for T24 cell line (88.70 ± 4.83 [2D] vs. 151.38 ± 4.72 [3D], $p < 0.01$) (148).

From another point of view, Baskaran and co-workers assessed the association between cell adhesion and 3D invasion concerning 2D migration and 3D invasion. The four proteins of ECM tested (collagen I, fibronectin, tenascin C, and collagen IV) drive a significant increase in invasion in MDA-MB-231 and MDA-MB-468 breast cancer cells, although MDA-MB-468 migrate more individually than the MDA-MB-231 cells. For the MDA-MB-231 alone and with this cell line in combination with MDA-MB-468, they did not discover a noteworthy correlation between the effect of collagen I, fibronectin, tenascin C, and collagen IV proteins on 3D invasion and either 2D migration or cell arear. Moreover, the Q^2 for each of these models was negative, showing a low expectation of 3D invasion using cell area, cell migration speed, or persistence. Generally, these results indicate that ECM-driven effects on 3D invasion may not associate with effects on the cell area and 2D migration (149).

2.2.2. 3D cell culture techniques for tumor models

3D tumor cells growing using 3D cell culture methods have drawn the attention of tumor cell biology research to the difficulty of replicating the *in vivo* environment of an *in vitro* tumor cell.

As explained before, ECM is a non-cellular component where the cells that constitute a tissue or a tumor exist. This component is a 3D meshwork constituted by several structural fibrous such as collagen, elastin, and fibrillin and bounded by glycosaminoglycans, proteoglycans, and glycoproteins that present biophysical, mechanical, and biological characteristics offering biophysical, structural, and biochemical

support and signals to the cells (98). According to the tissue type and the disease phase, the ECM composition can change (150). This change in composition is principally due to cancer and stroma cell-mediated ECM deposition or degradation, mostly through the enzymatic digestion of matrix metalloproteinases. Therefore, the deposition of collagen and hyaluronan affects the matrix thickening and increased the likelihood of tumor metastasis, angiogenesis, and drug resistance (150).

In that way, it is extremely important to find cell culture methods that mimic the ECM environment to the results obtained are closer to the real effect that we can have on the human body when it is subjected to disease treatment. So, nowadays, there are two main types of 3D models: scaffold-based models where the cells and the substrate interact, and scaffold-free models where the cells are incapable to attach to a surface, leading to the aggregation of cells forming spheroids.

In scaffold-based 3D cell cultures, cell growth happens with the attachment of the cells to a substrate that mimics the ECM. This classification includes different support types: hydrogel, paper, hydrophilic glass fibers, and organoids. On the other hand, scaffold-free models include hanging drop microplates, magnetic levitation, and ultra-low attachment coating spheroids microplates. Table 4 displays a comparison between all the previously described methods to better identify the characteristics, advantages, and disadvantages of all of them. The difficulty to apply to 3D models the mechanisms and devices used to assess the cytotoxicity, gene and protein expressions, and further endpoint assays compared to 2D cell culture methods is considered one of the key drawbacks behind the switch from 2D to 3D cell culture for the application in drug screening analysis (97, 151). Even so, some documented techniques in literature have been implemented to analyze the results obtained in 3D models (152) and those are also identified in Table 3.

From a different perspective, microfluidics is at the forefront of the improvement of micro- and nano-scale fabrication in the elaboration of several devices for cell analysis. Microfluidic devices are mostly made of poly(dimethylsiloxane) (PDMS) polymer. This polymer presents some characteristics that make it the chosen polymer: low toxicity, optical transparency, the possibility of a wide range of stiffness, chemical inertness, not get corroded by the aqueous solutions used in cell culture or affected by pressure or temperature, being the cells able to survive and grow. One of the major boundaries is the absorbance of small molecules not being appropriate for long-term drug studies (153, 154).

Newly, there have been created several microfluidic devices that allow their progression of them to mimic organs' functions. Organs-on-chips are considered microfluidic devices that mimic the function of organs. This skill allows the performance of new drug studies before testing them in animals or clinical studies. To engineer the microfluidic model, it is

essential to take into consideration: cancer progression stages, tumor microenvironment complexity, and the characteristics of the specific tissue and heterogeneous tumor that we want to study (77, 155).

Table 3 – Summary of 3D cell culture techniques.

Type of 3D technique	Technique name	Purpose	Properties	Endpoint assay	Advantages	Disadvantages	Ref.
Scaffold-based	Hydrogel-based support	- ECM mimic according to the nature, porosity, and viscoelastic properties - Loading with water and biological fluids - Osmoregulation	- Hydrogel composition: possibility to cells and molecules to move across the pores due to polymerization pattern of the hydrophilic polymers - Diversity of hydrogels: enable the refinement of the porosity, stiffness, and matrix degradation.	- Viability and cytotoxicity assay: Calcein AM/ ethidium homodimer). - Quantitative Real-time Polymerase Chain Reaction (qPCR). - Immunofluorescence (IF).	- Response to environmental stimuli using smart hydrogels such as variations in temperature, pH, ionic strength, radiation, electric field, and so on. - Matrigel is widely used in cancer research. - High cell viability level. - Possible to apply to co-culture assays. - High reproducibility	- Viscosity can be a challenge for the automation of liquid handling. - Temperature and environment control is required to the manipulation and polymerization. - Supplementary phases to disperse cells from the matrix can affect colorimetric and fluorimetric detection signals.	(99, 156-160)
	Paper-based support	- Paper created by micro-and nanocellulose fibers mimic the ECM. - Activate the cellular mechanotransduction through the formation of focal adhesion and intracellular cytoskeleton to improve cell contractility, spreading, migration and differentiation.	- Physical properties: topography, permeability, stiffness, and mechanical. - Structural properties: space for cell growth and allow the paper adjustment with biological ligands (e.g., peptides and nucleotides). - Possibility of modifying the chemical and physical characteristics of paper to improve mimic the cell environment pretended.		- Biocompatibility - Eco-friendly - Low cost - Large scale production	- Need additional ECM proteins. - Paper fibers are incomparable with body fibrils (1 mm in diameter instead of 500 nm)	(161-165)

Scaffold-free	Fiber-based support	- Mimic the scale and organization of collagen fibrils. - The adherence and elongation of cells along the fibers promotes the cell alignment and cultures directionality.	- High tuneable structural and strength properties	- High biological compatibility and reproducibility - Stability over the time and at a widespread temperatures range. - Topographic characteristics to cancer cells on 3D tumoroid development.	- High degradation capability of natural fibers compared with synthetic fibers.	(166-168)	
	3D bioprinting	- Mimic tissue models by the creation of a geometrically complex scaffold - Layer-by-layer cells deposition	- Enable the spacial control in 3D of several cells location, biomaterials, and bioactive factors.	- Viability and cytotoxicity assay: Calcein AM/ ethidium homodimer, - Cell counting kit-8 cell proliferation assay (CCK-8) - Alamar blue - Lactate dehydrogenase (LDH) - Matrix Metalloproteinases (MMP) Zymography assay kit. - Scanning electron microscopy (SEM) - Histology - Immunohistochemistry (IHC). - Immunofluorescence (IF) - qPCR	- High reproducibility, standardization, and assays accuracy - Compatibility to HTS and co-cultures approaches. - Allow the application of identical models for anticancer drugs screening.	- Need low viscosity bioinks for more effective outputs. - Emission of harmful chemicals during the bioprinting process. - Lack of droplet precision and size. - Difficult to adapt to HTS.	(169)
	Hanging drop microplates	- Create a 3D microenvironment role for developing tissues	- The accumulation of the cells at the tip of the drop and aggregates spontaneously by gravity, forming spheroids.	-Viability and cytotoxicity assays: CellTiter-Glo® 3D, Calcein AM/ ethidium homodimer. - Trypan blue. - Western Blot (WB). - Immunohistochemistry (IHC)	- Good replicabilities allow to be apply to a widespread range of uses.	- Difficulty in exchange the culture medium. - Cells within the spheroids are exposed to diffuse oxygen and nutrition source. - Difficult to control the necrotic damage to the	(151, 170-173)

			- Immunofluorescence (IF). - Light-sheet fluorescence microscopy (LS-FM).		cells at the spheroid core. - Difficult to control the spheroids size and composition.	
Magnetic levitation or bioprinting	- Two procedures with identical principles: employ a technique to form nanoparticle while inducing ECM synthesis. - Enables cell-cell interaction.	- Loading the cells with magnetic nanoparticles that by exposition to an outer magnetic field, causes cells aggregation into a spheroid. - If the magnet is positioned on top of the lid is magnetic levitation, if the magnet is underneath the plate is magnetic bioprinting.	- Viability and cytotoxicity assays: CellTiter-Glo® 3D, Calcein AM/ ethidium homodimer. - Trypan blue. - Reporter transgene. - Immunofluorescence (IF). - Light-sheet fluorescence microscopy (LS-FM). - Enzyme-linked immunosorbent assay (ELISA).	- Wide range of cell types could be used - Can synthesize ECM while forming. - A 3D cell culture can be formed without the consumption of an artificial protein substrate.	- Could require multiple steps - In assays to quantify enzymes, it predicts to be a long-time assay.	(159, 174-176)
Spheroid microplates with ultra-low attachment coating or forced floating	- The ultra-low attachment coating decreases the adherence of cells promoting spheroids formation. - Suppress cell-substrate interactions.	- Usually formed by polystyrene and treated with hydrophilic or hydrophobic coatings or with natural polymers such as agarose. - These plates wells have a well-defined shape (round bottom, V-shaped or conical), letting the location of a single spheroid. - Reliability in all of V-shaped bottomed wells in the formation of spheroid.	- Viability and cytotoxicity assays: CellTiter-Glo® 3D, Calcein AM/ ethidium homodimer; ViaLight™ Plus. - qPCR. - Western Blot (WB). - Immunohistochemistry (IHC). - Immunofluorescence (IF). - High-content imaging (HCI).	- Easy to use. - Compatibility. - In one study, spheroids formed by human breast cancer cells mimicked <i>in vivo</i> characteristics such as hypoxia, dormancy, anti-apoptotic effect, and drug resistance. - Needless the transference of spheroids to a new well-plate since the large volume of 96- or 384-well plates.	- In ultra-low attachment plates, some cancer cell lines do not form tight spheroids.	(136, 137, 157, 159, 173, 177-181)

The application of microfluidics presents a handful of advantages in oncologic research that includes fluid handling precision, 3D microenvironment with microvascular perfusion and diffusion, request of a small amount of sample, great cost-effectiveness, control of the microfluidic environment, and high-throughput (HT) multiplexing skills (182-185). Microfluidic devices have been applied to distinctive cancer types. The application of microfluidics in breast cancer has focused on fast and consistent specific biomarkers detection on fixed tissue samples and early detection of circulating cancer cells. Also, some of these have already been used to test new drugs for the treatment of breast cancer (186-188). In Figure 7, it is represented some microfluidic platforms developed to study mainly the extravasation of triple-negative breast cancer cells, the cytotoxic activity of anticancer drugs (in this specific case paclitaxel), and the migration and evasion of breast cancer cells (189-191).

3D cell cultures can replicate the three-dimensional complexity of a tumor, effectively mimicking the development of the 3D cell matrix. This enables the cell of the immune system to attack the tumor target cells, as *in vivo* models would. In literature, some studies indicated that 3D tumor cell cultures have greater resistance to cytotoxicity caused by the phenotypic variations that are not presented in 2D cancer cell cultures (192-194).

Dangles-Marie et al. discovered that in 3D lung carcinoma cell culture, there was a reduction in Heat Shock Protein-70 (Hsp70) and antigen presentation. This decrease was justified by the attacks of cytotoxic T lymphocytes that became less probable in the cells, proving that the 3D cell culture models resemble more accurately the *in vivo* tumoral microenvironment than the 2D cell culture models (195).

In another perspective, Müller and Kulms modeled a 3D spheroids cell culture as a melanoma tumor cell to study the mechanisms of molecular targets associated with immunotherapy treatments resistance nowadays (196). They used juvenile primary fibroblasts and keratinocytes and were able to grow melanoma spheroids *in vitro* to model tumor cells *in vivo*. These 3D melanoma spheroids were made via the handling drop method giving to the researchers the skills to analyze within the cell the drug resistance (197). Coupling these two methods makes it possible to produce tumor models on a patient-to-patient basis to assess treatments and develop a wide range of tumor models to study several non-patient-specific treatments.

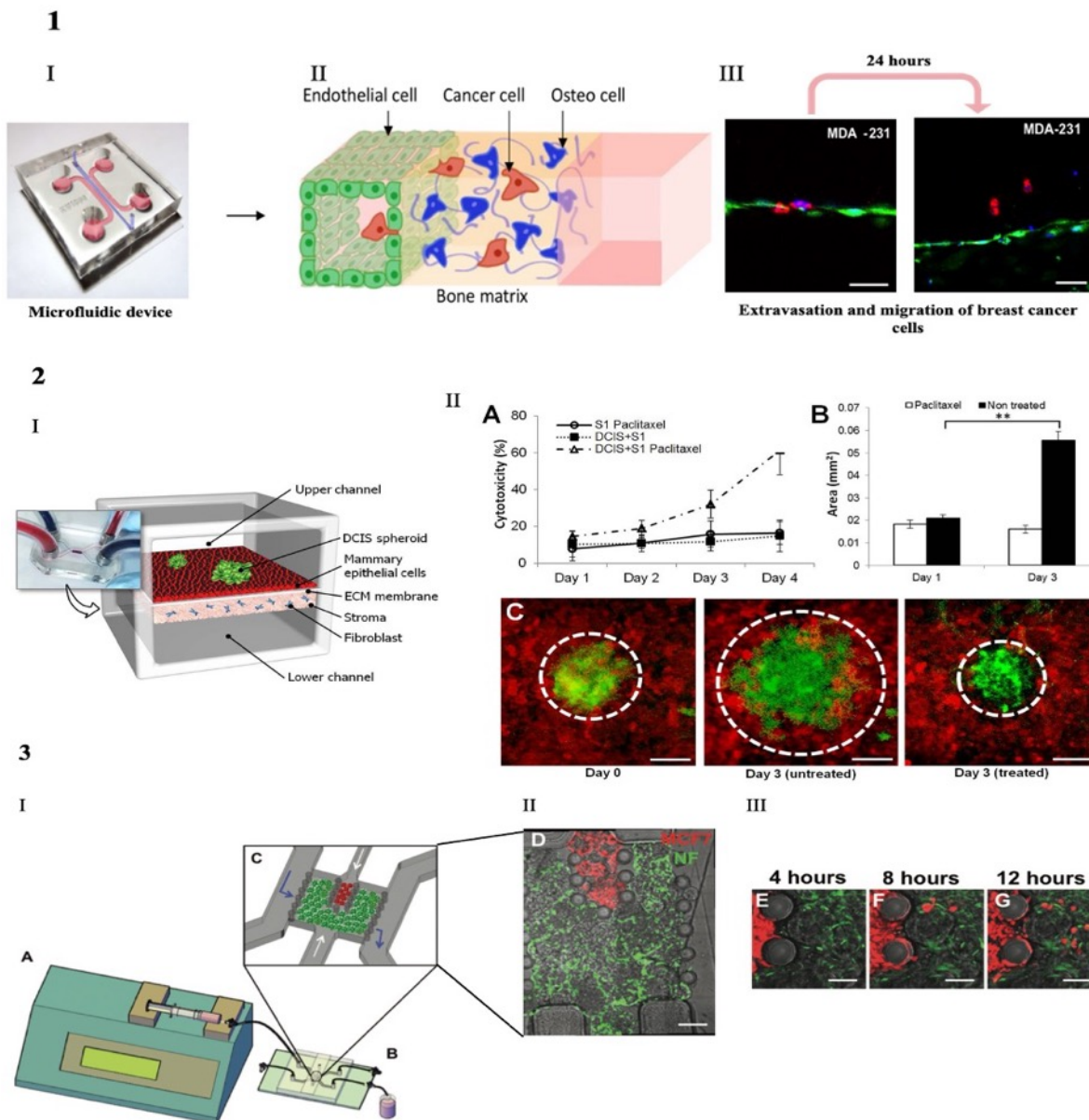


Figure 7 – Three examples of breast cancer microfluidic devices. **(1)** Characterization of a single-gel microfluidic device used to mimic extravasation of triple-negative breast cancer (TNBC); (I) and (II) schematic illustration of triculture microfluidic device to study the extravasation of cancer cell concerning a bone-like environment; (III) Representation of MDA-MB-231 extravasated into the extracellular matrix in acellular (left side) and BMi (right side). Figures adapted from (189). **(2)** A multi-layer perfusable human breast cancer-on-a-chip. (I) Schematic representation of compartmentalized DCIS spheroids constituted by a mammary epithelial on the top layer and fibroblasts in a collagen gel on the bottom layer. (II-A) Paclitaxel-treated cells showed percent cytotoxicity measured by the LDH production that increased over time in the DCIS-on-a-chip (open triangle) and presents a negligible cytotoxic effect on normal epithelial cells (open circle). The control group and the untreated DCIS-on-a-chip (close square) do not significantly differ. (II-B) Paclitaxel treatment was shown to avoid further DCIS spheroids growth in this model (** $p < 0.05$). (II-C) DCIS spheroids at day 0 and day 3 with and without paclitaxel. Figures adapted from (190). **(3)** Tumor-on-a-chip device for luminal-like breast cancer (I) Schematic representation of luminal-like model exploiting physical separation on-chip for tumor migration study with two compartments for tumor cells (red) and stromal cells. (II) Fluorescent image of the device with normal fibroblasts (NF) and MCF-7 cells. (III) Time sequence figures of MCF-7 cancer cell migration and invasion after 4, 8, and 12 hours of MCF-7 loading. Adapted from (191).

2D and 3D cell culture platforms could also be applied to study the diverse varieties of biomarkers in cancer cells. The production of biomarkers by tumors or the body could happen in reaction to the tumor's existence, being the enzymes considered one of the most currently used cancer biomarkers (198).

Biomarkers studies in breast cancer using further multifaceted 3D cell culture model systems have nonetheless to be performed, especially, for high-throughput drug screening (HTDS) methodologies with more elaborated breast cancer models since they have a huge potential in role performance in drug resistance and tumor dormancy (199, 200) can supply novel principals for the progress of biomarkers and treatment advances. Indeed, new methodologies and skills for 3D tissue culture systems that can be used in HTS assays have been developed to predict and describe genetic aberrations in cancer cells that are disease promoters. So, the 3D cell culture application is more significant when function mutations are lost and more accurately defines which pathways and mutations are found in the microenvironment signals given. This cannot be summarized in 2D models (201).

2.3. 2D and 3D cell culture assays to identify enzyme biomarkers

2D and 3D cell culture platforms have been used to analyze different parameters related for example metabolism and proliferation. Looking for some papers in the literature, it was proved that there are modifications in the whole cell as the cell adjusts from exponential growth to a dynamic equilibrium. Throughout this process, the cell creates a very well-organized structure allowing it to perform unconventional physiological functions representative of tissues.

So, comparing the 2D with 3D cell platforms, in 3D the enzymes involved in glucose, glycogen, and NADPH-pentose sugar metabolism are toughly augmented: glycolysis (2.92); glycogenesis (6.59); glycogenolysis (4.06) and pentose shunt (1.9). These increases provide the principal association between glycolysis and the TCA cycle. However, the enzyme's abundance in the ATP-producing TCA cycle does not increase. This could be justified by the high level of ATP in the cells. On the other hand, the proteins presented in the first three complexes of the TCA cycle are also unaffected. Nevertheless, the quantity of complex IV and both F0 and F1 subunits of ATP synthase decreased by nearly 20 % (202). This could be justified by the increase of ATP levels related to hepatocyte spheroids maturation (203) and cellular polarisation (204).

However, the great summary of these changes is that the cells grown in 3D regulate several protein levels to create an efficient structure and can preserve a physiological functioning comparable to what happened *in vivo* (205).

From a different point of view, the synthesis of fatty acids is more active in 3D cell platforms than in 2D, perhaps by the higher quantity of acetyl-CoA from glycolysis and NADPH from the pentose phosphate pathway and promoted by the response systems for the bile acids production. Moreover, acetyl-CoA acyltransferase is toughly augmented in peroxisomes, which are generally related to the synthesis of fatty acids, and reduced in mitochondria, which are routinely related to the oxidation of fatty acids (205).

Now, cholesterol and urea production are also related to changes in enzymes and in 3D cell platforms there is a lowly increase (1.37 and 1.50, respectively) of the enzymes involved in that, nonetheless, it should be enough to augment by factors of 10 and 3, cholesterol and urea, respectively to get physiologically corresponding *in vivo* levels (206). Tidwell and co-workers also found that the metabolism of glucose in 3D spheroids is meaningfully different from what is measured in 2D cultures, both in terms of glycolytic and oxidative phosphorylation metrics in colorectal cancer and pancreatic ductal adenocarcinoma. Also, they discovered that the 3D spheroids present higher levels of non-aerobic ATP in the lack of added glucose. In 3D spheroids, it was reported a decrease in the expression of mitochondrial transport protein (TOMM20). Still, the expression of the monocarboxylate transporter improved in 3 of the 4 spheroids models tested (HCT116, SW948, and Panc1). Considering all the results obtained by them, they concluded that in the study of cancer metabolism, it will be interesting to use more complex 3D *in vitro* cell models to increase the information within this field and achieve a better knowledge of the pertinency of the application in tumor biology. More important than that, is the possibility to apply these cell models to studies such as screening and retargeting of recognized metabolic drugs for cancer treatment to upgrade translation in tumor settings *in vivo* (207).

2.4. Biochemical assays and cell-based enzyme assays

Nowadays, enzyme activity direct detection *in vitro* is a noteworthy challenge.

Enzymology isolated cannot provide all the essential information needed in drug discovery early stage, so it is vital to combine enzymology with biophysical approaches and the analysis of protein structures for a consolidative pharmacological understanding of the thermodynamics and kinetics of enzyme catalysis and inhibition. Moreover, it is important to understand the comprehensive systematic classification in isolated enzyme assays coupled with cell-based assays, and finally, metabolic pathways and systematic data will continue to recognize enzymology as one of the most noteworthy quantitative subjects in drug discovery (208). This is also helpful since the most powerful compound in biochemical assays could not certainly be the best inhibitor in cell-based assays. One of

the advantages of testing the highlighted compounds in cell-based assays is understanding the target functionality and the compound cell permeability (209).

Conventional enzymology procedures used to study the enzyme activity as a function of time for well-known enzyme concentrations, typically use tissue homogenates or purified proteins, however, novel methodologies are desired to monitor enzymatic reactions in single cells. Unexpectedly, current research has headed to focus the scientific attention on biological heterogeneity and the technological capacity essential to study individual cells, leading to fast improvement in this field (210).

Biochemical assessment of individual cells is becoming more widespread as an appreciation of heterogeneity between apparently similar cells grows (211, 212). The heterogeneity of cells is acknowledged to cause a considerable effect at the organism level, so enzyme activity on single cell assays provides complete data about the variability in the individual biochemical stages that affect the results. From the point of view of the nucleic acid and protein levels, the differential enzyme activity between cells depends on transcriptional and translational regulation, epigenetic and post-translational modifications, protein degradation, and genetic mutations contributing to different enzyme activity among cells. All these processes impact enzyme activity firstly by the alteration of the chemical enzyme identity and the enzyme concentration and producing the observed biological output and the dissimilarity in every process leads to cellular heterogeneity (213).

In the cellular environment, the enzyme could act from different perspectives. Usually, it acts combined with further proteins and enzymes as part of multimeric complexes that are essential to direct the target activity temporally and positionally or can act at bigger signaling or metabolic cascade as a specific unit.

To be considered a model of single-cell enzyme assay, it should measure several enzyme activities with high spatiotemporal resolution and high throughput. For example, when we observe cellular responses that change in time, single-cell measurements elucidate the relative events of time. The growth of caspase-3 activity brings cell lysis since an apoptotic stimulus might be tolerated for the cells for hours before a sudden (e.g. tumor necrosis factor- α , staurosporine, or etoposide) (214). Collective of the same enzyme activity measurements demonstrate a gradual and steady growth unusual in the response of any cell. Likewise, cells treated with EGF present a quick increase in Src kinase activity. Concurrent measurements of membrane-type 1 matrix metalloprotease (MT1-MMP) in single cells exhibit that the activity of spike in Src kinase is associated with progressive growth in the activity of MT1-MMP (215). Ultimately, the analysis of single-cell may moreover identify enzyme activity heterogeneity due to variations in time in the cell cycle stage (216) or the cell age (217).

Moreover, enzymes often are present in several forms in cells rising from similar (splice variations) or diverse loci in the genome. On the other hand, in a reconstituted biochemical system, the enzyme is frequently isolated, without several or entirely of its natural binding partners, which can expressively affect the stability and activity of the enzyme *in vitro*. Furthermore, the enzyme expression and purification might be challenging in its natural form since its size and/or stability limitations in the lack of its associated proteins. To increase the production, activity, and stability of the protein, it is possible to truncate variants or tagged species to obtain the desired enzyme. An important warning about these mechanisms is that the more divergent from the native protein, the more probable the compound recognition that absences the physiologically significant mechanism and to miss compounds that work under physiological environments (218).

There are some reliable methods already reported for quantifying transcription such as northern blot and quantitative PCR and for quantifying translation, there was also reported western blot and enzyme-linked immunosorbent assay (219-221).

However, activity-based sensing (ABS) is considered a great methodology for the monitorization of post-translational since it gives straight enzyme activity read-out and adopts a biocompatible detection method (222). Initially, these probes were used in primary biochemical analyses, when the preparation of the substrate to measure wide-ranging enzymatic activities *in vitro*, e.g., esterases, lipases, phosphatases, and so on (223-225). In the last few years, the goal has been to provide biocompatible probes for the analysis of cellular and live animals. These enable the possibility of advanced approaches for biology discoveries and to be used as diagnostic tools. Looking at the probe application, generally, uses recognition and catalysis of the enzyme to reveal a measurable handle. The most used handles are fluorescent dyes; though, it is possible to use different detection methods such as chemiluminescence, photoacoustic spectroscopy, and magnetic resonance.

The design of the probe is crucial to provide the specificity and the signal development following the catalysis of the enzyme. The challenging parameter is maintaining the specificity since the goal is to transfer from the test tube assay into complex biological organisms, nevertheless, probes with suitable selectivity enable the straight progress of the activity of the enzyme *in vivo*. This property overcomes the drawbacks of the purification of the protein and the identification of the best assay conditions.

Considering that *in vivo* there are high sequences and structural and substrates resemblances, the skill to differentiate isoforms is demanding for distinguishing their function due to their different preferences in expression, activation, and substrate. Methods selective to the isoform are aimed to exploit the affinity, stability, and interactions

between the isoform and the substrate, and location to attain selective detection (Figure 8).

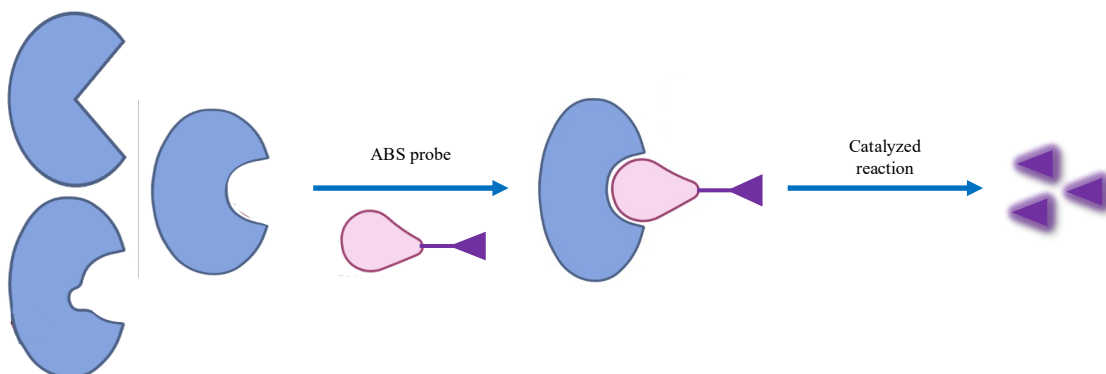


Figure 8 – ABS probe with activation of an isoform-selective in enzyme-catalyzed to obtain a fluorescent product.

In literature, some works reported the application of ABS probes to identify and study several enzymes within live cells.

CoxFluor was the first ABS methodology for COX-2 in live cell imaging with an intentional linkage among arachidonic acid and a fluorophore precursor. It was suggested as acting as a substrate for COX-2 activity to produce an intermediate such as PGG₂. CoxFluor exhibited a 40-fold activation after incubation with recombinant COX-2 although, under the same conditions, no cross-reactivity was observed against related enzymes or with several bioanalytes. Thus, CoxFluor has been validated as a system that can be used for the validation of inhibitors and COX-2 detection activity in live cells using confocal microscopy and flow cytometry. (226).

Kim et al. developed Probe 2, a two-photon compatible probe comprising a succinate ester to mimic 18 β -glycyrrhetic acid which is recognized as a selective inhibitor of carboxylesterase2. This alkyl ester was shown to be stable and optically confirmed the reduced activity in cancer compared to non-tumor tissues when 6-(benzo-[d]thiazol-2'-yl)-2-(N, N-dimethylamino)naphthalene (BTDAN) based probe was used (227).

On the other hand, some reports performed the biochemical assay for the corresponding enzyme that they want to study, and then they performed the cell-based enzyme assay. This is useful when the idea is a drug discovery assay, and we know that the enzymes are involved in the metabolism of compounds and this pathway could be overexpressed in a specific type of cancer.

Seegers et al. established an HTS assay for indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TPO) based on a new chemical probe, N-formylkynurenine (NFK) Green. The suitability of the HTS of the assay was established by the screening of an 87,000 compounds library in 384- and 1536-well formats, respectively. In addition, they

also used this assay to analyze the ability of cells to metabolize tryptophan and to assess the cellular potency of IDO1 and TDO inhibitors. Two biochemical assays were developed for each enzyme and then 44 cancer cell lines were screened for the enzyme's ability to catabolize tryptophan. TDO enzyme was only detected in colorectal carcinoma SW48 cells and just following an extensive incubation time (after 72 h). On the other hand, IDO1 was expressed in the MDA-MB-231 breast cancer cell line and three colon cancer cell lines (LS 174T, SW480, and HCT-116) after stimulation with IFN- γ . Moreover, in these three colon cancer cells, there was an increase in tryptophan conversion. Taking all of this into account, NFK Green proved to be an effective chemosensor to identify NFK production in cell assays for both enzymes (228).

From another perspective, Yanagi et al. developed a method to apply to HTS based on live cells targeting cellular metabolism according to the idea of coupled assays based on live cells (Figure 9).

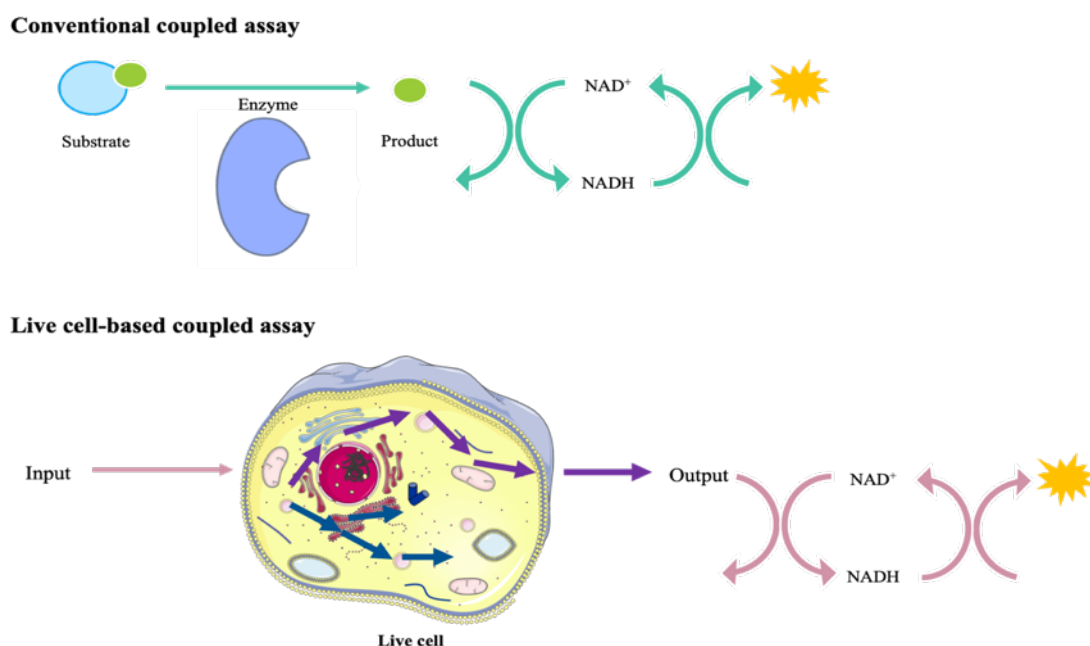


Figure 9 – Example of the concept of coupled assays based on live cells for metabolic pathway-oriented screening.

In this case, the authors tried to transform a coupled assay (generally performed *in vitro*) into a live-cell-based platform with two goals: to target the metabolic pathway of inner live cells instead of targeting specific enzymes and specify the metabolites that enter and leave the cells to monitor the pathway. They apply this methodology in two different domains: glycolysis inhibitors and amino acid metabolism. The selection of suitable input and output can extend this type of assay to several metabolic pathways in general. In the glycolysis inhibitors assay, they designed the platform using input and output D-glucose and L-lactate, respectively, to detect the extracellular L-lactate by fluorescence selectively.

Also, they used HL-60 leukemia cells in the experiment. With a 0.53 signal-to-noise ratio, they confirmed the ability of the assay to identify the influence of known inhibitors of glycolysis (2-deoxyglucose and iodoacetic acid). On the other hand, in the amino acids metabolism platform, they used A549 cells (lung adenocarcinoma) and normal human bronchovascular epithelial cells with several amino acids as inputs and considered the modifications in the subsequent extracellular outputs. As result, they were able to identify several candidates for inputs and outputs pairs. Specifically, comparing the tumor and the non-tumor cells, they noticed that the input of L-glutamine generated a significantly higher production of L-asparagine and L-serine in tumor cells. These could be justified by the enzymatic vias used to produce these outputs: L-asparagine was produced from L-glutamine via the tricarboxylic acid cycle, where L-glutamine is used as an energy source and L-serine was generated by the pathway that involves aminotransferases such as phosphoserine aminotransferases where L-glutamine is used as a co-substrate. For this second assay, they also developed a live cell-based coupled assay for these input/output pairs by producing H_2O_2 from L-asparagine and L-serine by suitable oxidoreductases and detecting them using aminophenyl sulfofluorescein (229).

3. Conclusions and perspectives

In this review, we have provided an extensive summary of the cancer microenvironment and the main hallmarks of cancer. We also elevated the main differences between 2D and 3D methodologies and we also analyse several of these methodologies to highlight their proposes, properties, advantages, and disadvantages. We know that in 3D cell cultures, spheroids resemble living tissue *in vivo* in terms of their structure and function properties compared to 2D planar cell cultures. However, the lack of typical approaches in 3D cell cultures makes 2D cell cultures more practice and comparable. So, in some cases, 2D cell cultures could be more feasible and meaningful, nevertheless, 3D cell cultures will provide more accurate results and can be joined with 2D analyses.

On the other hand, we also reviewed the changes in cellular metabolism in cancer cells, being this one considered a hallmark of cancer. The metabolic differences in cancer promote the anabolic metabolism, which can be responsible for the changes in the cancer phenotypes. The glycolytic pathway is the most critical phase to create the energy needed by cancer, responsible for enzymes' high involvement. We also realize that further investigation needs to be done to realize how each metabolite and its levels affect signaling and metabolism. From a drug discovery point of view, molecular enzymology has been an extensive influence on the interpretation of cancer processes and their variation. Consequently, mechanistic enzymology could be interesting to evaluate in the

initial drug discovery stages, detecting the exact target, the correct reagents, the perfect mechanism, and the accurate assay to obtain the right compound. Hence, this understanding will help in finding new therapeutic advances, based on better-quality compounds and a better chance of clinical success.

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Chapter III

The theoretical background of the techniques used

This chapter presents all the techniques developed and used to perform this work to identify the best synthesized ruthenium-based compounds or known anticancer compounds in enzymes overexpressed in cancer or in breast cancer cells.

1. Enzyme kinetic parameters

Enzymes are known as biocatalysts that accelerate biochemical reactions in living organisms. Enzyme activity can be measured by determining the conversion of substrate molecules into product molecules.

Leonor Michaelis and Maud Menten (1) were the researchers responsible for realizing that enzyme activity depends definitively on defined conditions such as concentration, temperature, pH, nature, and ions ionic strength. The comparison of enzyme assays is only possible when these conditions are strictly observed. Enzymes exhibited their highest activity with optimal conditions of the previous parameters. So, the optimization of these parameters is really important to perform the enzymatic assay in optimal conditions since the enzyme is presented in small amounts and their activity in cells can often be detected only under these conditions. In addition to these parameters, the addition of ions interactions (e.g., metal ions, hydrophobic substances), and detergents to the assay mixture can improve or limit the enzymatic reaction. For example, ATP reaction is dependent on Mg^{2+} as a counter ion. On the other hand, the presence of complexing compounds, e.g., EDTA or inorganic phosphates can become limiting (2).

Enzyme kinetics analyses the features that determine the velocity of enzyme-catalyzed reactions. The measurement of enzyme activity can be performed using discontinuous or continuous methods. Discontinuous methods comprise the substrate and enzyme mixing and measuring the product formed after an established time, being these methods very quick and easy to implement. Instead, in continuous enzyme assays, the study of enzyme-catalyzed reaction rate is done by mixing the enzyme with the substrate and determining the product formation by continuously measuring over time (3).

The Michaelis-Menten model is one of the best-known approaches to studying enzyme kinetics and predicts a hyperbolic relationship (Figure 1) between the substrate concentration and the initial velocity of the reaction. This model is characterized by the Michaelis-Menten derivation equation (Eq. 1):

$$v_0 = \frac{V_{max}[S]}{[S] + K_m}, \quad (\text{equation 1})$$

where v_0 is the initial velocity of the reaction, V_{max} is the maximum velocity achieved by the reaction at maximum concentrations of substrate, $[S]$ is the concentration of substrate, and K_m is the substrate concentration at which the reaction velocity is half of the V_{max} (Michaelis-Menten constant).

There are two important conditions that it is mandatory to assume to apply this equation: it is necessary to assume the v_0 when the product concentration will be negligibly small (i.e. $[S] \gg [P]$), overlooking the possible reversion of product to the substrate; and the substrate concentration should significantly exceed the enzyme concentration (i.e. $[S] \gg [E]$). According to these parameters, it is possible to assess the affinity of the substrate to the enzyme to study. If the K_m is low, this means that the enzyme needs a small substrate amount to become saturated. In this case, the V_{max} is achieved at low substrate concentrations, so the substrate has more affinity to the enzyme. On the other hand, when the K_m is high, this means that is necessary a higher substrate concentration achieves the V_{max} . So, in this case, this substrate does present a lower affinity to the enzyme.

While the Michaelis-Menten plot is useful to describe the enzymatic reaction rate, the Burk plot is a graphical representation useful to compare and identify the competition type of inhibitors with enzymes. To obtain the K_m and V_{max} from the Lineweaver-Burk plot, it is necessary to compare the Lineweaver-Burk equation to a linear regression analysis (Eq. 2):

$$\frac{1}{v_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}, \quad (\text{equation 2})$$

Where, compared with linear regression analysis: $\frac{1}{v_0}$ is as y ; $\frac{K_m}{V_{max}}$ is m , $[S]$ is x and $\frac{1}{V_{max}}$ is c .

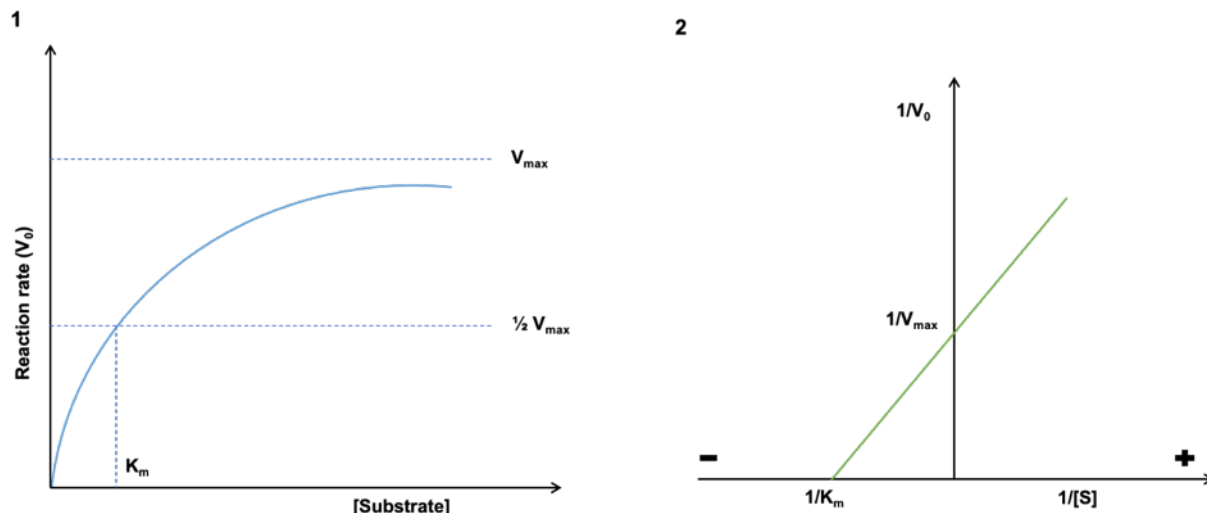


Figure 2 – The study of rates of catalysis reactions involving substrates and enzymes. **1.** Michaelis-Menten plot (enzyme + substrate to create a product); **2.** Lineweaver-Burk plot.

Another parameter that can interfere with the enzyme kinetics is the presence of inhibitors and activators. Considering the goal of this thesis, the next point is to talk about enzymatic inhibition, the effect of inhibitors on enzyme kinetics, and the type of inhibitors that exist.

1.1. Enzymatic inhibition

The enzymatic inhibition is determined by the calculation of the IC_{50} value, which corresponds to the inhibitor concentration needed to decrease the rate of an enzymatic reaction by 50%. The IC_{50} value varies depending on the enzyme, inhibitor, and substrate concentrations amongst other experimental conditions (4). The IC_{50} values in Chapter IV – works 1, 2, and 3 were obtained using GraphPad Prism software.

Enzyme inhibitors are substances that change the catalytic activity of the enzyme and therefore decrease the activity of the enzyme or even, stop the catalysis. These inhibitors mostly act in the active site of the enzyme, preventing the connection of the substrate with the enzyme. However, in some cases, the inhibitor can also be attached to the co-factor site. According to that, there are three enzyme inhibition types: competitive, uncompetitive, and non-competitive or mixed.

Looking at the Lineweaver-Burk plots for enzyme inhibition (Figure 2), competitive inhibition is characterized by an increase in K_m and an unaffected V_{max} . In this case, the inhibitor presents a very similar structure when compared with the substrate, so it binds to the active site and avoids binding to the substrate. In uncompetitive inhibition, there is a decrease in K_m and V_{max} , since the inhibitor binds to the complex formed between the enzyme and substrate. On the other hand, in non-competitive or mixed inhibition there is an unaffected K_m and a decrease in V_{max} . This type of inhibition is characterized by a

reduction of enzyme activity by the inhibitor binding at an allosteric site separated from the active site or as well as by the equally well binding to the substrate, avoiding the binding of substrate to the enzyme.

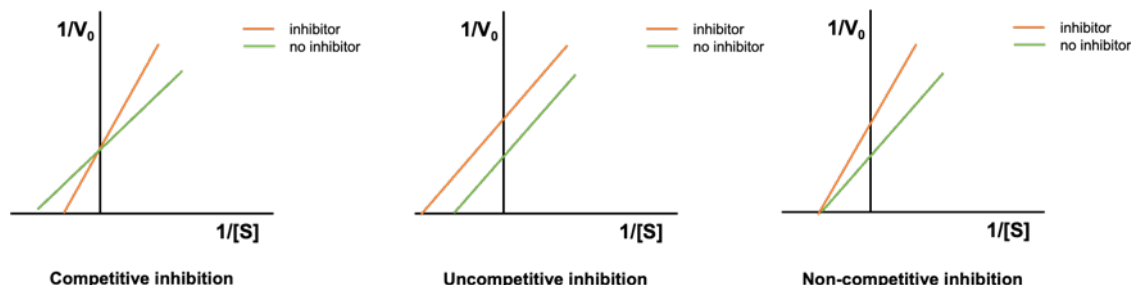


Figure 2 - Enzymatic inhibition types.

2. Flow apparatus and instrumentation

2.1. General features of flow techniques

Flow techniques are based on the introduction of an aliquot of sample into a flowing carrier, where subsequent contact with one or more reagents occurs. This contact will result in a reaction zone that will be moved to a detection system, giving rise to an analytical signal, whose intensity is related to the physical and chemical properties of the analyzed solution.

In 1975 appeared the first non-segmented and continuous flow technique named flow injection analysis (FIA). After some improvements and overcoming FIA limitations, sequential injection analysis (SIA) emerged in 1990 (5). SIA is based on the sequential aspiration of precise volumes of sample and reagents from a selection valve into a holding coil that, after reversing the flow direction, they are propelled into the reactor tube, being the product formed monitored by the change of a certain physical parameter (absorbance, fluorescence, chemiluminescence, electrode potential, etc.) at the detector.

In the SIA system, there is a reproducible overlapping of sample and reagent zones, through the combination of stop periods and changes in the flow direction (6).

Mixing the different sequentially aspirated zones depends on the operating parameters of the assembly itself and affects the extent of dispersion suffered by the reaction zone. In this type of system, dispersion is also related to the processes of transport by convection, which occurs with the flow, and molecular diffusion at the contact interfaces of adjacent zones, which affect their interpenetration (7).

Since the flow is mainly laminar, a segment introduced in the system will first be subject to a parabolic velocity profile, preferring the concentration gradients responsible for radial and axial diffusion. The inversion of the flow direction causes a decrease in segment axial

dispersion, creating a relatively symmetric concentration profile, which leads to an analytical signal of approximately Gaussian form, by passing through the detector (7).

In the SIA assembly, contrary to the FIA system, after reversing the flow direction, the overlapping of the sample zones and adjacent reagents remains, usually, only partial. This fact constitutes one of the limitations of this type of technique because there is a limitation as to the number of zones that can be mixed by flow inversion, in a tubular reactor despite the possibility of sequentially aspirating a greater number of reagents. Nevertheless, the SIA technique, due to its computer control and huge versatility, presents characteristics that enable the achievement of complex analytical procedures without physical reconfiguration of the assembly (8).

The fluids circulation inside the system starts from the central orifice of the valve, which is connected to the holding coil, and this same port can be connected to each of the lateral ports by the electrical rotation of the selection valve.

The SIA system assembly is controlled by a computer, so that the propulsion device and the selection valve work in a synchronized way, making it possible to define the volume, direction, and flow speed of the different solutions. The volumes are defined by the flow rate and aspiration or propulsion time, so the propulsion device must provide precise movements of starting, stopping, and reversing the flow direction, which guarantees that the aspiration and propulsion of the solutions are reproducible.

Also introduced by Ruzicka and been considered the third generation of flow injection analysis is the lab-on-valve (LOV) (Figure 3) (9). LOV works automatically with the sequential injection of micro volumes of aliquots, gases, and/or beads by stopping, reversing, and accelerating flow rates. This system presents the same components of SIA with two main improvements: the addition of a transparent or colored LOV platform that is attached to the selection valve, enabling the aspiration and propulsion of smaller volumes, and generating miniaturization of SIA (μ SI-LOV), and the possibility to integrate optical fibers to detect the final product inside the LOV platform.

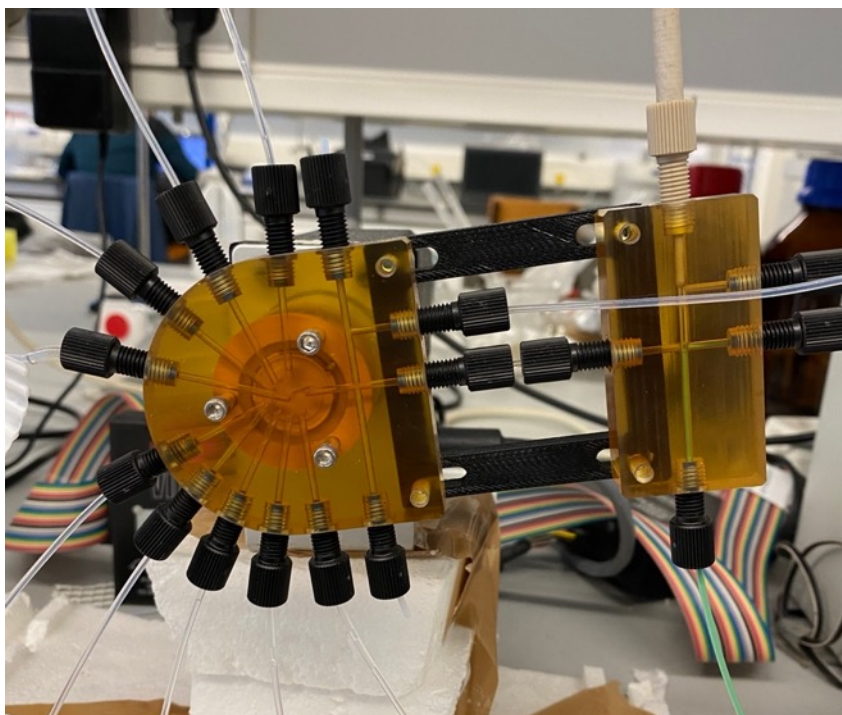


Figure 3 - Lab-on-valve setup.

2.2. Components of flow systems

The description of the main components and the configuration of the developed SIA and μ SI-LOV systems to perform the biochemical determinations under the aim of this thesis will be presented in Chapter IV – works 1, 2, and 3.

2.3. Optimization and analytical parameters of flow systems

The selection of the most suitable values for the variables under study in each flow system was carried out using the univariate method, which consists of varying the parameter to be studied within a certain interval, with the other parameters remaining fixed. The optimization of the different system parameters was carried out considering several factors that affect the performance of the analytical system, namely sensitivity, sampling rate, detection and quantification limits, precision, accuracy as well as sample and reagent consumption.

The range of concentrations in which there was a linear relationship between the concentration of the species and the mean value of the analytical signal from the detector was defined by plotting calibration curves, with solutions of increasing concentration of the species to be determined. The concentration of the samples was obtained by interpolation in the calibration curves, previously established, of the analytical signal obtained in the analysis of each sample (Chapter IV – works 1, 2, and 3).

The detection limit was defined by IUPAC as the minimum concentration value (C_{LD}) for which an analytical response (Y_{LD}) significantly different from the value of a blank or background noise can be obtained (14). The quantification limit (also known as determination limit) is defined as the minimum concentration value (C_{LQ}) for which a quantitative and precise response is obtained (Y_{LQ}). Thus, the limit of detection and the limit of quantification values was obtained by the application of the following equations, respectively (Eq. 3 and Eq. 4):

$$CLD = \frac{3 \times \delta^y/x}{b}, \quad (\text{equation 3})$$

$$CLQ = \frac{10 \times \delta^y/x}{b}, \quad (\text{equation 4})$$

Where δ^y/x is the residual standard deviation of the calibration curve, and b is the slope of the calibration curve.

2.4. Calculation of normalized activity

The evaluation of the inhibition curves was performed using GraphPad Prism 7 software using the equation defined by [Inhibitor] vs. normalized response—variable slope, where X values should be concentrations, not transformed to logarithms, and the Y values of the curve went from 100 down to 0. This model was referred to in Chapter IV- work 3, and corresponds to equation 5:

$$Y = \frac{100}{1 + \left(\frac{IC50}{X}\right)^{Hillslope}} \quad (\text{equation 5})$$

To obtain the normalized activities for each inhibitor concentration, we assume that 100% is the maximum activity of the reaction without the presence of an inhibitor. 100% is equal to 1, so each percentage of inhibition is converted into a normalized activity (a number between 0 and 1, being 0 and 1 equal to 0% and 100%, respectively).

3. High-Throughput screening assay

High-throughput screening (HTS) is a crucial method used in drug discovery to promptly assess thousands to millions of compounds from chemical libraries to evaluate the pharmacological and biological activity at the molecular, pathway, cellular, and organism levels to identify hits that may become clues to medicinal chemistry. In addition to compounds, chemical combinations, natural extracts, oligonucleotides, and antibodies can also be screened. These substances are usually assayed against well-characterized targets to elucidate the specific mechanism of action, e.g., enzymes inhibition or activation, radioligands binding inhibition to receptors, modulation of biomacromolecules interaction such as proteins and nucleic acids, and intracellular signal transduction and responses interferences (10). To perform these experiments, it is critical to use simple and automation-compatible assay designs, robotic-assisted sample handling, and automated data processing analysis (11). These analytical assays are usually performed in 96-, 384-, and 1536- well plate formats.

In Chapter IV – work 5, it was performed a 384-well plate assay to identify enzymatic GST-P1 inhibitors. However, during the optimization of this assay, some changes had to be made in order to achieve an effective HTS assay.

First, it was used the dispenser BioTek MultiFlo (Agilent®) (Figure 4) with two peristaltic pumps and uses cassettes. In this work, one cassette was used for the enzyme and the other one for the substrate. However, this dispenser was given up since there is no reaction due to the absorption of the enzyme or substrate into the silicon tubes. So, to solve this problem, a new dispenser was tested.

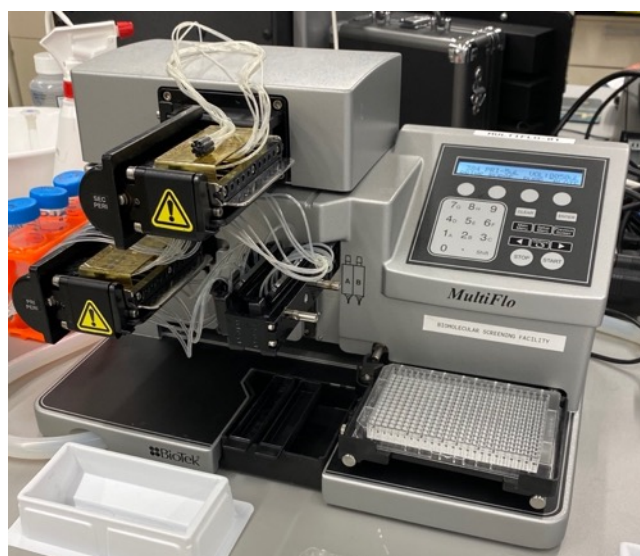


Figure 4 - BioTek MultiFlo setup.

The Gyger CERTUS FLEX (Figure 5) was the dispenser used to dispense the enzyme and the substrate for the final validation of the assay (Z'-factor) and for libraries screening. This dispenser has PTFE tubes that are more compatible with the substrate and the enzyme solutions.



Figure 5 - Gyger CERTUS FLEX setup.

3.1. Signal-to-noise and Z'-factor calculation

In the HTS systems, generally, each compound is tested in duplicate. Unsurprisingly, all the determinations from an assay contain a variability degree due to the perturbation introduced by instrumental and human random inaccuracies. Naturally, the smaller the measurement variance, the greater the confidence that an identified hit is reconfirmed after retesting. An HTS considered of high quality should be efficient to identify those few compounds with a high level of confidence that show significant biological activity. Hits are recognised as the active compounds that are responsible for the activation or inhibition of the assay signal beyond a defined threshold value from the sample mean signal. The signal-to-noise ratio (S/N) is one of the expressions that indicate the quality of an HTS assay, and this value is obtained by the application of the following equation (Eq. 6):

$$S/N = \frac{\text{mean signal} - \text{mean background}}{\text{standard deviation of background}} \quad (\text{equation 6})$$

In a typical HTS assay validation, the Z'-factor is a parameter to assure the quality of the assay itself, without the intervention of compounds, being this parameter used for quality

evaluation in the development and optimization of the assay. This parameter considers the assay signal dynamic range and the data variation associated with the sample and reference control measurements. So, the Z'-factor can hit identification for each given assay at the defined screening conditions. The separation band, also called the signal window, is between three times the standard deviation of positive control (with reference inhibitor) and three times the standard deviation of negative control (without reference inhibitor), and hits are shifted away (usually by a defined standard deviation (SD) from the mean of the general sample population. The calculation of the Z'-factor is based on the following equation (Eq.7) (12):

$$Z = 1 - \frac{3SD \text{ of sample} + 3SD \text{ of control}}{|mean \text{ of sample} - mean \text{ of control}|} \quad (\text{equation 7})$$

Where SD is the standard deviation.

The control data for inhibition assays is the negative control (lower signal) data.

For every assay, the Z'-factor value is just significant within the range of $-1 < Z \leq 1$. To approve the optimization and perform the compounds screening, the Z'-factor should be $1 > Z \geq 0.5$, where the separation band is large and is considered an excellent screening assay. When the Z'-factor is $0 > Z > 0.5$, the separation band is small and is considered a double assay (12).

4. *In vitro* cell viability and cytotoxicity assays

Proliferation and viability cell rates are worthy signs of cell health. Cell health and metabolism can be affected by physical and chemical factors through several mechanisms such as the destruction of the cell membrane, protein synthesis inhibition, irreversibility of receptor binding, enzymatic reactions, and so on.

To evaluate the cytotoxicity of drugs and chemicals is commonly used *in vitro* cell viability and cytotoxicity with cultured cells. These assays are extensively applied in oncology research to assess compound cytotoxicity and tumor cell growth inhibition during drug development in the first stages of pre-clinical trials since they do not require the use of animals and are fast and low-cost. However, these assays are not technically advanced enough to substitute animal tests (2).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 6) is one of the most extensively used colorimetric assays to evaluate cytotoxicity or cell viability (13). This assay establishes cell viability over the mitochondrial function determination of cells by assessing the activity of mitochondrial enzymes such as succinate dehydrogenase (14). After the application of MTT in cell culture, MTT is reduced to purple

formazan by NADPH. This product is quantified by absorbance measurements at 570 nm. This method presents several advantages when compared with others. It is easy to perform, safe, presents high reproducibility, and can be applied to determine both viability and cytotoxicity cell experiments. (14, 15) Nevertheless, some drawbacks can also be addressed to this technique. As formazan crystals are insoluble in water, and it forms purple needle-shaped crystals in the cells, it is required the use of organic solvents e.g., dimethylsulfoxide (DMSO) or isopropanol to solubilize the crystals. Furthermore, due to the cytotoxicity of MTT formazan, it is difficult to remove the cell culture medium from the plate wells since floating cells with MTT formazan needles, give significant well-to-well error. To avoid this problem and to reduce the false positives or false negatives caused by the background interference by the particle's inclusion, it is mandatory to substrate the background absorbance of the cell in the presence of the particles but without the assay reagents. (14, 16).

The description of *in vitro* cytotoxicity methodology to perform the inhibitory assays in breast cancer cells under the aim of this thesis will be presented in Chapter IV – works 4 and 5.

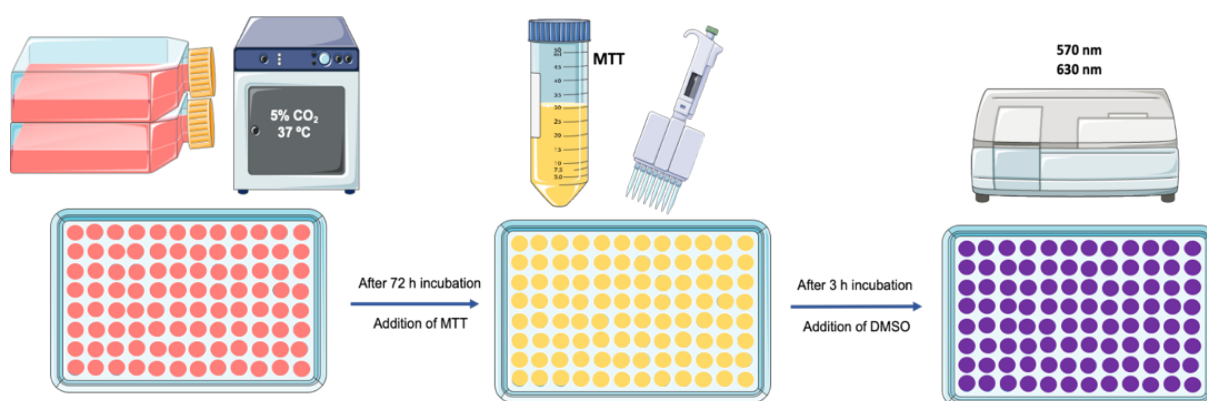


Figure 6 - *in vitro* cytotoxicity methodology to perform the inhibitory assays.

5. Apoptosis analysis

Apoptosis is a programmed cell death mode extensively used in multicellular organisms, enabling the remotion of dying cells without releasing cell contents, thereby minimizing the impact on surrounding cells (17). Basically, cells undergoing apoptosis present the integrity of the plasma membrane until almost the end of this process, however, some specific changes happen such as nuclear condensation and fragmentation, cell rounding and retraction, and plasma membrane blebbing. These changes cause the activation of the cysteine aspartyl-specific proteases named caspases (17). After apoptosis activation, several protein substrates for proteolysis are targeted by these enzymes (17, 18). Finally,

apoptotic cell death results in controlled cell breakdown and apoptotic forms development. Significantly, phagocytes can differentiate between healthy and apoptotic cells, and then quickly engulf the apoptotic cells to fastly remove them and prevent the possibility of involuntary leakage of cellular contents (19). This recognition can be justified by the plasma membrane changes, since phosphatidylserine (PS), a phospholipid usually located in the inner leaflet of the plasma membrane is transported to the outer plasma membrane leaflet, after receiving pro-apoptotic signals (19, 20) (Figure 7). This mechanism is still unknown (20-23), nevertheless, it is thought that the ATP-binding cassette transporter (ABC1) can be involved in this mechanism in mammals (24).

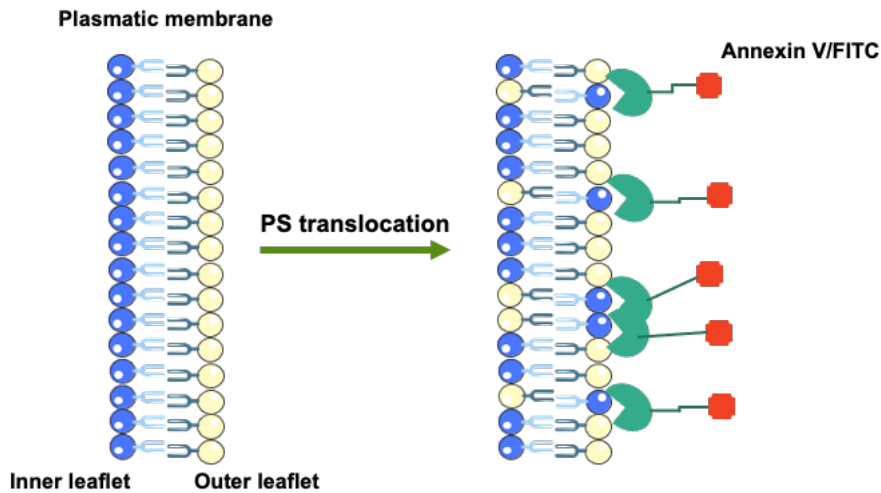


Figure 7 - Membrane changes in apoptotic cells.

A phospholipid-binding annexin family, Annexin V, efficiently binds PS being a selective probe to detect and quantify apoptotic cells (25). According to this, the Annexin V binding assay has become a recognized procedure for the identification and quantification of apoptotic cells. This method presents several advantages including being a fast, precise, unbiased, and reliable technique that involves slight sample preparation. The binding of Annexin V with plasma membrane PS can be assessed by microscopy or flow cytometry analysis. Although it is not possible to identify other characteristics of apoptotic cells, flow cytometry analysis allows a fast and highly quantitative technique for evaluating apoptosis. Also, in combination with 7-amino-actinomycin D (7-AAD), it is possible to quickly differentiate apoptotic and necrotic cells (20) (Figure 8).

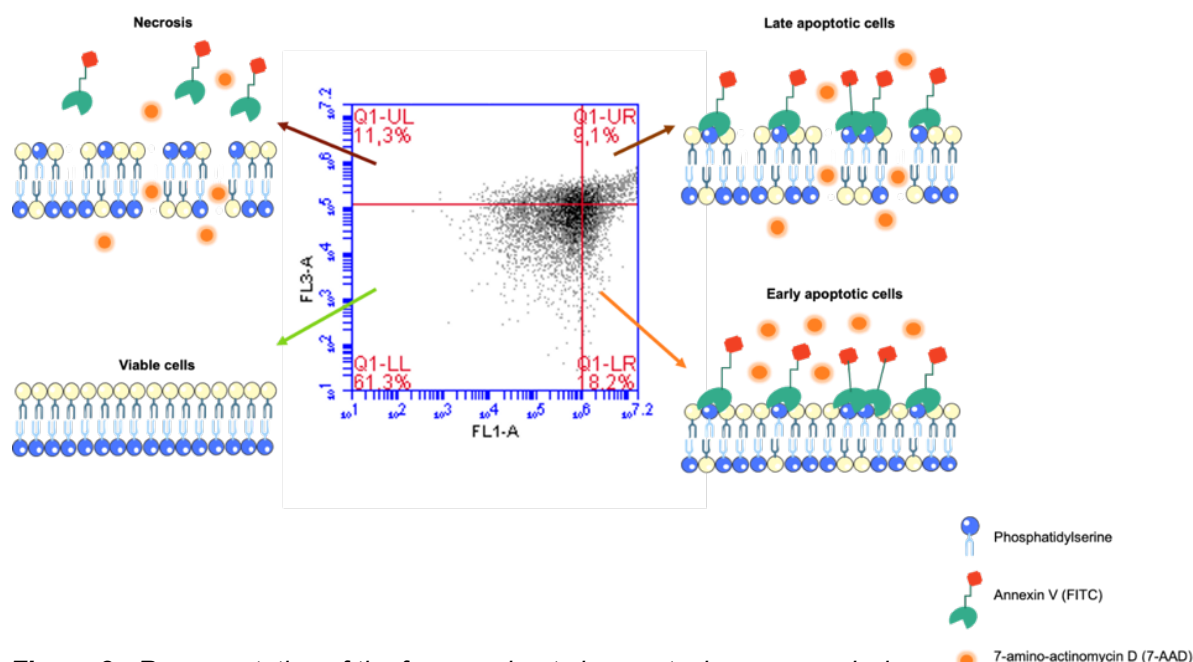


Figure 8 - Representation of the four quadrants in apoptosis assay analysis.

The description of *in vitro* apoptosis assay to evaluate the apoptosis rate of ruthenium-based compounds in breast cancer cells under the aim of this thesis will be presented in Chapter IV – work 4.

6. Cell uptake assay

Cellular uptake is an important assay to study the bioavailability of drugs. The internalization and the accumulation of metal-based drugs into cancer cells are essential to identify the therapeutic effect against tumors. The exchange and signaling of substances by cells are strictly regulated by the plasma membrane, a thin layer with 4-10 nm. The plasma membrane presents an outer boundary responsible for the protection of cells from the surrounding environment and stabilizing the intracellular environment, enabling several biochemical reactions to happen safely. Even with all these benefits, the plasma membrane is considered the first barrier for drugs to get into cells, limiting the internalization of several potential drugs. There are three ways to internalize substances into cells: passive or facilitated diffusion, and active transport (Figure 9) (26).

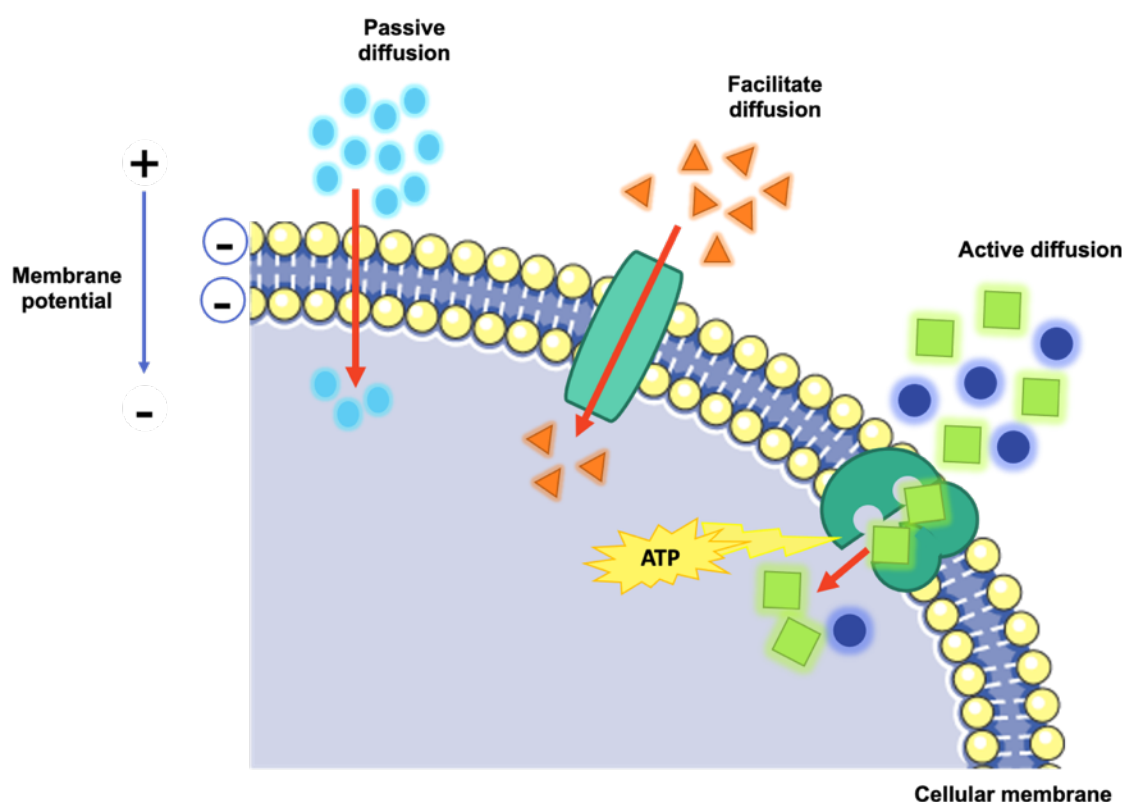


Figure 9 - Internalization of types of substances into cells.

Comparing breast cancer cell lines used in this thesis, MCF-7, and MDA-MB-231, these two cell lines present different cell membrane components or the same component but in different proportions. In terms of neutral glycolipid, there are more twice in MCF-7 than in MDA-MB-231. The main neutral glycolipids in MDA-MB-231 and MCF-7 cells were identified as Gal α 1-4Gal β 1-4Glc β 1-1'Cer (CTH or Gb3Cer) and globoside, however, MCF-7 also presents two other glycolipids which were identified as galactosylgloboside and fucosylgalactosylgloboside. Gangliosides are sialic acid-containing glycosphingolipids localized in the outer leaflets of plasma membranes. These glycosphingolipids are four-fold higher in MDA-MB-231 cells compared to MCF-7 cells, however, the most abundant gangliosides in both cell lines are GM3 (monosialodihexosylganglioside), GM2 (monosialoganglioside), GM1 (monosialotetrahexosylganglioside), and GD1a.

7. Gel electrophoresis

Gel electrophoresis is an analytical separation method for the analysis of biomolecules including nucleic acids (DNA and RNA) and proteins. Their fragments are separated based on their size and charge. These biomolecules are separated by the application of an electric field to move negatively charged molecules through a gel matrix (usually

agarose or acrylamide) to the cathode. According to the size of the molecules, the smaller ones move faster and migrate further than the longer molecules since the small molecules migrate more straightforwardly through the gel pores, so their migration rate is proportional to the logarithm of the length of the DNA fragment (27).

In Chapter IV – work 4, it was applied an agarose gel electrophoresis technique to assess the DNA fragments separation in contact with the ruthenium-based compounds. In this work, it was used an agarose gel matrix. Agarose forms a gel matrix by hydrogen bonding when heated in a buffer (Tris-HCl EDTA buffer) and allowed to cool. Agarose gels present several advantages, including being simple and fast to prepare, so they are considered the most popular gel matrix used for separation, it is possible to recover the samples by melting the gel, digesting them with enzyme agarose, or treating them with chaotropic salts. Nevertheless, there are also some disadvantages such as fuzzy and poor separations of low molecular weight bands due to the relatively low-resolving agarose powder, and the high costs of agarose (28, 29). The agarose percentage used depends on the biomolecules fragments size to be resolved. The agarose concentration is referred to as the agarose percentage to the buffer volume (w/v), being usually prepared in the range of 0.2% to 3% (30).

The loading buffer is added to the DNA fragment that will be electrophoresed. This buffer includes glycerol or sucrose to rise the DNA solutions density, otherwise, the samples would dissolve in running buffer and not sink into the gel matrix. The gel loading buffer also includes dyes to observe the samples during gel loading and electrophoresis, such as bromophenol blue or xylene cyanol (28). The 6X loading dye concentrations and components commonly used are 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol; or 0.25% bromophenol blue, 50 mM EDTA, and 0.4% sucrose.

Considering the voltage applied, the higher voltage is applied, the faster biomolecules migrate. However, high voltage causes a remarkable increase in buffer temperature. This effect can lead to gel melting, smiling DNA bands, and decreasing DNA bands resolution. So, it is greatly suggested not to exceed 5-8V/cm and 75 milliamperes (mA) for standard-size gels or 100 mA for mini gels (27).

After the electrophoresis, several methods can be applied to visualize the separated bands by the human eye. The most used method is ethidium bromide staining (EBS). In this case, low concentrations of the intercalating fluorescent ethidium bromide (EB) dye is stained into the gel for some time (usually 30 minutes), and then the gel is observed under ultraviolet (UV) light or blue light. Instantaneous photos are taken from the gels in a dark room for a permanent record. It is extremely important to a reminder that EB is a potent mutagen and moderately toxic after acute exposure, so this experiment should be performed with significant carefulness (27).

All these steps are summarized in Figure 10.

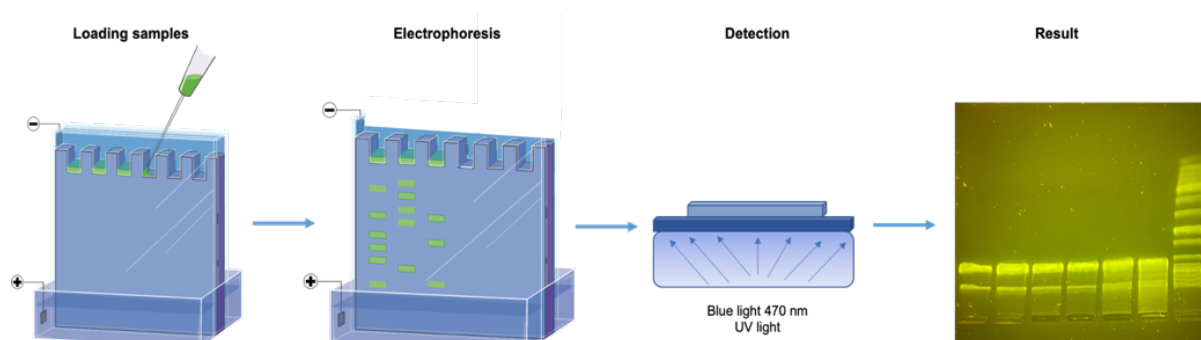


Figure 10 - Gel electrophoresis setup and analysis.

8. Circular dichroism

Circular dichroism (CD) is an absorption spectroscopy methodology that uses polarized light to assess differential absorption of left and right circularly polarized light. UV-vis CD is widely used to study the secondary structure and conformational properties of chiral biomolecules including DNA, RNA, amino acids, and proteins (31). According to DNA-binding agents, there are some that are achiral and optically inactive, nevertheless, by noticing the changes at certain wavelengths in the UV spectrum, it is possible to detect drug-DNA interactions with extreme sensitivity. The CD profile of right-handed B-DNA displays two positive bands at 220 nm and 268 nm; and two negative bands at 210 nm and 246 nm elliptical signals. The CD is usually determined to identify the preferred binding mode, such as minor groove, major groove, and intercalation (Figure 11), compared with untreated DNA (32).

This technique presents several advantages when compared with NMR and X-ray crystallography since it is an inexpensive technique that requires small sample amounts allowing a fast and highly sensitive analysis (33).

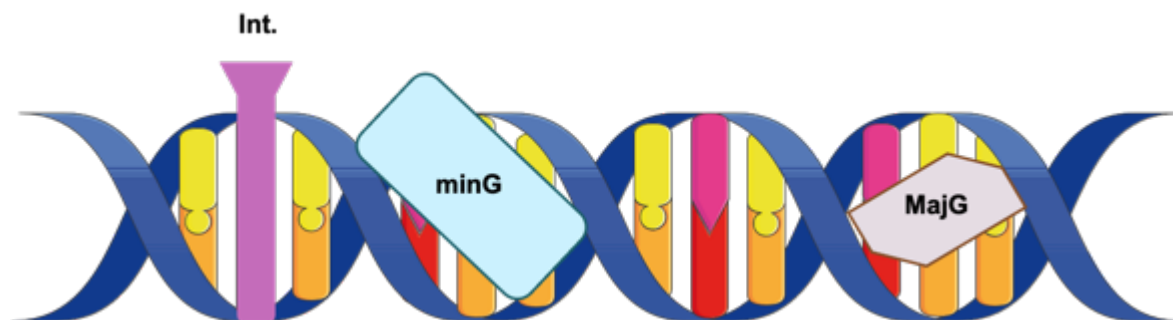


Figure 11 - Different DNA binding modes. Int - intercalation; MinG - minor groove; MajG - major groove.

To study the different binding modes, it is possible to study DNA probes with characteristics of each binding mode: 4',6-diamidino-2-phenylindole (DAPI), methyl green (MG), and propidium iodide for, minor groove, major groove, and intercalation, respectively, being the changes in the respective bands reported in Table 1, where it is possible to observe the differences in wavelength and energy shifts for each band.

Table 1 - Binding modes changes at different wavelengths.

Probe	210 nm		220 nm		246 nm		268–280 nm	
			Hydrogen bonding		Helicity		Base-pair stacking interactions	
DAPI Minor groove	Hypochromic, $\Delta \downarrow$	Red shift, $\lambda \uparrow$	Hyperchromic, $\Delta \uparrow$	Blue shift, $\lambda \downarrow$	Unchanged	Unchanged	268 nm – Hyperchromic, $\Delta \uparrow$ 280 nm – Hypochromic, $\Delta \downarrow$	Unchanged
MG Major groove	Hypochromic, $\Delta \downarrow$	Blue shift, $\lambda \downarrow$	Hyperchromic, $\Delta \uparrow$	Blue shift, $\lambda \downarrow$	Hypochromic, $\Delta \downarrow$	Unchanged	Unchanged	Unchanged
PI Intercalation			Unchanged	Blue shift, $\lambda \downarrow$	Hyperchromic, $\Delta \uparrow$	Unchanged	Hyperchromic, $\Delta \uparrow$	Unchanged

According to this information, in Chapter IV – work 4, this technique was applied to identify the DNA binding mode of the ruthenium compound (**11Cl**).

9. Western blot

Western blot, also called immunoblotting, is a commonly used method in molecular biology to identify specific proteins in a complex protein mixture extracted from cells using specific antibodies. Before starting this experiment, it is mandatory a suitable lysis of the cells to extract the biggest quantity of interest protein needed to identify and quantify it. After that, the proteins are separated in a denaturing polyacrylamide gel using electrophoretic equipment. The proteins are separated under an electrical field based on the molecular weight as distinct bands in the gel. Then, the proteins are transferred onto polyvinylidene fluoride (PVDF) or nitrocellulose membrane to continue the experiment and analysis. Next, the membrane with the bound proteins is treated with a blocking solution for decreasing nonspecific signals. The membrane is subsequently washed with washing buffer followed by incubation with the primary antibody specific for the interested protein. The primary antibody could be straightforwardly conjugated with horseradish peroxidase (HRP) or other reported modules for detection (34). This process is exemplified in Figure

12. Nevertheless, most protocols use a secondary HRP antibody specific to the primary antibody and are visualized using a chemiluminescence imaging platform.

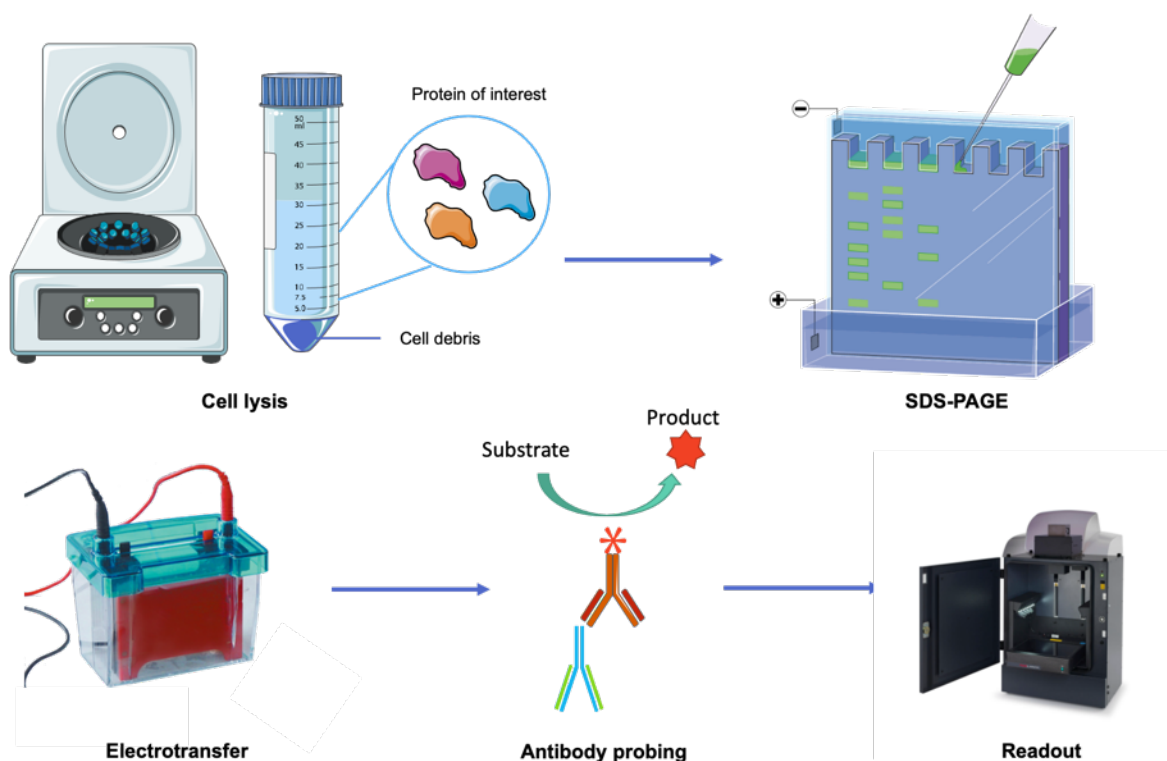


Figure 12 - Western blot setup.

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Chapter IV

Progress beyond the state of art

This chapter presents the articles that this work originated:

1 – Automatic evaluation of cyclooxygenase 2 inhibition induced by metal-based anticancer compounds

2 – Assessment of metal-based dihydrofolate reductase inhibitors on a novel mesofluidic platform

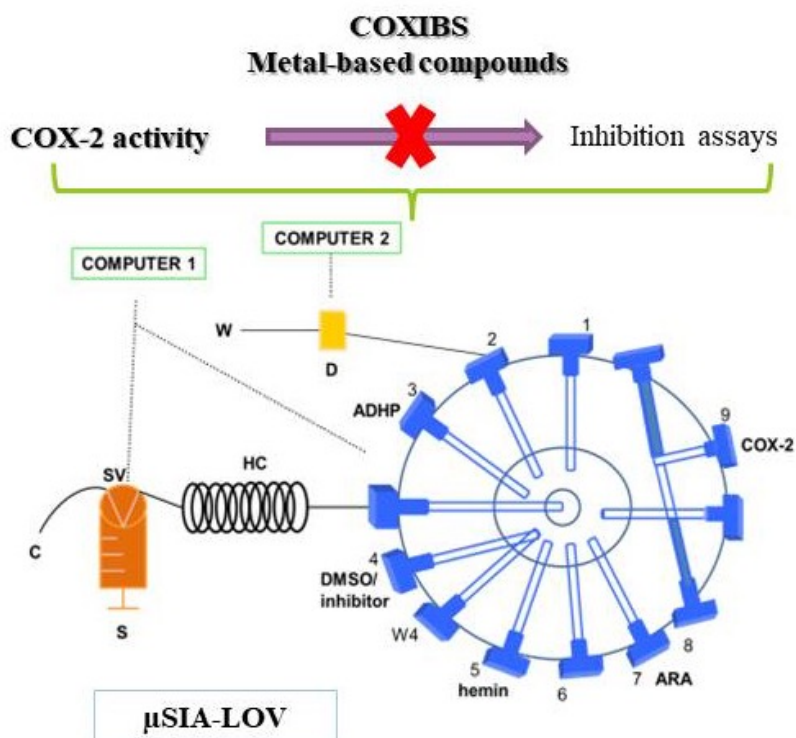
3 – Automated approach for the evaluation of glutathione-S-transferase P1-1 inhibition by organometallic anticancer compounds

4 – Tailoring the cytotoxicity of ruthenium(II) arene compounds for enhanced activity against breast cancers

5 – Identification of glutathione transferase (GST P1) inhibitors via a high-throughput screening assay and implications as new treatment options for breast cancers

1 – Automatic evaluation of cyclooxygenase 2 inhibition induced by metal-based anticancer compounds

In this work, I developed and optimized the μ SIA-LOV technique, to test the metal-based compounds, and calculate the IC₅₀ values for each compound. Also, I validated the μ SIA-LOV by the application of the batch method and performed the statistical data analysis. In the end, I wrote the original draft of the manuscript for later publication.





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Automatic evaluation of cyclooxygenase 2 inhibition induced by metal-based anticancer compounds

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ABSTRACT

An automatic methodology based on micro sequential injection analysis coupled to a lab-on-valve system (termed μ SIA-LOV) was developed and used to determine the ability of metal-based anticancer compounds to inhibit cyclooxygenase 2 (COX-2) activity. COX-2 may be involved in pathogenesis of cancer and it is overexpressed in several types of solid tumors. Since platinum-based compounds are extensively used in the treatment of cancer, and ruthenium compounds are considered as promising candidates for next generation of non-targeted anticancer drugs, it is interesting to establish whether COX-2 inhibition is relevant to their mode of action. The μ SIA-LOV system was optimized and the IC_{50} values of each compound were calculated. All the results present RSD values less than 2.5%. IC_{50} values of $9.7 \pm 0.6 \mu\text{M}$ to $207 \pm 3 \mu\text{M}$ were obtained, with the most active inhibitor for COX-2 being rofecoxib with the metal compounds exhibiting IC_{50} values in the range 13.7 ± 1.6 to 207 ± 3 . The results obtained in this work provide significant information about the mechanism of the studied compounds, mostly ruthenium-based compounds, and the role of COX-2 in their mode of action. Moreover, this work confirmed the potential of the μ SIA-LOV system as a simple, versatile, robust, and rapid analytical tool for automating the determination of IC_{50} values of metal-based compounds.

1. Introduction

Cancer is a deadly global disease and the search for more effective chemotherapies remains important [1]. Some of typical anticancer drugs present serious side effects which disturbs the patient's life quality. Consequently, new treatment strategies such as new drug families have been introduced with the aim of reducing or even eradicate these side effects. Traditional cancer drugs damage DNA, but since cancer cells overexpress many enzymes and proteins [2–4], targeted drugs that selectively inhibit these enzymes have grown in importance in recent years. In the clinic, drugs that damage DNA are frequently used in combination with a targeted drug that inhibits a specific enzyme overexpressed in the cancer being treated [5]. The primary mode of action of platinum-based drugs involves DNA binding [6], but additional non-targeted enzyme binding also takes place which can both enhance the efficacy of the compound [7], or lead to unwanted side-effects [8].

COX-2 is commonly overexpressed in several types of solid tumors such as breast, colon, cervical and prostate [9–12] and is considerably less abundant in healthy cells and normal tissues [10,13]. In this way, COX-2 is considered an attractive and targetable enzyme to differentiate

cancer from normal cells.

Selective COX-2 inhibitors are good examples as they could be used as an adjuvant therapy with higher efficacy and lower side effects than current drugs [14]. Rofecoxib is one of the most selective inhibitors of COX-2 [15]. Celecoxib-like structures (COXIBS) have already demonstrated efficacy in tumor growth inhibition. Consequently, they are considered to be used in chemoprevention and in cancer therapy, although there is evidence of an increase in cardiovascular risk.

However, there are problems associated with platinum-based compounds, such as cisplatin and oxaliplatin including their strong side effects and incidents of drug resistance [16], they are still used in anticancer chemotherapy treatments. Consequently, significant research has been devoted to the development of alternative metal-based anticancer drugs that overcome the limitations of platinum compounds [17,18]. Ruthenium compounds are considered as promising candidates for the next generation of metal-based anticancer drugs [17].

Previously, it has been shown that conjugation of organic COX inhibitors to platinum and ruthenium complexes leads to compounds that can potentially overcome drug resistance [19,20]. However, it is not clear whether metal complexes exhibit residual COX-2 inhibition in their

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own right.

Therefore, we decided to evaluate metal-based anticancer drugs activity in COX-2 enzyme.

Currently, there are commercial assays for the evaluation of COX-2 activity based on spectrophotometric and fluorometric detection methods that are routinely employed [4,21–23].

In this work, we decided for the automation of the COX-2 assay to expedite the screening of COX-2 inhibition by metal-based compounds. For that, we resort to a microflow-based on sequential injection analysis methodology usually referred to as μ SIA Lab-on-valve (μ SIA-LOV) and optimized the fluorometric COX-2 enzyme reaction. The automatic, rapid and low volume features of the μ SIA-LOV system make it more superior than the tedious manual process of enzyme kinetic study which is prone to error and imprecision. The μ SIA-LOV system is a useful alternative system that combines the benefits of automation, rapidity, time and volume precision and the ability to perform real time initial rate studies [24]. LOV has been profitably applied in enzyme activity assays and proved to be a suitable choice due to its ability to guarantee precisely the reaction conditions through computer control of analytical parameters.

Briefly μ SIA-LOV is a mesofluidic platform that belongs to the third generation of flow analysis that involves the sequential aspiration of well-defined reagent and sample volumes to a holding coil by reversed flow and the reaction product is propelled into the detector. Sample processing channels being integrated into the mesofluidic platform promote the proximity between the injection port and the flow cell where the detection takes place by the use of optical fibers, responsible for the detection important to monitor real time evolution of the reaction [24].

This mode of function fits the purpose of proposed work for the study of kinetics of COX-2. Herein, this system was also used to evaluate the inhibition effect of a library of platinum- and ruthenium-based anticancer compounds in COX-2 enzyme activity.

2. Materials and methods

2.1. Reagents and solutions

All solutions were prepared using ultrapure water from Milli-Q water plus system with specific conductivity of less than $0.1 \mu\text{S cm}^{-1}$ and the chemicals were of analytical grade.

Cyclooxygenase 2 human recombinant (COX-2), 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), arachidonic acid (ARA) and hemin were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Merck. Ethanol and sodium hydroxide were purchased from Fisher Chemical. Rofecoxib was purchased from Tokyo Chemical Industry.

COX-2 vial was reconstituted from a solution comprising 80 mM Tris-HCl at pH 8 with 0.1% TWEEN® 20, 300 μM diethyldithiocarbamate and 10% glycerol.

A Tris-HCl buffer solution 0.1 mol L^{-1} (pH 8) was used as carrier solution for the LOV procedure.

ADHP, ARA and hemin were prepared daily in DMSO (500 μM), ethanol (2.25 mM) and NaOH 1 M solution (50 μM), respectively.

Rofecoxib, auranofin, cisplatin, oxaliplatin and carboplatin were purchased from Sigma-Aldrich. All other compounds were prepared according to literature methods – see Table 3 for the relevant references.

2.2. Analytical instrumentation

The μ SIA-lab-on-valve (μ SIA LOV) system is illustrated in Fig. 1. The flow manifold is composed of (i) a standalone 5000-step bidirectional micro-syringe pump Bu1S (Crison Instruments S.A., Allela, Barcelona, Spain) equipped with a 2.5 mL gastight glass syringe (Hamilton, Bonaduz, Switzerland) for automatic fluid handling (ii) a 10-port multi-position selection valve (Cheminert™) operating as an actuator and

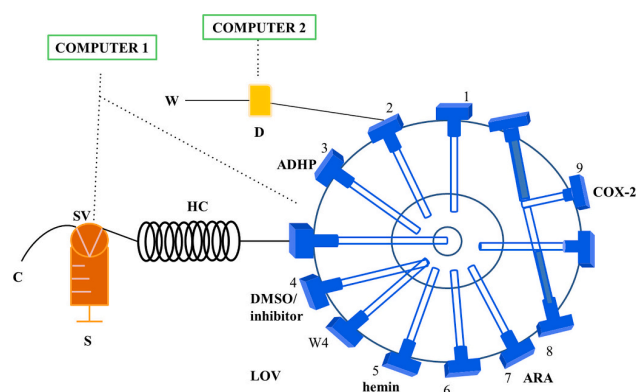


Fig. 1. Schematic representation of the μ SIA LOV manifold used. C – Carrier; SV – selection valve; S – syringe; HC – holding coil; ADHP – 10-acetyl-3,7-dihydroxyphenoxazine; DMSO – dimethylsulphoxide; ARA – arachidonic acid; COX-2 – cyclooxygenase 2; D – detector; W – waste; LOV – Lab-on-valve.

(iii) a customized LOV conduit platform mounted on top of the selection valve that acts as a valve stator.

Solenoid commutation head-valves allow interchange of the syringe either to the manifold or to the carrier. Computer control of the flow rate, flow direction, valve position, stop flow duration, sample, enzyme and substrate volumes, as well as data acquisition and processing, was used.

The LOV contains a central port that enables sequential commutation with any ten peripheral LOV channels and ports. The central port of LOV is connected to a holding coil (HC) with 2 m. All the manifold components were connected by 0.8 mm i.d. PTFE tubing.

A second computer with OOIBase32™ software version 2.0.6.5 of Ocean Optics was used to record the analytical signals. For detection of resorufin, it is necessary (i) a fluorometer USB4000 (from Ocean Optics Inc., Dunedin, FL, USA), (ii) a light source (a tungsten halogen lamp, LS-1-LL, from Ocean Optics Inc.) and (iii) a pair of optic fibers with a core diameter of 600 μm that connect the light source with the flow cell (integrated in the LOV) and the flow cell with the fluorometric detector were used for the detection.

2.3. LOV analytical procedure

The determination of inhibition percentage and half maximal inhibitory concentration (IC_{50}) of the ruthenium-based compounds using COX-2 enzymatic reaction were performed in the μ SIA-LOV system. Before starting the analytical cycle, it was necessary to fill the syringe with the carrier solution (Tris-HCl solution at pH 8) and then fill the central tube and the lateral tubes of the selection valve with the reagents. The tubes from positions 3, 7, 5, 4 and 9 were filled with ADHP, ARA, hemin, inhibitors and COX-2, respectively. After these filling steps, the analytical cycle (Table 1) was performed by aspiration of 20 μL of hemin, compound (inhibitor) and COX-2 solutions (step 1, 2 and 3). Then, the flow was stopped, the reagents remaining in the holding coil were kept there for a period of 5 min (step 4), in order to ensure sufficient contact between the enzyme and the inhibitor. After this, 20 μL of ARA and 20 μL of ADHP solutions were aspirated (steps 5 and 6) and by a flow reversal, the fluids were propelled to the detection cell (a cell between the optic fibers placed between LOV and waste) (step 7). The flow was stopped again inside the detection cell during 5 min to obtain continuously the fluorescence signal of the reaction product (step 8). After this stop period, the detection cell was cleaned (step 9). In order to take advantage of the volume in the syringe, the analytical cycle was repeated twice, without having to fill the syringe completely. All the assays were conducted at 25 °C and each assay was carried out in triplicate.

Table 1

Analytical cycle used to perform the COX-2 inhibition assays.

Step	Position valve	Volume (μL)	Time (s)	Flow rate (mL min ⁻¹)	Event
1	5	20	2.4	0.5	Aspiration of hemin
2	4	20	2.4	0.5	Aspiration of inhibitor
3	9	20	2.4	0.5	Aspiration of COX-2
4	9	0	300	0	Stop period in holding coil
5	7	20	2.4	0.5	Aspiration of ARA
6	3	20	2.4	0.5	Aspiration of ADHP
7	2	200	22.5	0.5	Propulsion to the detector
8	2	0	300	0	Stop period in detector
9	2	625	37.5	1	Cleaning detector

2.4. Batch procedure

The enzymatic activity of COX-2 (0.25 UN) was assayed fluorimetrically at 576/586 nm by measuring the rate of ARA conjugation to COX-2 as a function of time based on COX-2 assay kit from Cayman Chemical Company, Ann Arbor, MI, USA. The assay mixture contained 25 μM ADHP, 5 μM hemin and 37.5 μM ARA in 0.1 M of Tris-HCl buffer (pH 8). All the assays were conducted at 25 °C according to the manufacturers specifications and each assay was carried out in triplicate.

3. Results and discussion

The proof-of-concept applicability and feasibility of μSIA-LOV for the automatic establishment of the COX-2 enzymatic inhibition assay and the analysis of a library of metal-based anticancer compounds was demonstrated by the decrease activity of COX-2 enzyme as a model of biochemical target using a COX-2 fluorometric assay. It consists of two enzymatic reactions serially coupled. COX-2 enzyme present both COX activity converting arachidonic acid into a short-lived 15-hydroperoxide-Prostaglandin G₂, and prostaglandin hydroperoxidase activity, which is crucial in the process of reduction of prostaglandin G₂ into prostaglandin H₂.

The reaction, between PGG₂ and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound, resorufin. A schematic COX-2 reaction is represented in Fig. 2.

Before start the enzymatic assays in μSIA-LOV, the excitation and emission wavelengths were confirmed, and for all the assays, the excitation and emission wavelengths defined were 576 nm and 586 nm, respectively.

Initially, ARA, ADHP and the inhibitors were solubilized in DMSO. In order to decrease the concentration of this solvent in the reaction, ARA was solubilized in ethanol.

3.1. μSIA-LOV system optimization

In order to achieve the best performance with the μSIA-LOV system, the main chemical and physical parameters involved such as solutions concentrations and composition, volumes of the aliquots solutions and their order of aspiration, time of reaction were studied. All the main parameters optimized are described in Table 2, along with the studied range and the selected values.

In the μSIA-LOV system, since the reagents are aspirated sequentially to the holding coil and then, by flow reversal, they are propelled to the detector, their interdispersion is essential for the partial reagents sobrepone with the subsequent reaction. Volume minimization also supports the mixing between the different reagent zones.

The first parameter optimized was the reagent volumes where it was tested at several volumes from 10 μL to 25 μL (see Table 2). The fluorescence intensity (FI) is 3.5 times higher at a volume of 20 μL compared with 10 μL, and at 25 μL saturation is observed at the detector.

The aspiration order was also studied and the best results in terms of FI were achieved when ADHP solution was the last to be aspirated. Moreover, when COX-2 was in contact with ARA, the FI is higher 2.3 times than when COX-2 was in contact with ADHP.

As the reaction time was crucial for the method sensitivity and to increase the time contact between COX-2 and the inhibitor, without increasing the dispersion of the reaction zone, two stop periods were added to the analytical cycle. A stop period of 5 min was used in the holding coil, the same time used in batch assays and defined in the protocol by Cayman Chemical (COX Fluorescent inhibitor screening assay kit). A stop period of 5 min was also included at the detector in order to acquire more data.

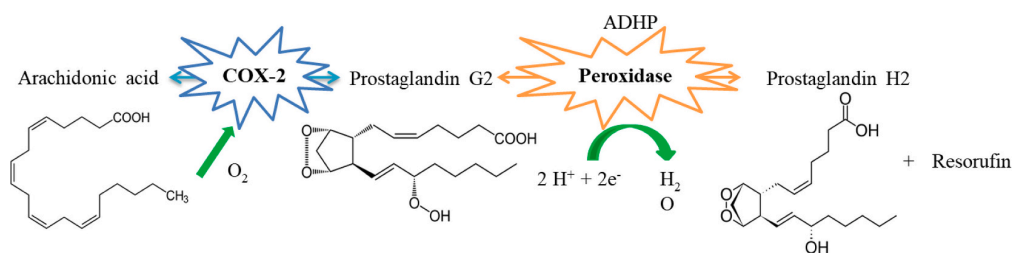
The concentration of the ARA, COX-2 and ADHP solutions was also studied.

COX-2 was evaluated in the concentration range 0.01 U – 0.65 U (Unit – one unit of enzyme activity is defined as that catalysing the conversion of 1 μmol substrate or the formation of 1 μmol product in 1 min) [25]. An optimum COX-2 concentration of 0.05 U was selected as at this concentration there is a direct proportionality with the FI.

Table 2

Tested conditions and results of the optimization.

Parameter	Range	Selected values
Reagents volumes	10 μL – 25 μL	20 μL
Aspiration order	hemin – COX2 – inhibitor – ADHP – ARA hemin – COX2 – inhibitor – ARA – ADHP hemin – inhibitor – COX2 – ARA – ADHP inhibitor – COX2 – hemin – ARA – ADHP	Hemin – inhibitor – COX2 – ARA – ADHP
ARA concentration	50 μM – 1000 μM	250 μM
COX-2 concentration	0.01 U – 0.65 U	0.05 U
ADHP concentration	13.9 μM – 55.6 μM	55.6 μM
Propulsion volume to the detector	193.75 μL – 212.5 μL	200 μL

**Fig. 2.** COX-2 enzymatic reaction.

Hemin and ADHP are the cofactors of the COX-2 enzyme reaction. The hemin concentration was fixed in 5.5 μM . The ADHP concentration was also considered and in this case, the higher ADHP concentration, greater is FI obtained, since the intensity of this fluorescence is proportional to the amount of resorufin, which is proportional to the amount of PGG₂ present at the end of the reaction.

The concentration of ARA, the COX-2 substrate, was then studied. As the substrate concentration increases, the rate of enzyme activity increases up to the point where the enzyme becomes saturated by the substrate. The ARA concentration was used to obtain a calibration curve. The calibration curve was $\text{FI} (0.003 \pm 0.001) \text{ C } (\mu\text{M}) + (0.181 \pm 0.322)$; $R^2 = 0.99$, where FI is the fluorescence intensity and C is the concentration of ARA in μM , respectively, with 95% confidence limits for the intercept and slope.

The Michaelis-Menten constant (K_m) was also calculated to be 366 μM using GraphPad Prism 7 software. K_m is numerically equal to the substrate concentration at which the reaction rate is half of maximum velocity (V_{max}) and to ensure high COX-2 activity in the inhibition assays a value of 250 μM was chosen.

3.2. Evaluation of half maximal inhibitory concentration (IC_{50}) of new ruthenium-based compounds

After the optimization of $\mu\text{SIA-LOV}$, it is now possible to determine the inhibition profiles of all the metal-based compounds tested. The IC_{50} were calculated using GraphPad Prism 7 software and are presented in Table 3 and the inhibition profiles of each one are represented graphically in Fig. S1 of Supplementary material. The RSD values are less than 2.5%. As shown in Fig. S1 of Supplementary material, all the compounds inhibited COX-2 in a dose-dependent manner.

Rofecoxib is a selective COX-2 inhibitor containing a methylsulfone group. The sulphur-containing phenyl rings of these compound binds in the side pocket of the cyclooxygenase catalytic channel of COX-2. The rofecoxib IC_{50} values in literature are very different dependent the assay performed. For example, according to Mitchell et al. the IC_{50} of rofecoxib in intact cells is $2.67 \times 10^{-3} \mu\text{M}$ [26]. On the other hand, Kato et al. [27] described an inhibitory COX-2 assay in human peripheral monocytes where the IC_{50} value obtained for rofecoxib was $25 \pm 18 \mu\text{M}$. The rofecoxib structure contains a methanesulfonyl moiety with 4-sulfonylmethylphenyl substitution at position 1 on the pyrazole ring resulting in stronger inhibitory effects against COX-2 [28]. In our study, the IC_{50} value obtained for rofecoxib was $9.7 \pm 0.6 \mu\text{M}$. In this case, as we explained before, the assay was performed directly on the COX-2 human enzyme.

Half of the compounds have IC_{50} values between 20 μM to 50 μM (compounds 4, 5, 6, 7, 8, 10, 11, 13, 16, 21, 23). It has previously been shown that ruthenium compounds are more prone to binding to proteins than platinum compounds [29], and from the data presented in Table 3, the ruthenium compounds tend to be better COX-2 inhibitors than the platinum compounds studied, although there is some overlap. Several complexes of formula $\text{Ru}(\text{arene})\text{Cl}_2\text{PTA}$ were assayed in which the arene varies, i.e. RAPTA-B 11 where the arene is benzene, RAPTA-T 12 where the arene is toluene, RAPTA-C 14 where the arene is *p*-cymene and 15 which has a hexamethylbenzene ring. The bulk of the ring increases in the series: 11 < 12 < 14 < 15, with the IC_{50} values being 26.9 ± 1.2 , 53.6 ± 0.6 , 64.4 ± 0.3 and $52.2 \pm 1.5 \mu\text{M}$, respectively. From these values it is difficult to ascertain a clear trend although RAPTA-B 11 with the least bulky arene is the most effective inhibitor. Other complexes with the same generic formula, but with functionalized arene rings were also studied, i.e. 19, 20, 21 and 23, with IC_{50} values for COX-2 inhibition of 50.3 ± 0.3 , 57.2 ± 1.5 , 41.7 ± 1.3 and $27.7 \pm 0.9 \mu\text{M}$, respectively. There does not appear to be a clear trend with respect to the functionality attached to the ring, although the most hydrophobic compound, i.e. 23, is the most effective inhibitor. In this respect, a comparison of RAPTA-C 14 with the analogue in which the amphiphilic PTA ligand is replaced by a hydrophobic PPh_3 ligand, i.e. 13, shows a similar trend in

that the latter is a more effective COX-2 inhibitor (cf. $64.4 \pm 0.3 \mu\text{M}$ for 14 vs. $22.3 \pm 1.1 \mu\text{M}$ for 13).

The platinum compounds tested here, i.e. 3, oxaliplatin and carboplatin are more effective COX-2 inhibitors than $[\text{PtCl}_2(\text{L}^{\text{EF}})]$, $[(\text{L}^{\text{EF}}): \text{ethacrynic acid} + \text{flurbiprofen}]$ a platinum compound reported in the literature [30] (cf. IC_{50} values between 40.2 and 56.6 μM versus 77 μM for $[\text{PtCl}_2(\text{L}^{\text{EF}})]$).

Auranofin, a gold-based drug, is a relatively poor COX-2 inhibitor ($55.8 \pm 1.3 \mu\text{M}$), and is much more effective at inhibiting the activity of other enzymes [31]. Notably, the IC_{50} values observed for the ruthenium compounds is potentially relevant to their mode of anticancer action, and COX-2 inhibition might explain, at least in part, why ruthenium compounds are generally much less toxic (leading to reduced side effect) than platinum-based drugs [32].

Comparing compounds 16 and 21, which differ by a halide ligand (Br and Cl, respectively), the COX-2 inhibition activity order observed is $\text{Br} > \text{Cl}$, potentially due to more facile loss of the bromide ligand which may be required for enzyme inhibition by allowing binding to the enzyme. Note that some of the compounds were tested using a batch procedure and the IC_{50} values obtained in both methods were calculated and compared in Table 4.

The agreement between both methods was evaluated using the *t*-test, carried out as a bilateral coupled test. The tabulated *t* value of 2.015 when compared to the calculated *t* value of -0.087 showed the absence of statistic differences at the 95% confidence level. The *p*-value was also calculated and according to the 95% confidence level ($p = 0.05$), the *p*-value obtained was 0.9, hence there are no statistic differences between both methods.

A linear relationship was also established, as described by the equation:

$$\text{IC}_{50}\text{LOV} = (0.7 \pm 0.5) \text{IC}_{50}\text{BATCH} + (9.8 \pm 25.0)$$

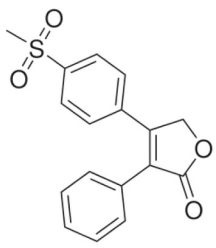
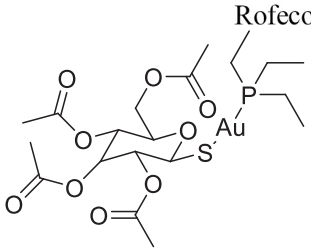
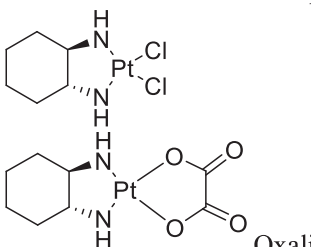
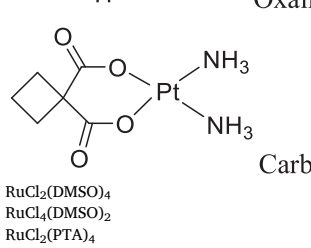
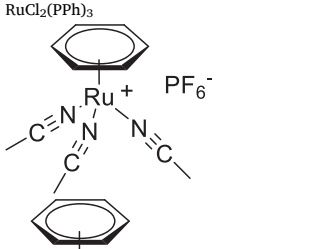
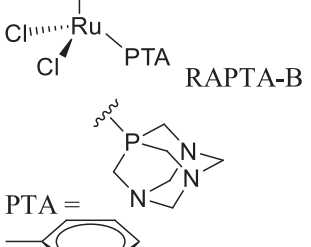
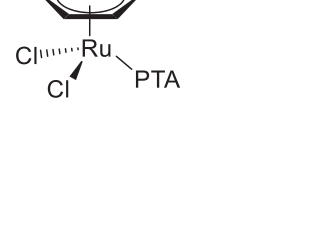
where IC_{50}LOV and $\text{IC}_{50}\text{BATCH}$ correspond to the IC_{50} values obtained in LOV and batch methods, respectively, with 95% confidence limits for the intercept and slope. The estimated intercept and slope values do not differ significantly from 0 and 1, respectively, which confirms the agreement between the LOV and batch methods. The Pearson correlation coefficient was also calculated (~ 0.9) and shows a good correlation between the two sets of results obtained by the new methodology and by the batch procedure. The LOV methodology showed a set of advantages compared with batch method. LOV allows a significant reduction in solvents, samples and reagents consumed, less operator intervention is required and the method is suitable for routine analysis.

4. Conclusions

A $\mu\text{-SIA LOV}$ system was developed to determine the inhibitory effect of metal anticancer compounds on COX-2. From the series of ruthenium and platinum complexes studied, and the gold-based drug auranofin, it would appear that COX-2 inhibition might be relevant to the mode of action of the ruthenium complexes, contributing to their lower toxicity and, as a consequence, lower side effects compared to the other complexes.

This automatic system is a useful tool for the application of enzymatic reactions promoted by COX-2, specifically for the evaluation of the inhibition profile of metal-based compounds. The results obtained are reliable, confirming the usefulness of this methodology for the implementation of enzymatic reactions. The automation and the miniaturization lead to the reproducibility improvement and also a temporal reproducibility since the aspiration, propulsion and analysis happen in the same time in all the assays performed. Consequently, there are fewer operator failures. Furthermore, this system reduces the consumption of reagents and minimizes waste. These advantages are relevant when compared to other automatic methodologies based on the flow analysis. In this specific case, the main advantages include the reduced incubation

Table 3
COX-2 inhibition data expressed as IC₅₀ values.

Number	Structure	IC ₅₀ (μM) ± RSD	Reference
1		9.7 ± 0.6	
2	 Rofecoxib	55.8 ± 1.3	
3	 Auranofin	56.6 ± 1.7	
4	 Oxaliplatin	40.2 ± 0.8	
5	 Carboplatin	46.5 ± 0.4	
6	RuCl ₂ (DMSO) ₄	30.7 ± 0.8	[33]
7	RuCl ₄ (DMSO) ₂	35.5 ± 1.3	[33]
8	RuCl ₂ (PTA) ₄	22.3 ± 1.8	[34]
9	RuCl ₂ (PPh) ₃	13.7 ± 1.6	[35]
10	 PF ₆ ⁻	26.5 ± 0.8	[34]
11	 RAPTA-B	26.9 ± 1.2	[36]
12	 PTA = 	53.6 ± 0.6	[36]
13		22.3 ± 1.1	[36]

(continued on next page)

Table 3 (continued)

Number	Structure	IC ₅₀ (μM) ± RSD	Reference
	RAPTA-T		
14		64.4 ± 0.3	[36]
15			
	RAPTA-C		
16		52.2 ± 1.5	[36]
17		29.9 ± 2.3	[37]
18		66.1 ± 0.6	[38]
	DAPTA-C		
19		73.3 ± 0.4	[39]
20		50.3 ± 0.3	[40]
21		57.2 ± 1.5	[41]
22		41.7 ± 1.3	[42]
23		207 ± 3	[41]
	Oxali-RAPTA-C		
24		27.7 ± 0.9	[43]

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Table 3 (continued)

Number	Structure	IC ₅₀ (μM) ± RSD	Reference

Table 4

Comparison of batch and LOV IC₅₀ values.

Compound	IC50 ± RSD values obtain in μ-SIALOV	IC50 ± RSD values obtained in batch
Rofecoxib	9.7 ± 0.6	6.2 ± 3.7
RAPTA-T	53.6 ± 0.6	46.7 ± 6.5
Oxaliplatin	40.2 ± 0.8	69.70 ± 0.01
9	13.7 ± 1.6	10.8 ± 2.4
10	26.5 ± 0.8	13.4 ± 1.6
18	73.3 ± 0.4	73.4 ± 5.3

time (5 min versus up to 60 min in manual assays reported previously) and the possibility to apply this automatic system to other enzymes than COX-2 without reconfiguration of the μ SIA-LOV.

In general, the assay has promising features that make it suitable for rapid and reliable screen of putative drug candidates.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinorgbio.2021.111399>.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

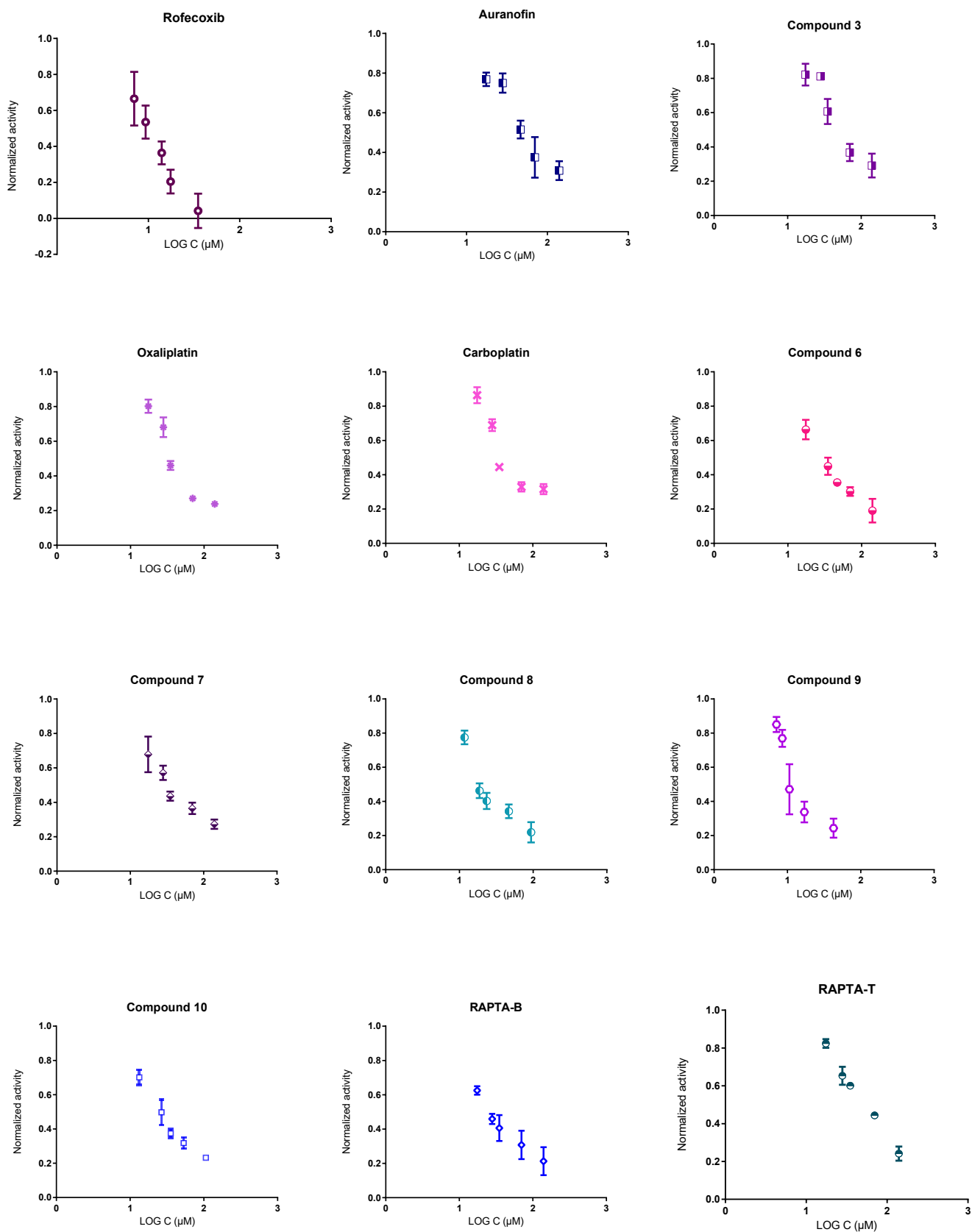
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Supplementary material



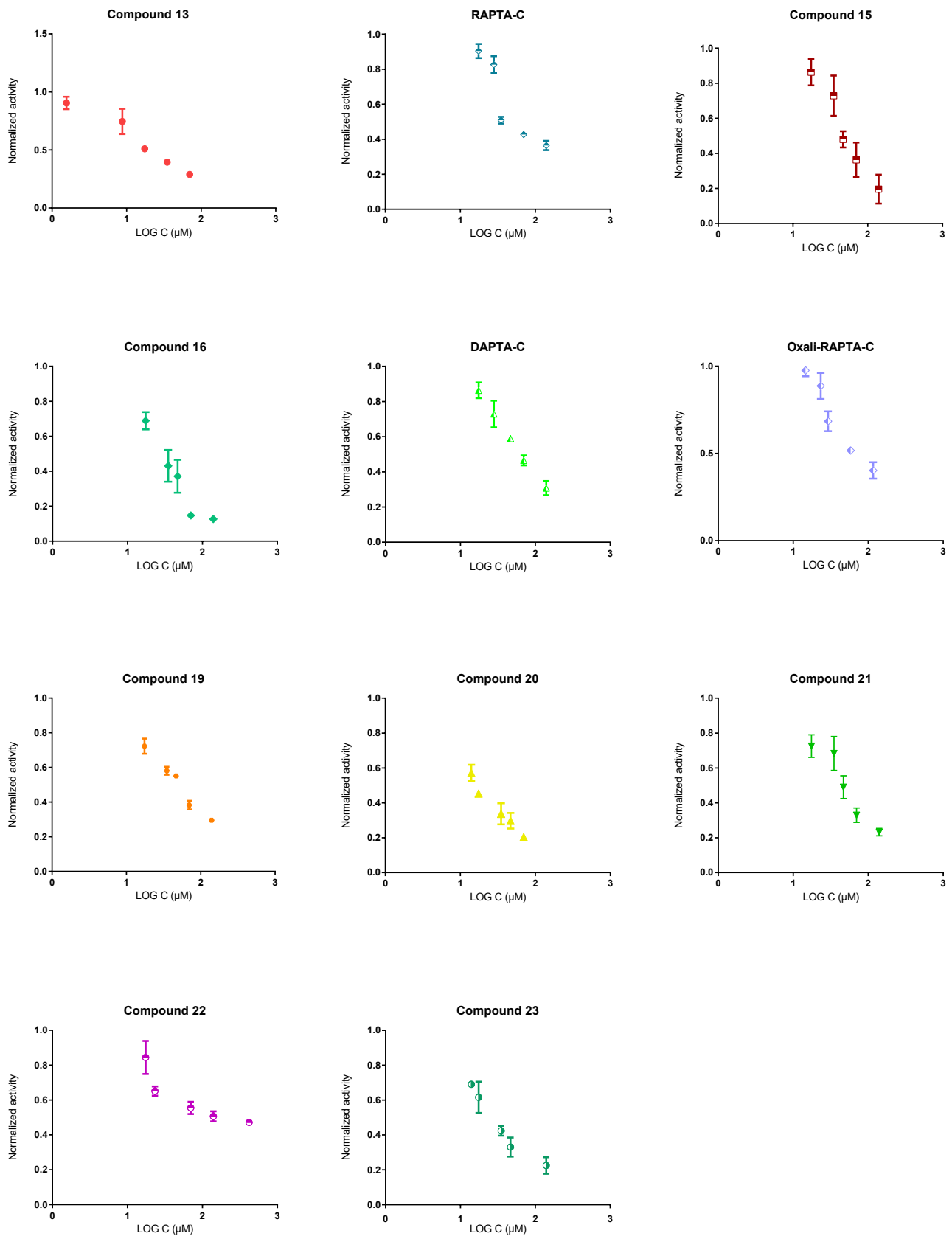
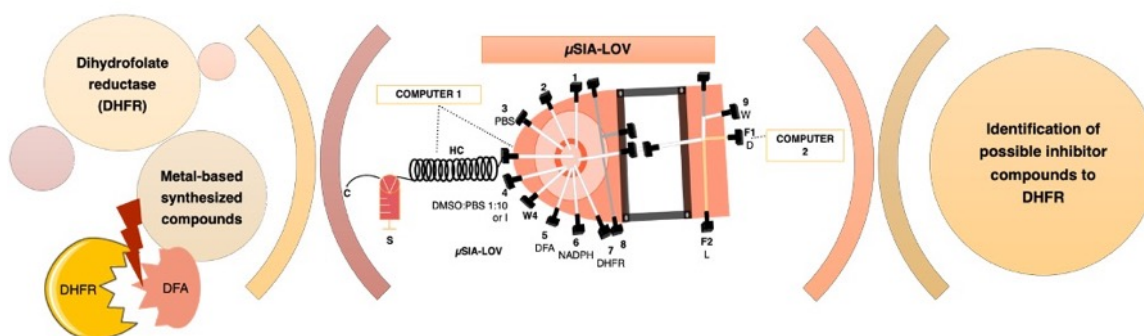


Figure S1 – Experimental inhibition data of the compounds tested in the COX-2 inhibition assays.

2 - Assessment of metal-based dihydrofolate reductase inhibitors on a novel mesofluidic platform

The CRediT author contribution statement is described in the following paper.





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Assessment of metal-based dihydrofolate reductase inhibitors on a novel mesofluidic platform

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ABSTRACT

A new miniaturized micro sequential injection coupled with the lab-on-valve (μ SIA-LOV) technique was full-blown to perform inhibitory studies on dihydrofolate reductase (DHFR). The system was used to evaluate the DHFR inhibition activity of metal-based anticancer compounds. The metal complexes exhibited IC_{50} values in the range 1.3 ± 0.3 – 108 ± 7 μ M, with half of the complexes lying in the low μ M range, i.e., 1.3 ± 0.3 – 4.4 ± 0.2 μ M. For comparison, methotrexate (MTX), a known inhibitor of DHFR, has an IC_{50} value of 0.38 ± 0.06 μ M. The μ SIA-LOV is a versatile, robust, rapid, and easy to operate, allowing automated determination of DHFR inhibition. Moreover, the automated system requires very little sample (approximately 40 μ L per analysis), uses minimal reagents (5 times less than the batch procedure used), and generates very little waste (around 1.2 mL per analysis) compared with batch methods, considerably reducing costs.

1. Introduction

The third generation of flow analysis, also termed the lab-on-valve (LOV) concept, is an advanced mesofluidic approach that provides huge potential for instrumental miniaturization, automatization, and continuous monitoring of chemical and biochemical assays combined with automatic treatment of sample [1]. The LOV concept merges all the intrinsic benefits of flow methods with micro-flow injection analysis (μ FIA) channels, using an integrated machined structure [2].

The resulting mesofluidic platform enables unattended chemical and biochemical analysis performance, independent of the matrix, chemistry, and samples involved, based on programmable pressure user-friendly software whose flow direction and rate are controlled [3].

Several detections could be associated with LOV platforms for several applications. There are some studies that reported the detection outside the LOV platform, using optic fibers that are connected to the flow cell [4] or also by chromatographic detection [5,6]. On the other hand, there are others LOVs where the detection is inside the flow cell. This configuration is mainly used for absorbance detection but also for fluorimetry and chemiluminescence detection [7–10].

Micro sequential injection coupled to lab-on-valve (μ SI-LOV)

platform based on the programmable flow, has been used to perform biomolecular assays, including enzymatic assays [11,12].

The study of enzyme kinetics allows both substrates and inhibitors to be characterized [13]. Standard enzyme kinetics studies typically employ manual bench techniques. However, the irregularity of the mixing time prior to detection and the time consumed by manual operation can lead to errors. Consequently, to improve accuracy and reduce the analysis time, LOV provides some advantages. The stopped-flow injection procedure has been successfully useful to the determination of reactions rate [14–16]. Basically, the flow can be stopped for a chosen period allowing the reaction to be continuously monitored as a function of time. Hence, we decided to develop a μ SIA-LOV system with a new coupled detection module using optical fibers in 90° based on stopped-flow analysis to evaluate the ability of metal-based compounds to inhibit dihydrofolate reductase (DHFR).

DHFR enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate in an NADPH-dependent manner [17]. This enzymatic reaction interferes with the various amino acids, thymidylate, and purines biosynthesis and is also an important target for anticancer drugs such as methotrexate. [18].

In recent years, significant efforts have been devoted to developing

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alternative metal-based drugs to platinum-based complexes currently extensively used to treat cancer [19–21]. One of the leading classes of compounds under intensive evaluation comprises ruthenium-arene complexes, which have been extensively evaluated *in vivo* and show highly promising properties [22]. Iron, due to its bioavailability, is another attractive element for a metal-based drug that noticeably decreases the toxic consequences of these compounds, and the chemical redox potential in physiological media, typically involving the oxidative states of Fe^{II} and Fe^{III} . Some monoiron complexes have been studied due to their anticancer effect and functionalized ferrocenes have emerged as promising candidates [23,24]. Recently, different categories of diiron compounds based on the $\{\text{Fe}_2\text{Cp}_2(\text{CO})_x\}$ scaffold ($x = 2$ or 3) have revealed potential in this field. They display ideal characteristics for a drug candidate, such as straightforward gram-scale synthesis from cheap precursors, generally noticeable water solubility, amphiphilicity, and wide structural variability by varying the substituents on the bridging hydrocarbyl ligand (i.e., hetero-carbyne or vinyliminium). These diiron complexes exhibit cytotoxicity against cancer cells ranging from low micromolar to inactive, and a marked selectivity towards noncancerous cells [25–27]. It has been outlined that aminocarbyne complexes probably exert their action by inhibition of thioredoxin reductase enzyme [25].

Herein, we describe a simple $\mu\text{SIA-LOV}$ method for the automated determination of DHFR activity and its application to a selection of metal-based compounds with a previously established anticancer activity. Results of DHFR inhibition are discussed with respect to the structure and the antiproliferative activity of the compounds.

2. Materials and Methods

2.1. Reagents

Dihydrofolate reductase (DHFR), dihydrofolic acid (DFA), Nicotinamide adenine dinucleotide phosphate (NADPH), and methotrexate were purchased from Sigma. Dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck and Fisher Chemical, respectively. Milli-Q water plus system was used to get ultrapure water with specific conductivity less than $0.1 \mu\text{S cm}^{-1}$. This specific water was used to prepare all the solutions and the reagents were weighed in analytical grade.

The carrier solution used in the $\mu\text{SIA-LOV}$ method was phosphate

buffer solution (PBS) 0.1 mol L^{-1} at pH 7.4. DHFR, DFA, and NADPH were prepared every day in PBS 0.1 mol L^{-1} pH 7.4 at concentrations of 0.03 U, 0.74 mM, and 2.6 mM, respectively. These solutions were protected from light. DHFR and NADPH were incubated in an ice bath during the procedure.

Compounds **2a–d** [28], **3a** [29], **3b** [30], **4a–d** [25,31], **5a–e** [26, 32], **6a–b** [33,34] were synthesized as described in the literature. The compounds were dissolved in a DMSO solution diluted 10-fold with PBS 0.1 mol L^{-1} pH 7.4.

2.2. Flow and detection setup

The $\mu\text{SIA-LOV}$ concept was developed with the purpose of improving the analytical performance since all the sample handling steps are integrated nearby to the measurement area. The developed system is represented in Fig. 1. In Fig. S1 (supplementary material), it is presented the real image of this new LOV platform.

The flow manifold consisted of a microsyringe pump model Bu1S with the bi-directional flow (Crisson Instruments S.A., Allela, Barcelona, Spain) with a 2500 μL syringe of gastight glass (Hamilton, Bonaduz, Switzerland) for programmed liquid handling; a holding coil with 2 m, a LOV platform fixed on the top of a ten-port multi-position selection valve that acts as a valve stator and two optical fibers, one with the light source and the other one with the optical detector. The central $\mu\text{SIA-LOV}$ port is connected to the “out” position of the syringe pump. To fill the syringe with carrier solution it is necessary to move to the “in” position of the syringe pump. All system components are connected by PTFE tubing with 0.8 mm of internal diameter.

The valve position, flow direction, flow rate, duration of stop flow, the volumes of enzyme, sample, and substrate, as the acquirement and process of data analysis were controlled by a computer using MCF (Microsoft Foundation Classes) software.

To record the spectroscopic signals, it was used another computer with OOIBase32TM software version 2.0.6.5 of Ocean Optics. The decrease of NADPH was detected using an Ocean Optics USB4000 handheld (from Ocean Optics Inc., Dunedin, FL, USA) which requires an optical detector for online photometric measurements and an LS-1-LL tungsten halogen light source (Ocean optics) that interfaces with the detector on the 3D LOV platform operating as an adaptive flow cell. For that, a couple of optical fibers with 600 μm of diameter were attached to

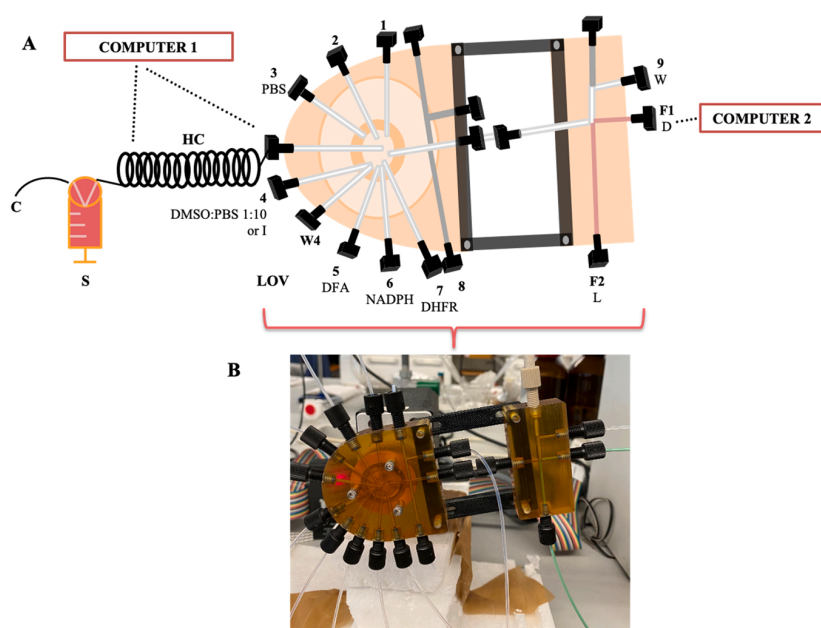


Fig. 1. A) Illustration of the $\mu\text{SIA-LOV}$ system used. C – Carrier; S – syringe; $\mu\text{SIA-LOV}$ – micro sequential injection analysis coupled to lab-on-valve; HC – holding coil; DMSO: PBS – dimethylsulfoxide: phosphate buffer solution; I – inhibitor; PBS – phosphate buffer solution; DFA – dihydrofolic acid; NADPH – β -Nicotinamide adenine dinucleotide 2'-phosphate reduced; DHFR – dihydrofolate reductase; W4 – waste of Port 4; F2 L – tungsten halogen lamp; F1 D – detector; W – waste. B) Photograph of the LOV platform used in this work.

the LOV platform and they were attached to the LOV platform in a 90° position to measure the fluorescent intensity of the reaction.

2.3. LOV analytical technique

Before starting the analyses of DHFR enzymatic inhibition in μ SIA-LOV, it was confirmed the excitation and emission wavelengths of DHFR reaction. Thus, the excitation and emission wavelengths defined were, respectively, 357 nm and 472 nm.

The inhibitory capacity of the compounds tested was determined by the inhibition percentages and half-maximal inhibitory concentration (IC_{50}) against DHFR enzyme using this automatic system. Previously, the syringe and the central tube of the selection valve were filled with the carrier solution (PBS 0.1 M at pH 7.4) and the selection valve adjacent tubes were filled with the reagents. The conduits at positions 3, 4, 5, 6, and 7 (see Fig. 1) were filled with PBS pH 7.4, DMSO 10-fold diluted or test compound, DFA, NADPH, and DHFR, respectively. Afterward, the analytical cycle described in Table 1 was implemented by aspiration of 10 μ L of DHFR, test compound, NADPH, DFA, and PBS (steps 1–5). Then, using flow reversal, the fluids were propelled to the flow cell (step 6) and the flow was stopped inside the flow cell for 90 s to acquire endlessly the reaction product fluorescence (step 7). After that, the detection cell was cleaned (step 8). To decrease the determination time, it was possible to repeat the analytical cycle twice without filling the syringe between determinations. All the determinations were led at room temperature and each assay was performed in triplicate.

2.4. Batch method

The activity of DHFR (3.9×10^{-5} U) was evaluated fluorimetrically at 357/472 nm by measuring the reversible catalysis of NADPH-dependent reduction of DFA to tetrahydrofolic acid by DHFR (Fig. 2) based on DHFR assay kit from Sigma-Aldrich, St. Louis, MO, USA. The assay contained 50 μ M DFA and 60 μ M NADPH in 0.1 M PBS (pH 7.4). The assays were led at room temperature considering the manufacturing specifications and each condition was performed in triplicate.

3. Results and discussion

A μ SIA-LOV system was developed for the automatic determination of DHFR activity and applied to a set of metal-based compounds with anticancer activity. DHFR catalyzed the reaction of dihydrofolic acid (DFA) with tetrahydrofolic acid (THF) using NADPH as a cofactor, and the fluorescence intensity (FI) of the reaction decreases as NADPH is converted to $NADP^+$.

3.1. μ SIA-LOV method optimization

The main parameters of the μ SIA-LOV system, i.e., order of aspiration, the volume of the aliquots, propulsion time to the μ SIA-LOV

Table 1
 μ SIA-LOV analytical cycle defined to carry out the DHFR inhibition assays.

Step	Position valve	Volume (μ L)	Time (s)	Flow rate (mL min ⁻¹)	Event
1	7	10	1.2	0.5	Aspiration of DHFR
2	4	10	1.2	0.5	Aspiration of inhibitor
3	6	10	1.2	0.5	Aspiration of NADPH
4	5	10	1.2	0.5	Aspiration of DFA
5	3	5	1.2	0.5	Aspiration of PBS
6	9	117	7	1	Propulsion to the flow cell
7	9	0	90	0	Stop period in flow cell
8	9	1000	60	1	Cleaning flow cell

detector, reagents concentrations, and volume were studied (Table 2). Initially, the aspiration order of the reagent aliquots was studied and defined as DHFR – inhibitor – NADPH – DFA, since this aspiration order provides a 5 times higher analytical signal than the other conditions tested. In this case, the DHFR and NADPH will be close to the inhibitor aliquot (that will substitute the DMSO 10-fold diluted aliquot) and DFA has a lower system dilution as it is the last reagent to be aspirated. To further enhance the reagents mixture, the total volume of the reagent's aspiration was reduced to 40 μ L, to decrease the reagents dispersion while preserving the low aspiration volumes without compromising the repeatability and the reliability of the assay.

In order to facilitate mixing of the aliquots to increase the reproducibility of the assay, the holding coil was placed near to the LOV platform. Since small reagent volumes are aspirated, these aliquots reach the holding coil (HC in Fig. 1) in shapes of 8 which promotes better mixing and hence reaction. The propulsion time to the detector was also optimized towards obtain the best analytical signal in the detector. Comparing all the conditions tested, a propulsion time of 7 s provided the higher signal of NADPH in blank (15 times, 5 times and 3 times higher than 5.1 s, 6 s, and 8.1 s, respectively). All the propulsion times were tested with a flow rate of 1 mL min⁻¹.

After establishing the volume of the reagents zone, the concentrations of DHFR, DFA and NADPH were optimized. DHFR was studied in the concentration range 9.8×10^{-5} to 3.9×10^{-4} U (Unit - is described as a unit of enzyme activity that catalyse 1 μ mol substrate conversion or the 1 μ mol product formation in 1 min) [35]. An optimal DHFR concentration of 2×10^{-4} U was selected as direct proportionally with the FI is observed. The NADPH concentration (give range) was also optimized simultaneously with the DFA concentration (0.006–0.25 mM), with the NADPH concentration fixed at 0.32 mM.

Lastly, the concentration of DFA was then studied. The enzyme activity rate increases with increasing substrate concentration until the enzyme gets saturated with the substrate. A calibration curve was performed using the DFA concentration. The obtained calibration curve is $F = (77.4 \pm 60.7) C - (3.3 \pm 4.1)$; $R^2 = 0.99$, where F corresponds to the intensity of fluorescence and C corresponds to the concentration of DFA in mM, with a confidence limit for the intercept and slope of 95%. The limit of detection and the limit of quantification is 0.01 mM and 1.07 mM, respectively.

The Michaelis-Menten constant (K_m) and half of the maximal velocity (V_{max}) were also calculated to be 0.25 mM and 10.76 mmol s⁻¹, respectively, using software GraphPad Prism 7. The concentration of substrate at which the enzymatic reaction rate is half of the maximal velocity (V_{max}) is K_m . To guarantee the higher DHFR activity in the proposed inhibition assays, a 0.09 mM DFA concentration was defined.

3.2. Application of the μ SIA-LOV system to determine metal-based compounds IC_{50} values

The outcome of diverse concentrations of each compound was evaluated in triplicate on the enzymatic activity using a 0.09 mM DFA solution which was selected from the linear concentration range of the calibration curve. In Fig. S2 of Supplementary material, it is represented the acquired polynomial relations including the normalized activity and each compound logarithm concentration. The whole compounds library tested inhibited DHFR in a dose-dependent manner and the IC_{50} values are presented in Table 3.

Methotrexate (MTX) is a known DHFR inhibitor and was used as a positive control. In the literature, IC_{50} values for methotrexate vary with the type of assay performed. El-Naggar et al. evaluate the inhibition of DHFR enzyme using methotrexate and the in vitro IC_{50} obtained was $0.14 \pm 1.38 \mu$ M [36] based on a different DHFR inhibition assay [37]. Abdelaziz et al. also determined the IC_{50} value of MTX by enzyme-linked immunosorbent assay (ELISA) technique and they obtained an IC_{50} value of 5.57 μ M [38]. Using the μ SIA-LOV system we obtained a IC_{50} of $0.38 \pm 0.06 \mu$ M.



Fig. 2. DHFR enzymatic reaction.

Table 2
Optimization conditions and results.

Parameters	Range	Values selected
Volume of reagents	5–10 μL	10 μL
Order of aspiration	DHFR - NADPH - inhibitor - DFA DFA - inhibitor - NADPH - DHFR DHFR - inhibitor - NADPH - DFA DFA - NADPH - inhibitor - DHFR	DHFR - inhibitor - NADPH - DFA
NADPH concentration	0.16–0.65 mM	0.32 mM
DHFR concentration	9.8×10^{-5} U - 3.9×10^{-4} U	2×10^{-4} U
DFA concentration	0.06–0.5 mM	0.09 mM
Volume propelled to the detector	85–135 μL	117 μL

All the metal compounds tested were found to be less efficient DHFR inhibitors than methotrexate. Nevertheless, several complexes (**3a**, **5b**, **5c**, **5e**, **5f**, **6a** and **6b**) showed IC_{50} values in the low μM range, similar to that of their antiproliferative activity on cancer cells (IC_{50} after 72 h incubation) [25,26,29,31], indicating that DHFR inhibition might contribute to their antiproliferative mechanism.

The RAPTA derivatives (**2a–d**) showed a weak inhibitory activity, with IC_{50} values within the 30–100 μM range. These complexes are also exhibiting limited antiproliferative effects on cancer cells [39]. Conversely, the Ru(II) η^6 -arene complex RUCYN (**3a**) was found to be a much more effective DHFR inhibitor ($\text{IC}_{50} = 3.9 \pm 0.8$) and this compound is also cytotoxic to cancer cells. It is interesting to note that while RAPTA-C (and its derivatives) easily undergo Cl^- displacement in aqueous solution [40], and many protein adducts are known [41–43], RUCYN is comparatively inert in water and not particularly reactive with biosubstrates like Bovine Serum Albumin and Calf Thymus DNA [44].

Regarding diiron cyclopentadienyl complexes, the aminocarbyne derivatives (**4a–d**) showed moderate DHFR inhibition activity IC_{50} in the range 9–30 μM , whereas the vinyliminium **5a–f** and thiocarbyne **6a–b** complexes revealed the highest inhibitory properties against DHFR, with IC_{50} values all-around 3 μM , respectively. The higher effect provided by **4a** ($\text{IC}_{50} = 15.4 \pm 4.8$ μM) compared to **4c** ($\text{IC}_{50} = 30.0 \pm 1.9$) evidence that DHFR inhibition maybe not necessarily correlated with cytotoxicity, on considering that the former compound is inactive against various cancer cell lines, while the latter exerts considerable cytotoxicity even in 3D models [25]. With reference to vinyliminium complexes **5a–f**, the biological activity for DHFR inhibition, in comparison with cytotoxicity data [25,26,31,32], is consistent within a given subset of compounds, and the influence of different substituents is minor. The widely used anticancer drug carboplatin was also evaluated and exhibits an IC_{50} value of 8.4 ± 0.8 μM , suggesting that DHFR inhibition may contribute, at least in part, to its anticancer effect.

3.3. Validation of $\mu\text{SIA-LOV}$

In order to validate our new approach to evaluate the inhibition of metal-based compounds using DHFR enzyme, some compounds were also analyzed using a batch method, with good agreement found between the two methods (see Table 4).

Three statistical tests were carried out to verify that the developed method is not statistically different from the batch method. In agreement with the student's t-test, the tabulated t value (2.57) was compared to the calculated t value (−0.81). Comparing the results, there are no statistical differences at the confidence level of 95% [45]. It was also considered the p-value and considering the confidence level of 95% ($p = 0.05$), the p-value of 0.91 was obtained and, consequently, it confirmed the absence of statistical differences when both methods are compared. Also, a linear relationship was determined, and the equation was defined as:

$$\text{IC}_{50} \text{ LOV} = (0.99 \pm 0.29) \text{IC}_{50} \text{ BATCH} - (0.64 \pm 4.38)$$

where IC_{50}LOV and $\text{IC}_{50}\text{BATCH}$ are, respectively, the IC_{50} results acquired in $\mu\text{SIA-LOV}$ and batch methodologies with intercept and slope confidence limits of 95%. The expected intercept and slope values were not considerably different, respectively, from 0 and 1, therefore, it approves the agreement of $\mu\text{SIA-LOV}$ and batch methodologies. Additionally, the coefficient of Pearson's correlation was considered (~ 0.96) validating the worthy correlation among both series of results obtained by batch and $\mu\text{SIA-LOV}$ methods.

Comparing this new $\mu\text{SIA-LOV}$ method with batch, in $\mu\text{SIA-LOV}$, the analytical signal is obtained in less than 3 min instead of the 5 min of the batch procedure. Furthermore, there is a significant reduction in solvents in the SIA system since we aspirated 5 times less of DHFR solution and 5 times less of NADPH and DFA solutions than in the batch method. In general, there is a decrease of 5 times in samples and reagents consumed. Taking this into account and all the other advantages of $\mu\text{SIA-LOV}$, it is possible to obtain precise and reliable results using this new methodology.

4. Conclusions

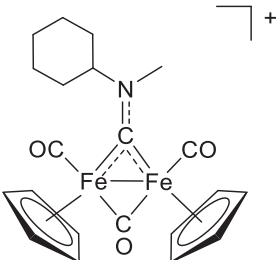
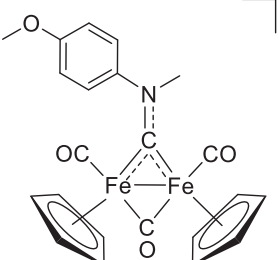
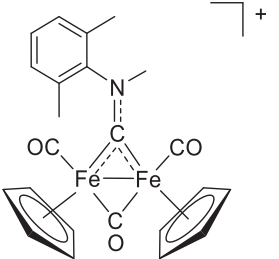
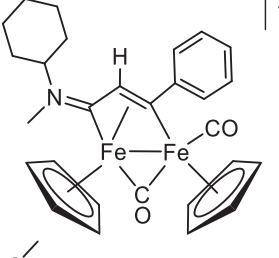
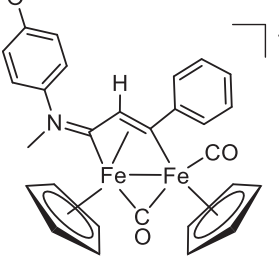
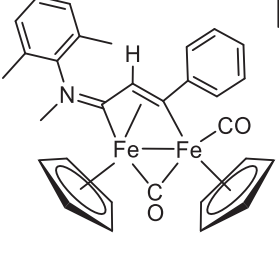
A new automatic $\mu\text{SIA-LOV}$ methodology was developed for the assessment of DHFR activity and used to determine the inhibitory effect of selected metal-based compounds endowed with anticancer activity. The $\mu\text{SIA-LOV}$ system offers several advantages over batch methods, including a noteworthy reduction in reagents, solvents, and samples consumed and less operator involvement. Notably, the system was found to be suitable for routine analysis. The application of the $\mu\text{SIA-LOV}$ method to study the inhibitory effect of a panel of metal-based anticancer compounds demonstrated that the inhibition of DHFR could be taken into consideration as a possible contribution to their antiproliferative activity.

Table 3IC₅₀ values of the compounds tested for DHFR inhibition.

Compound	Structure ^a	IC ₅₀ (μM ± SD)
1 Methotrexate		0.38 ± 0.06
2a RAPTA-C		96.3 ± 7.5
2b RAPTA-T		108 ± 7
2c RAPTA-B		66.4 ± 9.7
2d Oxali-RAPTA-C		30.2 ± 1.6
3a RUCYN		3.9 ± 0.8
3b		4.1 ± 0.9
4a		15.4 ± 4.8

(continued on next page)

Table 3 (continued)

Compound	Structure ^a	IC ₅₀ (μM ± SD)
4b		9.2 ± 3.5
4c		30.0 ± 1.9
4d		9.9 ± 0.1
5a		3.2 ± 0.5
5b		3.1 ± 0.1
5c		4.4 ± 0.2

(continued on next page)

Table 3 (continued)

Compound	Structure ^a	IC ₅₀ (μM ± SD)
5d		2.9 ± 0.2
5e		2.5 ± 0.2
6a		2.9 ± 0.4
6b		1.3 ± 0.3
7 Carboplatin		8.4 ± 0.8

^a Cationic complexes as CF₃SO₃[−] salts, except 3a (RUCYN) as NO₃[−] salt.

Table 4

Assessment of μSIA-LOV and batch IC₅₀ values.

Compounds	IC ₅₀ μSIA-LOV ± SD (μM)	IC ₅₀ batch ± SD (μM)
Methotrexate	0.38 ± 0.06	1.90 ± 0.07
4a	15.4 ± 4.8	17.6 ± 5.0
4b	9.2 ± 3.5	5.4 ± 0.5
4c	30.0 ± 1.9	30.5 ± 11.4
5b	3.2 ± 0.5	4.9 ± 0.6
6a	2.9 ± 0.4	5.4 ± 0.7

CRediT authorship contribution statement

Sarah A. P. Pereira: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Lorenzo Biancalana:** Resources, Writing – review & editing. **Fabio Marchetti:** Resources, Writing – review & editing. **Paul J. Dyson:** Conceptualization, Resources, Writing – review

& editing, Supervision **M. Lúcia M. F. S. Saraiva:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.131978.

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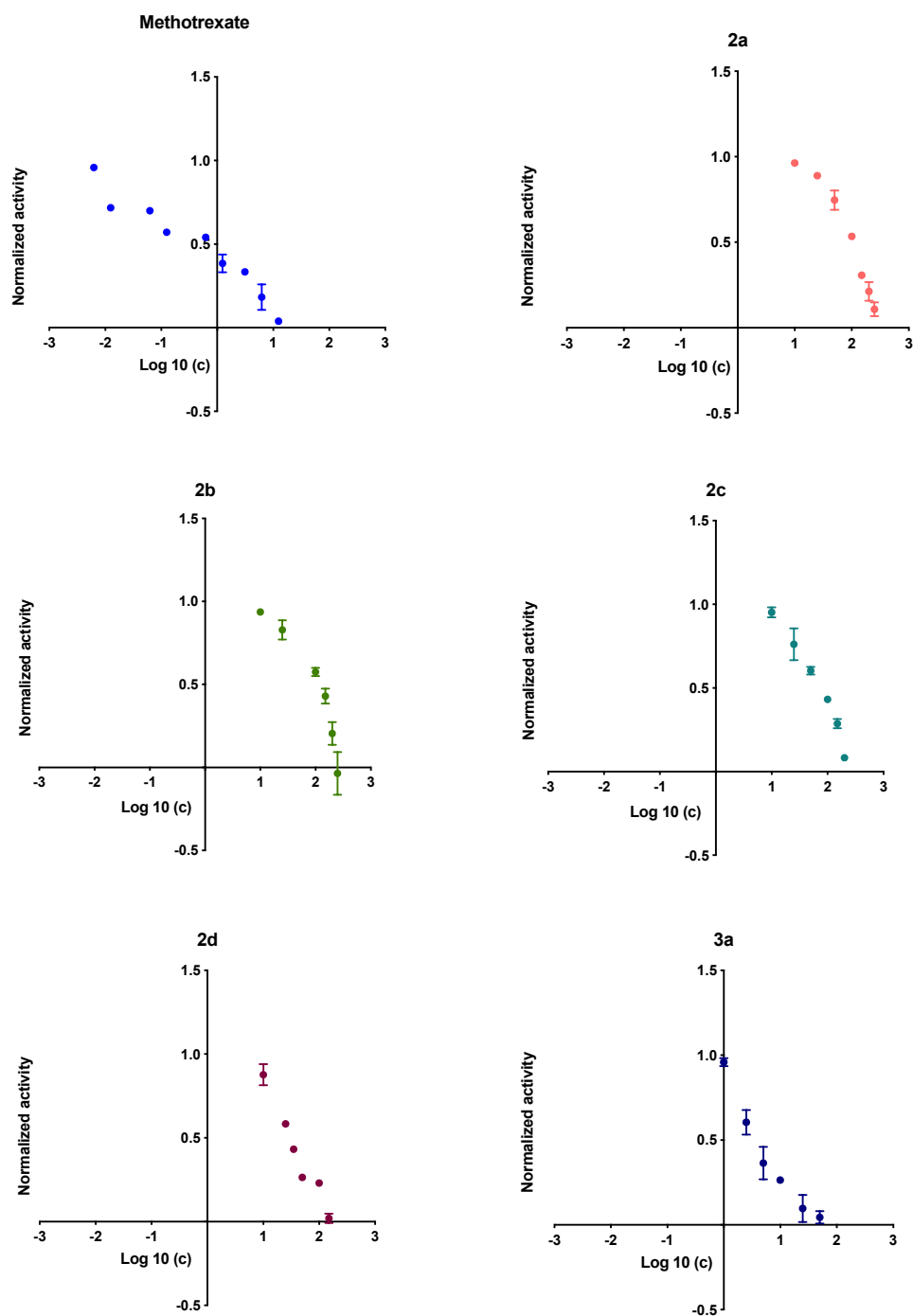
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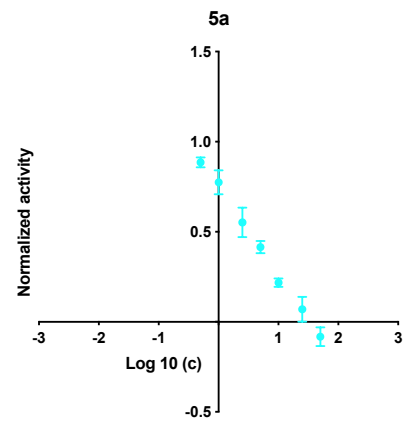
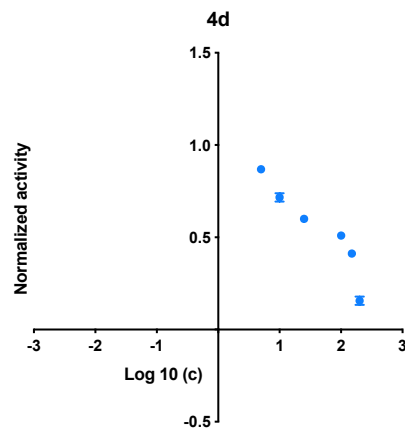
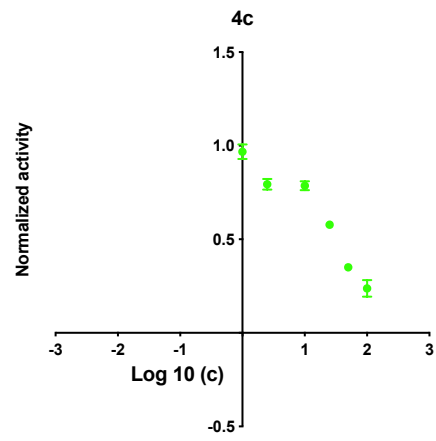
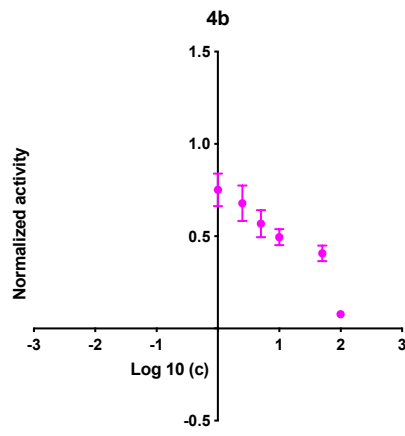
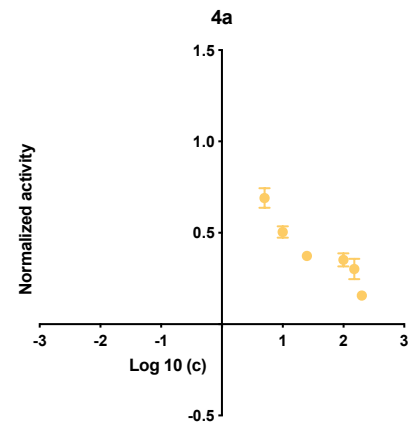
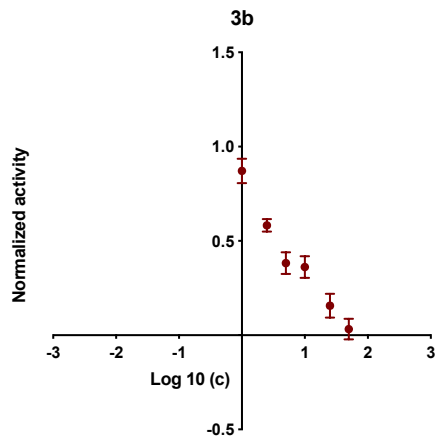
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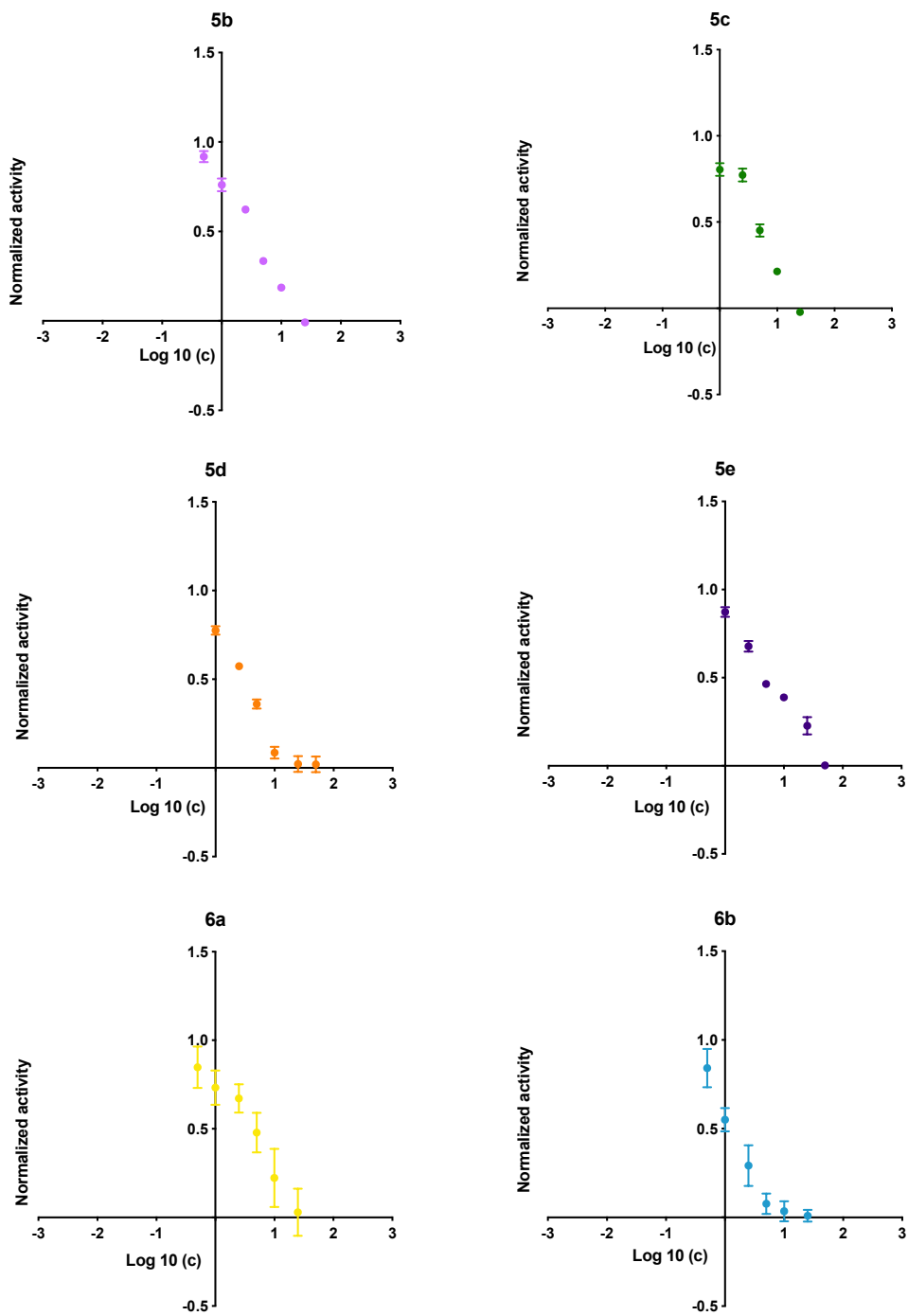
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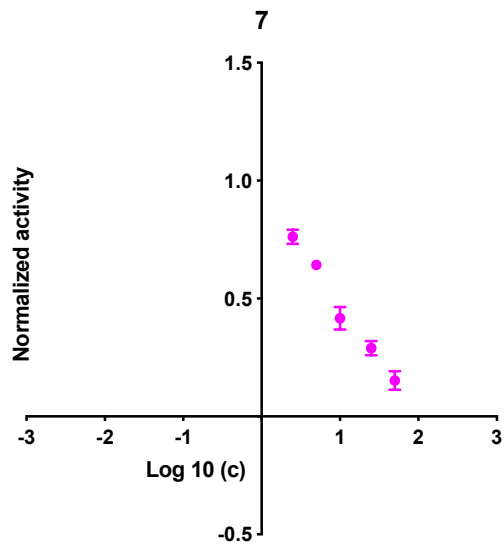
Supplementary material

Figure S1 – Experimental inhibition data of tested compounds in the DHFR inhibition assays.



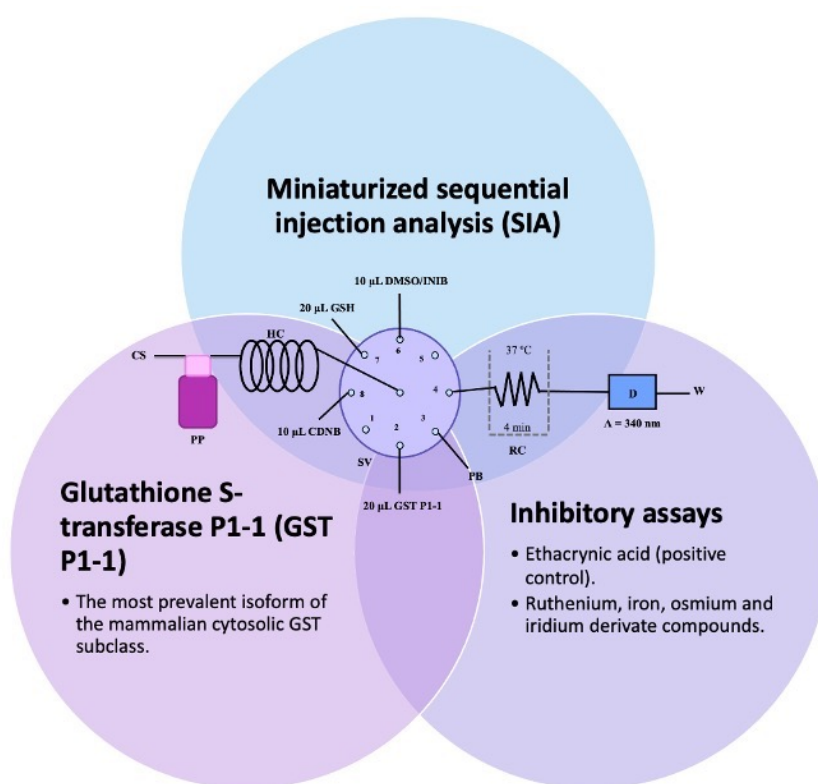






3 – Automated approach for the evaluation of glutathione-S-transferase P1-1 inhibition by organometallic anticancer compounds

In this work, I developed and optimized the SIA technique, to test the metal-based compounds, and calculate the IC_{50} values for each compound. Also, I validated the SIA by the application of the batch method and performed the statistical data analysis. In the end, I wrote the original draft of the manuscript for later publication.



RESEARCH PAPER

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Automated approach for the evaluation of glutathione-S-transferase P1-1 inhibition by organometallic anticancer compounds

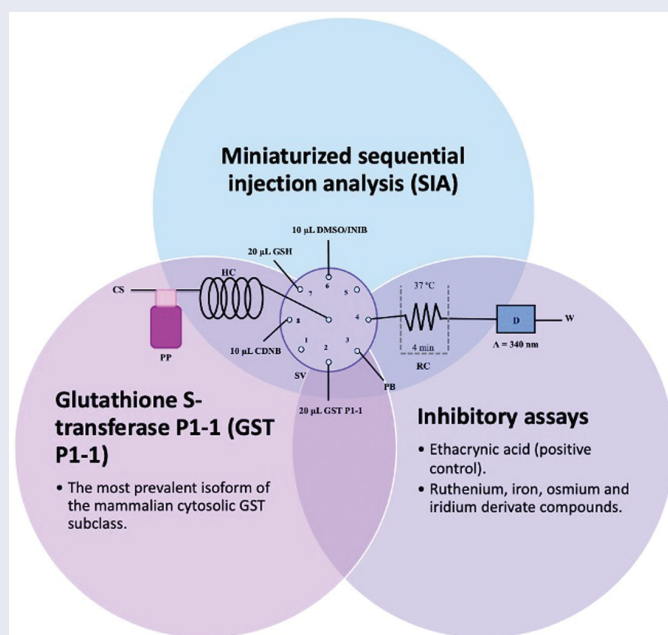
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ABSTRACT

A novel automated method based on sequential injection analysis (SIA), a non-segmented flow injection technique, was developed to evaluate glutathione S-transferase P1-1 (GST P1-1) activity in the presence of organometallic complexes with putative anticancer activity. The assay is based on the reaction of L-glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of GST P1-1 to afford the GS-DNB conjugate and the reaction may be monitored by an increase in absorbance at 340 nm. A series of ruthenium, iron, osmium and iridium complexes were evaluated as GST P1-1 inhibitors by evaluating their half-maximal inhibitory concentration (IC_{50}). An iridium compound displays the lowest IC_{50} value of $6.7 \pm 0.7 \mu M$ and an iron compound displays the highest IC_{50} value of $275 \pm 9 \mu M$. The SIA method is simple to use, robust, reliable, and efficient and uses fewer reagents than batch methods and each analysis takes only 5 minutes.

GRAPHICAL ABSTRACT



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KEYWORDS

Sequential injection analysis (SIA); glutathione S-transferase P1-1; enzyme inhibition assays; anticancer metal complexes

1. Introduction

Glutathione S-transferases (GSTs) are a superfamily broadly distributed in phase II metabolism enzymes that catalyse the

conjugation of extensive diversity of reactive electrophiles to the nucleophilic sulphur atom of tripeptide glutathione (γ -L-glutamyl-L-cysteinyl glycine, GSH). After formed, the hydrophilic GSH

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 Supplemental data for this article can be accessed [here](#).

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conjugates are successfully removed from the cell, inducing the detoxification of the organism^{1,2}. The greatest predominant isoform of the GST subclass in mammalian cytosolic is GST P1-1, and its overexpression can be directly correlated to carcinogenesis and chemotherapeutic drug resistance^{3,4}. This isoform is overexpressed in human tumours such as ovarian, kidney, and breast carcinoma^{5,6}, with its overexpression accelerating drug metabolism leading to a decrease in therapeutic efficacy⁷.

Several GST inhibition batch assays have been reported resorting to a different mode of detection, such as an electrochemical assay using a glassy carbon electrode with differential pulse voltammetry to evaluate GST kinetic parameters⁸, or an immunocytochemistry technique to evaluate the cellular reactivity of GST π ⁹. With a higher level of mechanisation, a high-resolution screening (HRS) technique using two simultaneous enzyme affinity detection (EAD) systems for human GST P1-1 using reverse-phase high-performance liquid chromatography (HPLC). This system was first optimised and validated using a flow injection analysis (FIA) system and the optimised results were then used in HPLC mode¹⁰.

In this work, a sequential injection analysis (SIA) system was developed to assess GST P1-1 activity and evaluate several organometallic compounds with putative anticancer activity. SIA was chosen rather than FIA, as it is better suited to high-cost enzymes/reagents and complicated multi-step reactions since it is possible to use fewer volumes and present several reagents handling abilities¹¹ and minimises some of the drawbacks of batch assays by ensuring effective control of the reaction conditions¹², significantly impacting precision and accuracy¹³. In SIA, enzymatic activity is determined in the early stages of the reaction avoiding interference from low-affinity substrates. Compared to FIA, SIA is more versatile, with computer control mode, and the implementation of different analytical procedures without physical reconfiguration of the setting¹⁴.

SIA is an automatic approach that enables the performance of wet-chemistry procedures in a rapid, precise, and efficient manner. SIA systems have been broadly accomplished in the last decades for the application of enzyme-based assays aiming at the evaluation of enzyme activity, enzyme inhibition assays, and the determination of specific analytes.

The SIA method reported herein is based on the GST P1-1 catalysed reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) which results in an increase in absorbance at 340 nm. Following validation of the assay using ethacrynic acid (EA), a benchmark GST P1-1 inhibitor¹⁵, a selection of organometallic iron, ruthenium, osmium, and iridium complexes, currently investigated for their anticancer activity, were tested to evaluate the inhibition capacity against GST P1-1 enzyme. Iron is attractive for developing metal-based drugs due to its bioavailability and the feasible redox chemistry in physiological media^{16–18}. Recently, organometallic diiron compounds based on the $\{\text{Fe}_2\text{Cp}_2(\text{CO})_x\}$

scaffold ($x=2$ or 3) were shown to display selective cytotoxicity to certain cancer cells. Organoruthenium (half-sandwich) compounds have been extensively studied over the last two decades due to their promising anti-cancer properties¹⁹, with some even validated in vivo against cancers with a very poor prognosis^{20,21}. Related osmium and iridium half-sandwich complexes have received far less attention than those of iron and ruthenium concerning their application in medicinal chemistry, but several promising results have been reported^{22,23}. The conjugation of known enzyme inhibitors to metal-based drugs emerged as a prominent strategy to develop effective anticancer compounds²⁴, with early examples corresponding to half-sandwich ruthenium complexes modified with EA^{25,26}, and some of the organometallic compounds studied herein have pendant EA groups^{25,27}.

2. Materials and methods

2.1. Reagents and solutions

Glutathione S-transferase P1-1 (GST P1-1); 1-Chloro-2,4-dinitrobenzene (CDNB), glutathione (GSH), and ethacrynic acid (EA) were purchased from Sigma. Dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck and Fisher Chemicals, respectively. Ultrapure water obtained from the MILI-Q plus system with a specific conductivity of $< 0.1 \mu\text{S cm}^{-1}$ was used to prepare all the solutions.

CDNB and GSH were daily prepared in ethanol and phosphate buffer 0.1 mol L^{-1} pH 6.5 at 44 mM and 12 mM, respectively. GST P1 was reconstituted from a solution comprising 50 mM Tris-HCl at pH 7.5 with 50 mM of sodium chloride (NaCl), and 1 mM of 1,4-dithiothreitol (DTT), 5 mM of ethylenediaminetetraacetic acid (EDTA), and 50% of glycerol. The GST P1 solution ($0.2 \mu\text{M}$) used in the assays was incubated in an ice bath during the procedure. A 0.1 mol L^{-1} phosphate buffer solution (pH 6.5) was applied as a carrier solution for the SIA method. Compounds **2a–d**²⁸, **3a**²⁹, **4a–d**^{30,31}, **5a–f**^{32–34}, **6a–b**^{35,36}, **7a–e**^{37–40} were prepared in agreement to literature methods and were dissolved in DMSO.

2.2. Analytical apparatus

The SIA system is represented in Figure 1 and consists of a selection valve Crison® module with 8 ports and a peristaltic pump Gilson® Mini plus 3 sets with a pumping tube of polyvinyl chloride with 1.30 mm i.d. All the system components are connected by Teflon tubes of 0.8 mm in diameter. A reactor coil of 50 cm in length was immersed inside the thermostatic bath to maintain the mixture at 37°C .

Measurements were performed using a Jenway® 6300 spectrophotometer detector, incorporating an $80 \mu\text{L}$ flow cell (Hellma Analytics®), connected to the reactor coil, with 10 mm of optical

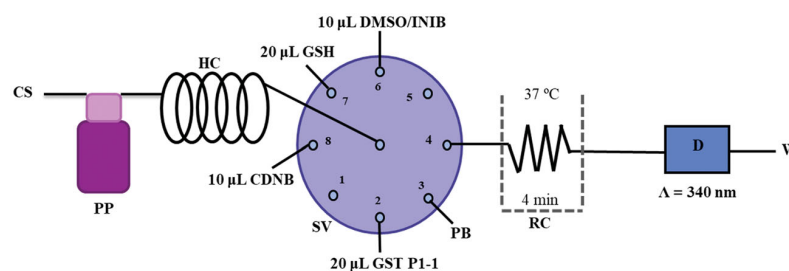


Figure 1. Illustration of the SIA manifold used. CS – Carrier solution; HC – holding coil; PP – peristaltic pump; SV – Selection valve; DMSO/INIB – dimethylsulfoxide/inhibitor; CDNB– 1-Chloro-2,4-dinitrobenzene; GSH – Glutathione reduced; GST P1-1 – Glutathione S-transferase P1-1; PB – Phosphate buffer; RC – Reaction coil; D – Detector and W – Waste.

path length. The absorption wavelength was fixed at 340 nm. Microsoft QuickBasic 4.5 software was used to control the flow system.

2.3. Sequential injection analysis procedure

The half-maximal inhibitory concentration (IC_{50}) determination of GST P1-1 activity of the compounds was performed using the SIA system as follows. Before starting the analytical cycle, all the system tubes were filled with the carrier solution (phosphate buffer at pH 6.5). Then the tubes from positions 2, 3, 6, 7, and 8 were filled with GST P1-1, phosphate buffer pH 6.5, inhibitor, GSH, and CDNB, respectively. Afterward, the analytical cycle, presented in Table 1, was carried out by the aspiration of 10 μ L of CDNB, 10 μ L of DMSO/inhibitor, 20 μ L of GST P1-1, and 20 μ L of GSH (steps 1–4). Then, the aliquots were propelled to the reaction coil (RC) by flow reversal (step 5) and the flow was stopped inside the RC for 4 min to promote the reaction product formation (step 6). After this stop period, the reaction product was propelled to the detection cell (step 7), and the analytical signal was recorded. All the determinations were carried out at 37 °C and each assay was performed in triplicate.

2.4. Batch procedure

To evaluate the enzymatic reaction, a concentration of GST P1 of 20 nM was spectrophotometrically determined at 340 nm by monitoring the reaction of CDNB (1 mM) with GSH (2 mM) (Figure 2) over 8 min in 0.1 M potassium phosphate buffer at pH 6.5 based on a previously reported protocol⁴¹. All the assays were performed

at around 37 °C and in triplicate. The IC_{50} values were acquired using GraphPad Prism 7 software.

2.5. Data analysis

The evaluation of the inhibition curves was performed using GraphPad Prism 7 software using the equation defined by [Inhibitor] vs. normalised response–variable slope, where X values should be concentrations, not transformed to logarithms and the Y values of the curve were go from 100 down to 0. This model corresponds to the equation $Y = \frac{100}{1 + \left(\frac{IC_{50}}{X}\right)^{Hillslope}}$.

To obtain the normalised activities for each inhibitor concentration, we assume that 100% is the maximum activity of the reaction without the presence of an inhibitor. 100% is equal to 1, so each percentage of inhibition is converted into a normalised activity (a number between 0 and 1, being 0 and 1 equal to 0% and 100%).

3. Results and discussion

3.1. Optimisation of the SIA methodology

The first stage of the SIA method development comprised the evaluation of the physical configuration and the chemical factors that affect the reaction. For this, it was used the univariate approach where each parameter is improved while the others are maintained constant. The main parameters studied include the reaction time, the reagents aliquots volume, their aspiration order, and the temperature. Table 2 lists these optimised parameters with the studied range and the chosen values.

Table 1. Analytical cycle used to perform the GST P1-1 inhibition assays.

Step	Position valve	Reagent	Volume (μ L)	Time (s)	Flow rate (mL min^{-1})	Action
1	8	CDNB	10	1.2	0.5	Aspiration of CDNB
2	6	DMSO/ inhibitor	10	1.2	0.5	Aspiration of DMSO/inhibitor
3	2	GST P1-1	20	2.4	0.5	Aspiration of GST P1-1
4	7	GSH	20	2.4	0.5	Aspiration of GSH
5	4	Mixture	333	20	1	Propulsion to the reactor coil
6	4	–	–	240	0	Stop period in reactor coil
7	4	Mixture	2000	60	2	Propulsion to the detector

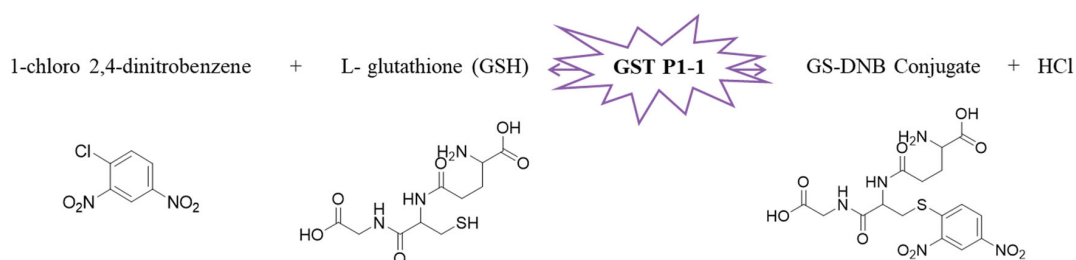


Figure 2. GST P1-1 enzymatic reaction.

Table 2. SIA system optimisation.

Condition	Range	Selected value
Stop period (min)	0–5	4
Aspiration order	GSH – DMSO/inhibitor – GST P1-1 – CDNB GSH – GST P1-1 – DMSO/inhibitor – CDNB CDNB – DMSO/inhibitor – GST P1-1 – GSH CDNB – GST P1-1- DMSO/inhibitor – GSH	CDNB – DMSO/inhibitor – GST P1-1 – GSH
Temperature (°C)	25–37	37
GST volume (μ L)	10–25	20
GSH volume (μ L)	15–25	20

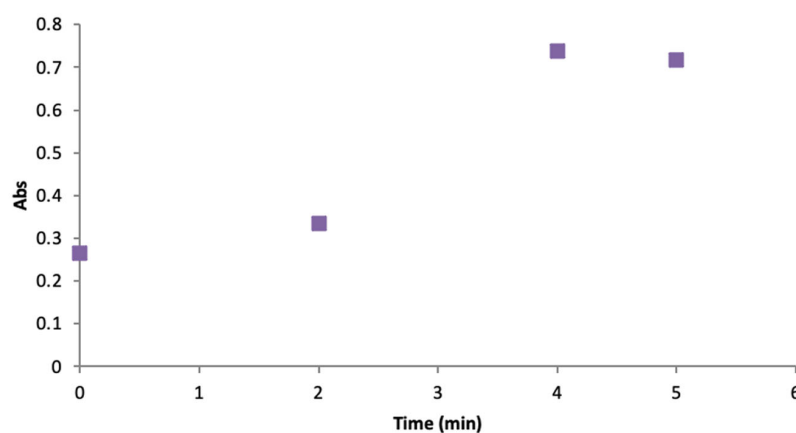


Figure 3. Optimisation of the GST P1-1 reaction time.

GST P1 activity was evaluated using a flow injection methodology, with a stopped-flow period at the reaction coil, enabling the GS-DNB product development without further increasing the dispersion. Stop reaction times of 0, 2, 4, and 5 min were assessed with a maximum increase in absorbance after 4 min of stopped time in the reaction coil (Figure 3).

The dispersion of the aliquots is essential for the partial zones overlap and consequent reaction. Also, the aspiration order is very important since the implemented sequence must ensure contact between the enzyme, the substrate, and the cofactor to maximise the chemical reaction. Hence, the aspiration order of the aliquots was also studied. The aspiration order CDNB – DMSO/inhibitor – GST – GSH was selected because the analytical signal is 4.3 times higher than the aspiration order CDNB – GST – DMSO/inhibitor – GSH and 1.7 times higher than the aspiration order GSH – GST – DMSO/inhibitor – CDNB/GSH – DMSO/inhibitor – GST – CDNB. Previously reported GST P1 assays are conducted at either 25 or 37 °C^{27,42,43}. To guarantee the best analytical signal and to simulate body temperature, 37 °C was used. Different GSH (12 mM) and GST P1 (5×10^{-6} g mL⁻¹) volumes were also tested with 20 µL being optimal for both. The flow rate of the propulsion to the detector was studied between 1- and 2-ml min⁻¹. It is evident that using the higher flow rate (2 ml min⁻¹), we obtained a higher absorbance of the final product (increases 1.5 times).

Using the optimised parameters, the analytical characteristics of the system were determined, to afford the concentration range in which there is a linear relationship between the CDNB concentration and the spectrophotometric signal. A calibration curve was obtained using standard solutions of increasing concentrations of CDNB. The obtained calibration curve was $\text{Abs} = (0.09 \pm 0.02) C \text{ (mM)} + (0.14 \pm 0.02)$; $R^2 = 0.99$, where Abs and C correspond to the absorbance intensity and the concentration of CDNB in mM, respectively, with a confidence limit for the intercept and slope of 95%. The linearity range of this method is between 0.85 and 44 mM. All the analytical features of this calibration curve are represented in Table 3.

3.2. Determination of GST P1-1 inhibition by organometallic compounds

The optimised GST P1-1 SIA method was used to determine the inhibition profiles of a library of organometallic compounds. Each concentration of each compound was performed in triplicate using a 1.8 mM of CDNB solution which was defined from the linear concentration range of the calibration curve.

Table 3. Analytical features of the calibration curve.

Analytical features	Values
Detection limit	0.26 mM
Quantification limit	0.85 mM
R^2	0.99
Slope	0.09
Intercept	0.14
Standard error of slope (Sb)	0.006
Standard error of intercept (Sa)	0.007
Standard error of regression	0.008
Sum of squares of the regression	0.01
Sum of squares of the residuals	0.0002

In Figure S1 in the Supplementary Material, it is represented the obtained polynomial relations depending on the normalised activity and each compound logarithm concentration. The resulting IC₅₀ values of the compounds are given in Table 4. The RSD obtained for all the IC₅₀ obtained with the new methods is around 7 ($n = 20$).

The known GST P1-1 inhibitor, ethacrynic acid (see Introduction), was used as a positive control. The literature reports different IC₅₀ values for **EA** ranging from 4.9 µM⁴⁴ to 12 µM²⁷, the latter being close to the IC₅₀ of 11.3 ± 0.8 µM obtained using the SIA system. The organometallic compounds display IC₅₀ values ranging from 6.7 ± 0.7 to 275 ± 9 µM with the results allowing some structure-activity relationships to be ascertained. RAPTA complexes **2a–d** showed a modest GST P1-1 inhibitory activity (average IC₅₀ 54 µM), albeit considerably higher than the related Ru(II)-arene compound **3a** (RUCYN, IC₅₀ 235 µM). Conjugation of **EA** to the Ru(II) and Os(II) η⁶-arene complexes via a modified triaryl phosphine ligand (complexes **7a–c**) does not result in effective GST P1-1 inhibitors with IC₅₀ in the range 137–181 µM. In this respect, modest GST inhibitory activity was previously ascertained for **7c** and related Os(II)-EA conjugates in ovarian cancer cell lines (A2780, A2780cisR)³⁸. In contrast, the Ru(II) (**7d**) and Ir(III) (**7e**) derivatives with a doubly-derivatized **EA** and flurbiprofen 2,2'-bipyridine ligand are potent GST P1 inhibitors. The iridium compound **7d** is the strongest inhibitor of GST P1 (IC₅₀ = 6.7 ± 0.7 µM) in the present work, more effective than **EA**. Compound **7e** exhibits significant cytotoxicity on a panel of cancer cell lines, with its biological activity benefiting from the combined action of the metal scaffold and the two enzyme inhibitors³⁷.

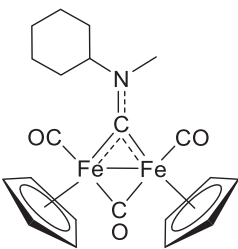
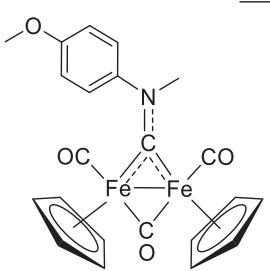
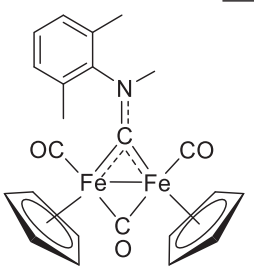
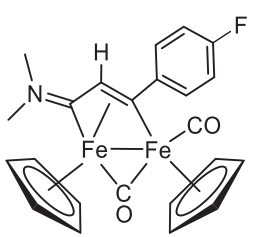
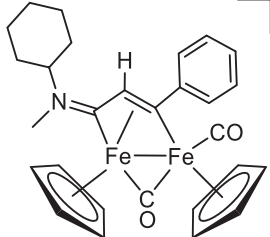
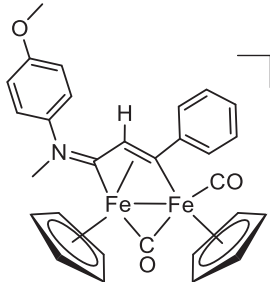
The diiron cyclopentadienyl complexes with aminocarbyne (**4a–d**) and vinyliminium (**5a–e**) ligands are either modest inhibitors of GST P1-1 or are essentially inactive, with IC₅₀ values in the

Table 4. GST P1-1 inhibition of ethacrynic acid and a series of organometallic compounds.

Compound	Structure ^a	IC ₅₀ (μM ± SD)
1 Ethacrynic acid		11.3 ± 0.8
2a		57 ± 4
2b		76 ± 6
2c		24.7 ± 1.4
2d		61 ± 5
3a		235 ± 2
4a		275 ± 9

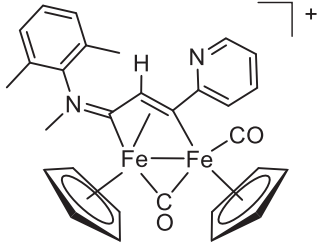
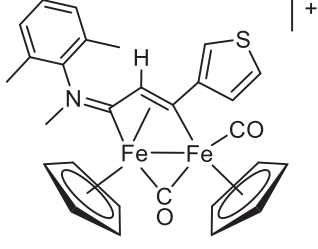
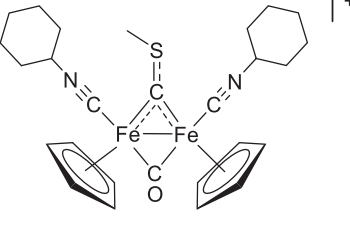
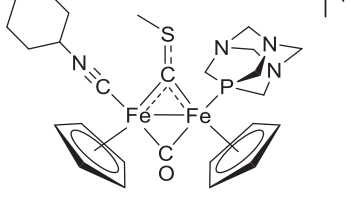
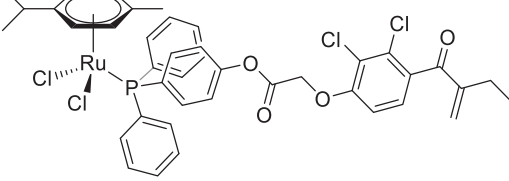
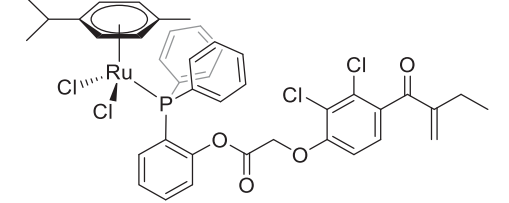
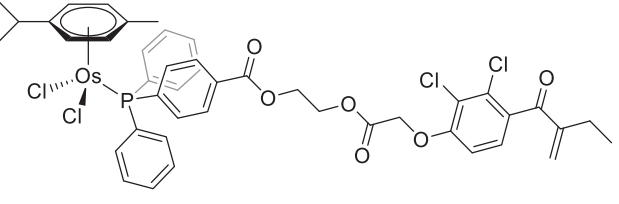
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Table 4. Continued.

Compound	Structure ^a	IC ₅₀ (μM ± SD)
4b		219 ± 8
4c		113 ± 5
4d		72 ± 5
5a		83 ± 9
5b		91 ± 9
5c		46.8 ± 2.8

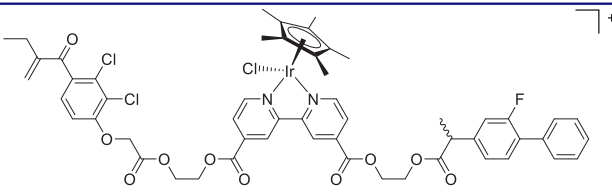
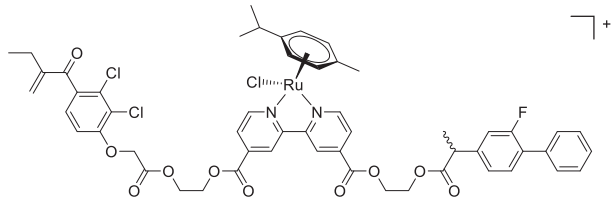
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Table 4. Continued.

Compound	Structure ^a	IC ₅₀ (μM ± SD)
5d		34.0 ± 4.3
5e		30.3 ± 8.3
6a		17.4 ± 2.8
6b		165 ± 4
7a		181 ± 7
7b		152 ± 6
7c		138 ± 12

(continued)

Table 4. Continued.

Compound	Structure ^a	IC ₅₀ (μM ± SD)
7d		6.7 ± 0.7
7e		12.1 ± 1.8

^[a] Cationic diiron complexes as CF₃SO₃[−] salts; cationic Ru/Ir complexes (**3a**, **7d**, **7e**) as NO₃[−] salt.

Table 5. Assessment of SIA and batch IC₅₀ values.

Compounds	IC ₅₀ batch ± SD (μM)	IC ₅₀ SIA ± SD (μM)
1	13.58 ± 0.02 ⁽¹⁾	11.3 ± 0.8
4a	226 ± 3	275 ± 9
4c	61 ± 3	113 ± 5.3
4d	33 ± 6	72 ± 5
6a	11.5 ± 0.7	17 ± 3
7e	11.6 ± 0.5	12 ± 2

IC₅₀ value obtained using the same batch method in reference⁴¹.

range of 30–275 μM. The presence of (hetero)aromatic substituents on the bridging ligand is correlated with an increase in inhibitory activity, e.g. compare the IC₅₀ values of **4a,b**, and **5a,b** with **4c,d**, and **5c,d,e**. The thiocarbonyl complex **6a**, with two cyclohexyl isocyanide ligands, is a comparatively good GST P1-1 inhibitor with an IC₅₀ value of 17.4 ± 2.8 μM. Notably, the introduction of a PTA ligand in **6b** dramatically impairs the ability of the compound to inhibit GST P1-1. Nevertheless, both **6a** and **6b** are effective DHFR reductase inhibitors.⁴⁵

2.3. Validation of the SIA system

To ensure the validation of the newly developed methodology, some compounds were also analysed using a batch procedure. The IC₅₀ values obtained were compared with those obtained from the SIA method in Table 5 and are in reasonable agreement, showing the same trend and similar values for the active inhibitors.

The results were evaluated using the *t*-test, carried out as a bilateral coupled test. In agreement with the student's *t*-test, the tabulated *t* value (2.57), is lower than the calculated *t* value (2.3). Thus, there are no statistical differences at the confidence level of 95%⁴⁶, further confirmed by a linear correlation as described by the equation:

$$IC_{50} \text{ SIA} = (1.2 \pm 0.3) IC_{50} \text{ BATCH} - (12.4 \pm 30.9) \quad (1)$$

where IC₅₀SIA and IC₅₀BATCH are, respectively, the IC₅₀ results acquired using the SIA and batch methods, with intercept and slope confidence limits of 95%. The predictable intercept and slope values were not considered significantly different, respectively, from 0 to 1, confirming the SIA and batch methods

agreement. The coefficient of Pearson correlation for the two methods is near 1 (~ 0.98).

According to the goal of this study and all the advantages of using the SIA methodology such as robustness, reproducibility, versatility, computer control, and reliability, the analytical signal is obtained in 5 min whereas 8 min are required for the batch procedure. The SIA system also requires fewer materials than in batch method, i.e. 5 times less GSH solution, 1.25 times less CDNB solution, and 2.3 times less GST P1-1 solution.

4. Conclusions

An SIA system was developed to evaluate the GST P1-1 inhibition capacity of organometallic complexes with putative anticancer activity. Some of the compounds tested exhibited good inhibition profiles with the low μM range of IC₅₀ values and were comparable to the benchmark organic inhibitor, EA. It is therefore expected that these compounds could be useful to treat cancers where GST P1-1 is overexpressed^{47–49}. The SIA method was found to be a good alternative to the batch method reducing the analysis time and the number of reagents required. Hence, the SIA method is considered an important automatic alternative for the analysis of GST P1-1 inhibitors.

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Disclosure statement

The authors declare no conflict of interest.

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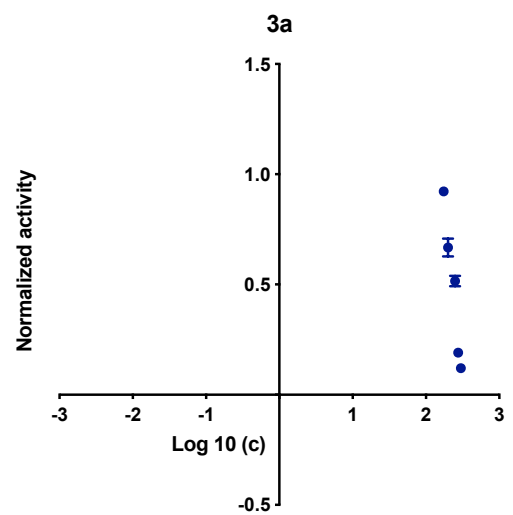
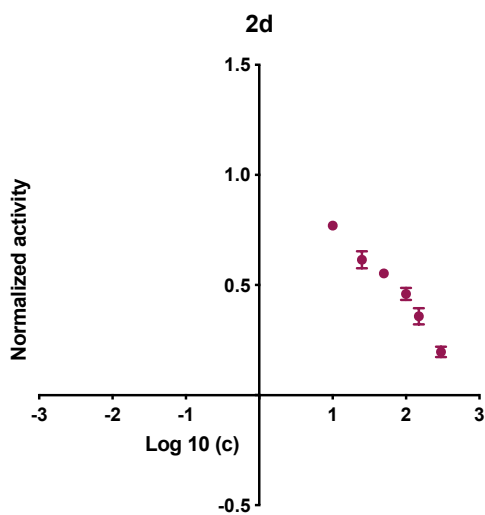
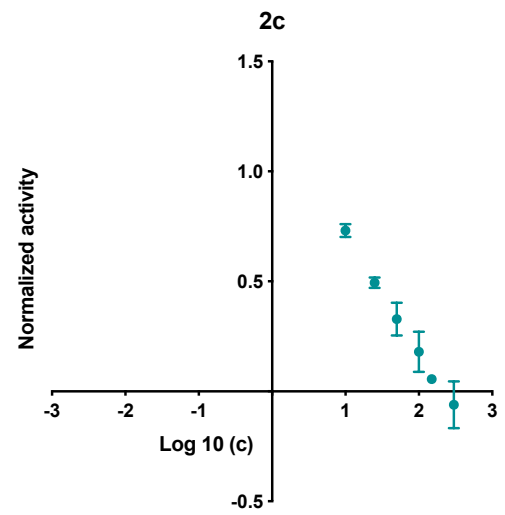
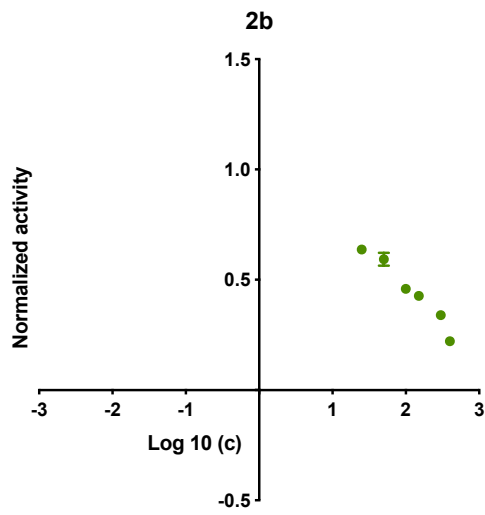
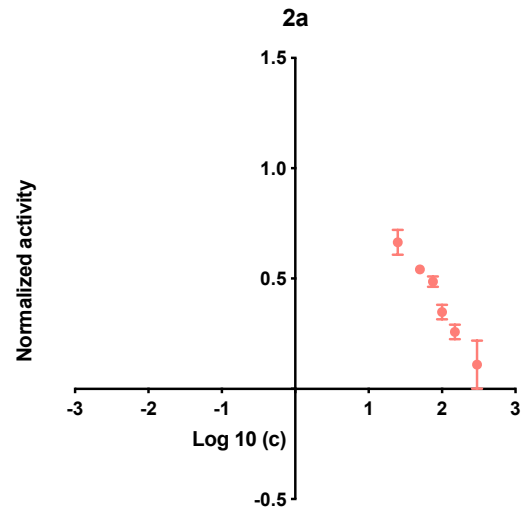
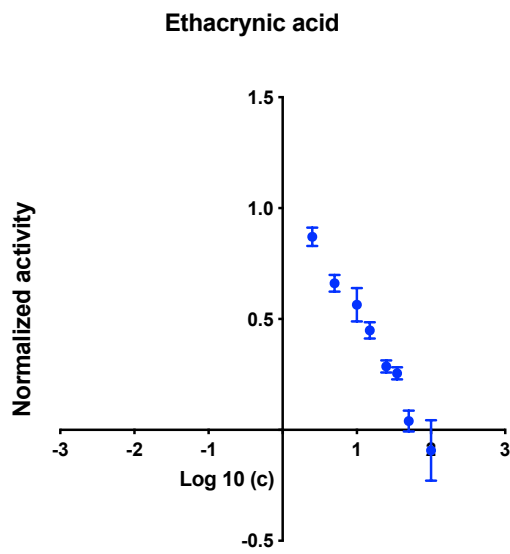
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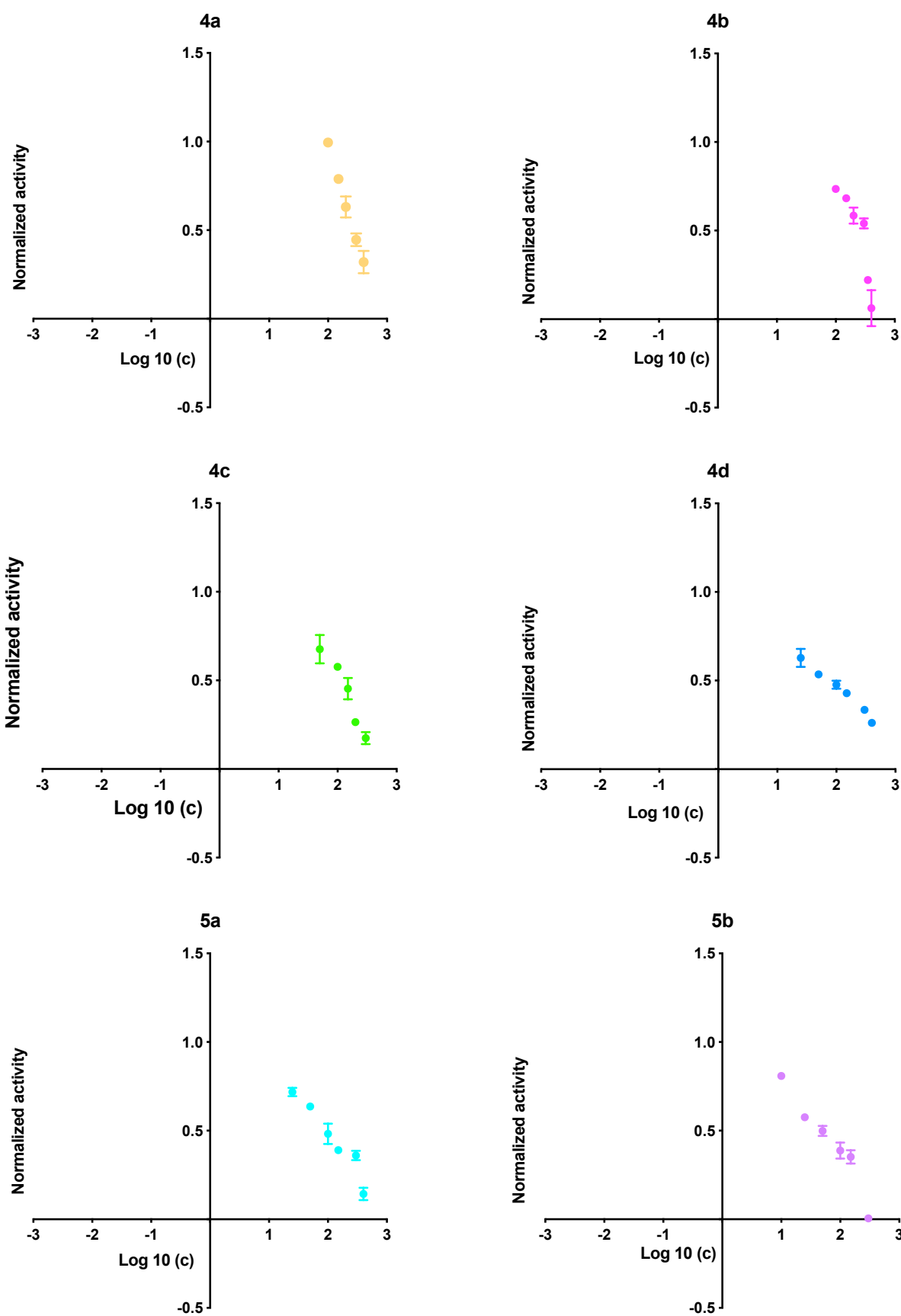
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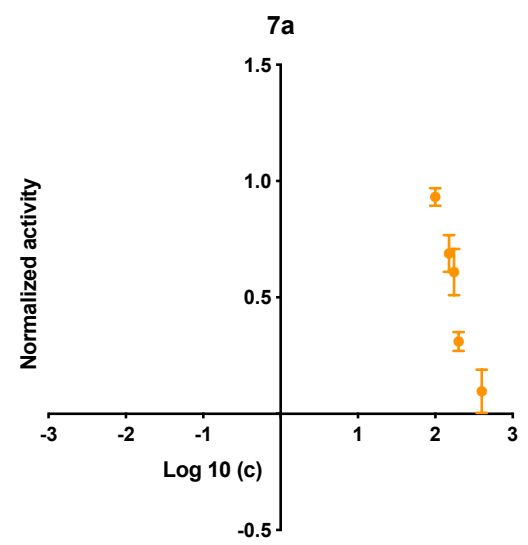
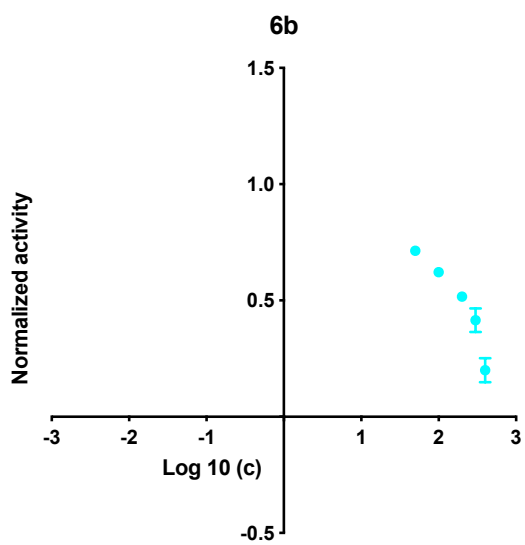
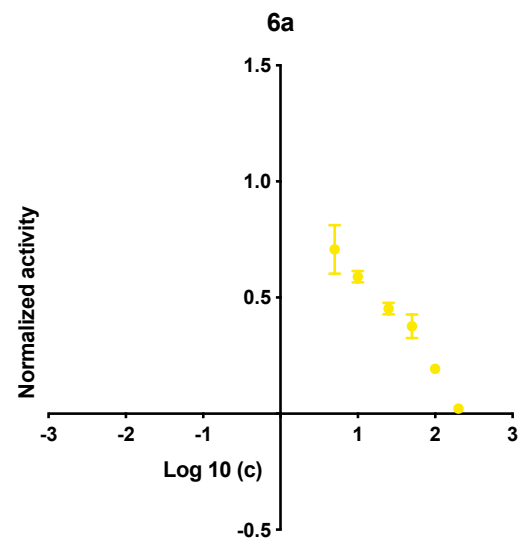
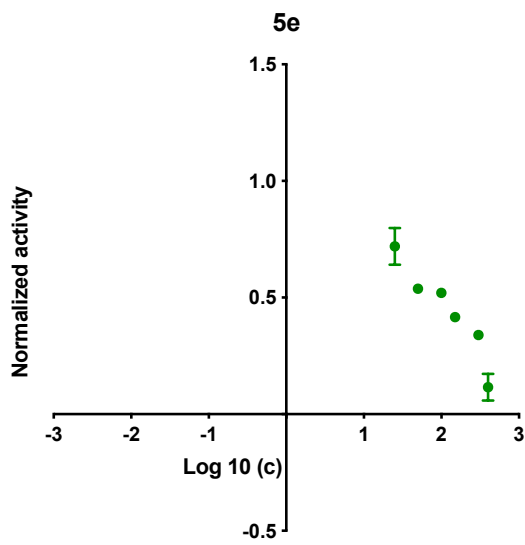
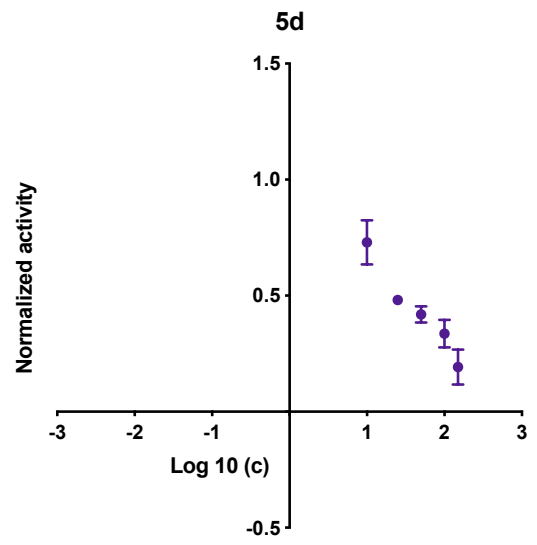
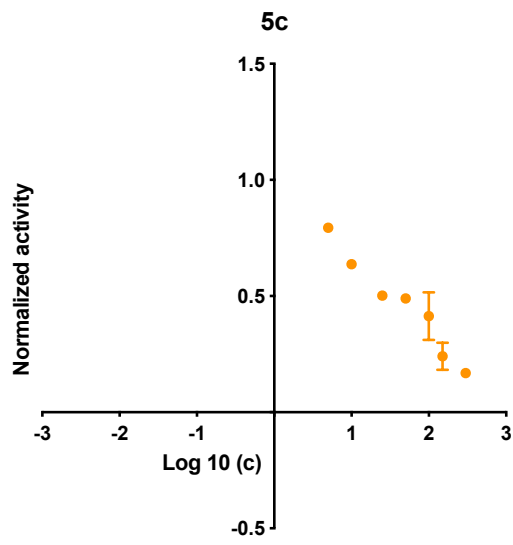
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Supplementary information







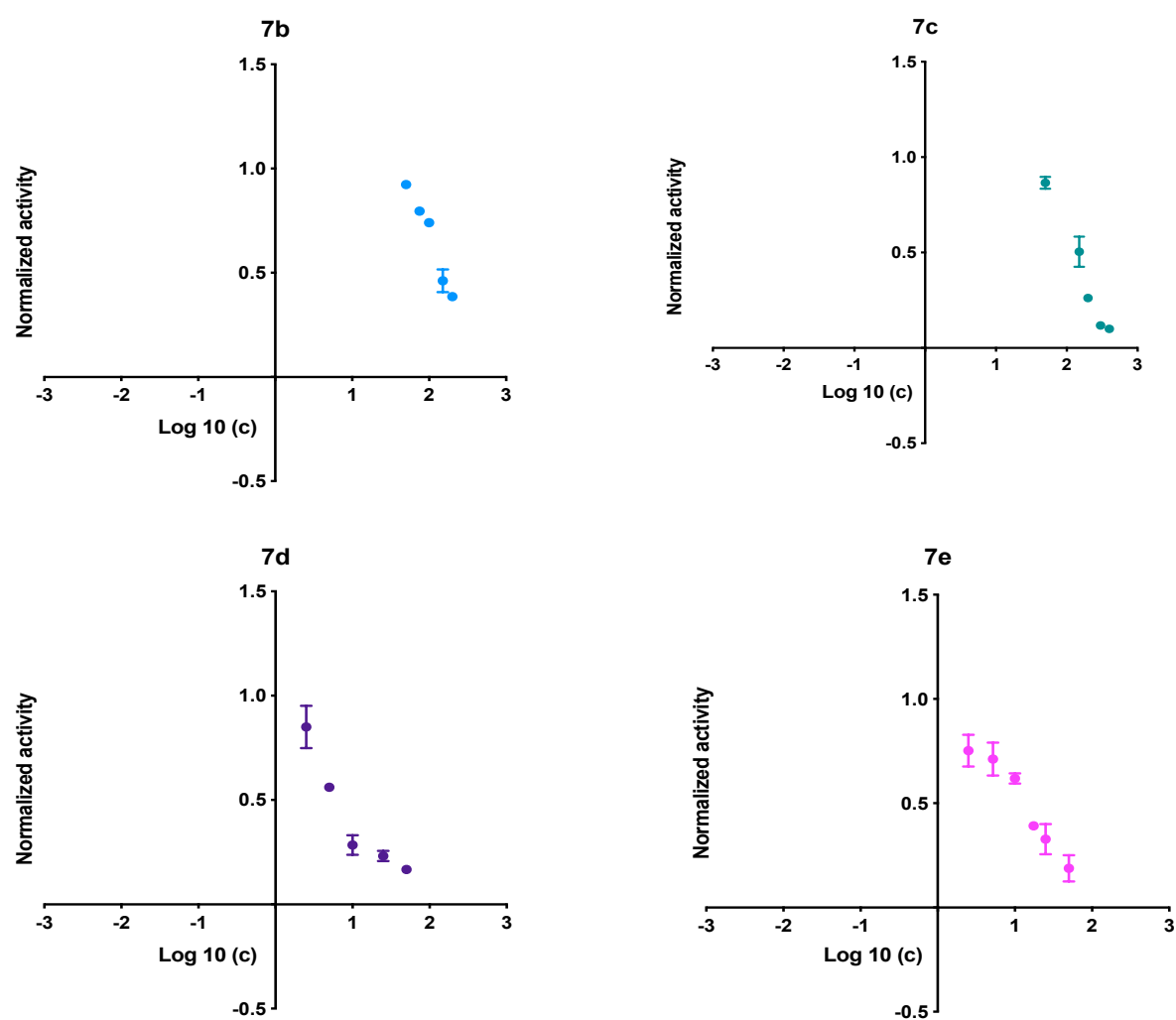


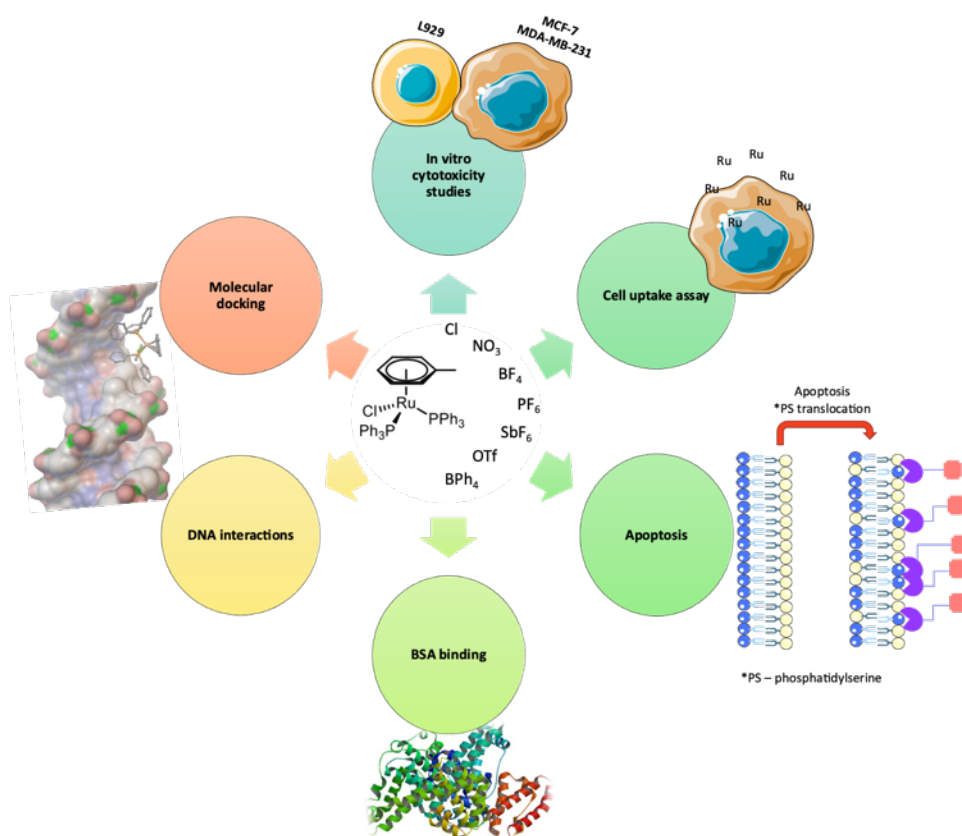
Figure S1 - Experimental inhibition data of the compounds tested in the GST P1-1 inhibition assays

Table S1 – Fitting parameters of each curve shown in Fig S1.

Compound	Coefficient of determination R ²
1 Ethacrynic acid	0.98
2a	0.98
2b	0.96
2c	0.99
2d	0.97
3a	0.95
4a	0.99
4b	0.78
4c	0.94
4d	0.98
5a	0.90
5b	0.93
5c	0.93
5d	0.95
5e	0.96
6a	0.98
6b	0.88
7a	0.96
7b	0.92
7c	0.97
7d	0.87
7e	0.94

4 – Tailoring the cytotoxicity of ruthenium(II) arene compounds for enhanced activity against breast cancers

In this work, I performed cytotoxicity, apoptosis, and cell uptake assays. Moreover, I also achieved gel electrophoresis, and circular dichroism techniques to identify the interaction between some compounds with DNA. The fluorescence measurements to identify the interaction of the most cytotoxic compound with DNA and BSA were also performed by me. In the end, I did the data analysis and statistical analysis of the cytotoxicity, apoptosis, cell uptake, interaction with DNA obtained by gel electrophoresis, and circular dichroism results and I was one of the people that wrote the original draft of the manuscript for later publication.



Tailoring the cytotoxicity of ruthenium(II) arene compounds for enhanced activity against breast cancers

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Abstract

Ruthenium-based complexes have been suggested as promising anticancer drugs with reduced general toxicity compared to platinum-based drugs. In particular, $\text{Ru}(\eta^6\text{-arene})(\text{PTA})\text{Cl}_2$ (PTA = 1,3,5-triaza-7-phosphaadamantane), or RAPTA, complexes have demonstrated efficacy against breast cancer by suppressing metastasis, tumorigenicity, and inhibiting the replication of the human tumor suppressor gene BRCA1. However, RAPTA compounds have limited cytotoxicity, leading to the evaluation of more cytotoxic RAPTA derivatives for enhanced activity against breast cancers. This study explored the activity of a series of ruthenium(II) arene compounds against MCF-7 and MDA-MB-231 breast cancer cell lines. A family of cationic ruthenium(II) toluene bistrisphenylphosphine chloride, viz. $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]^+$, were identified as promising candidates. Notably, $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]\text{Cl}$ was found to be remarkably stable, presented high cytotoxicity and selectivity for cancer cell lines, significant cellular uptake, and water solubility, and was shown to mainly interact with the minor groove of DNA.

Keywords: bioorganometallic chemistry; inorganic medicinal chemistry; metal drugs; breast cancer; cytotoxicity; DNA interactions; ruthenium-based complexes.

1. Introduction

Breast cancer is the most common malignant diagnosed tumor in women and the leading cause of cancer death in the world, and several factors contribute to its high mortality, i.e. acquired resistance during chemotherapy, active invasion, and advanced metastasis (1, 2). Despite drug resistance, chemotherapy is the main treatment option for breast cancer once it is in an advanced state, and cisplatin is the most frequently used drug used to treat malignant breast cancers, both as a single agent or in combination with other chemotherapeutics (3-5). Despite the undoubted success of cisplatin and its derivatives in breast cancer treatment, these metal-based compounds exhibit several side effects due to their lack of selectivity (6).

Ruthenium complexes have been attracting interest as promising alternatives to platinum-based anticancer drugs, apparently presenting lower side effects, and some show promising activity against different breast cancer cell lines (7-9). Although ruthenium-based compounds are not currently used in the clinic, two ruthenium(III) compounds, NAMI-A ((ImH)[*trans*-RuCl₄(DMSO-S)(Im)], Im =imidazole) (10) and KP1019 (IndH)[*trans*-RuCl₄(Ind)₂], Ind = indazole) (11), completed the phase I clinical trials and are nowadays in phase II trials.

Ruthenium (II)-arene complexes, Ru(η^6 -arene)(PTA)Cl₂ (PTA = 1,3,5-triaza-7-phosphaadamantane), frequently called RAPTA compounds, and related compounds, have been extensively studied for their anticancer properties (12). Although RAPTA compounds tend to display limited cytotoxicity to many cancer cell lines, Ru(η^6 -benzene)(PTA)Cl₂, Ru(η^6 -*p*-cymene)(PTA)Cl₂, and Ru(η^6 -toluene)(PTA)Cl₂ have an impact on metastatic tumors *in vivo* (13, 14), and also to exert an antiangiogenic effect (15). Subsequently, it was shown that Ru(η^6 -*p*-cymene)(PTA)Cl₂ reduces the growth of primary tumors in preclinical models for ovarian and colorectal carcinomas (16). Various RAPTA compounds can sensitize cancer cells towards other drugs when applied in combination and several promising *in vivo* studies against primary tumors were reported (17, 18).

Recently, BRCA1 gene damage in cancer cells has received much attention as a potential molecular target for metal-based anticancer compounds (19, 20), and RAPTA-T was shown to inhibit BRCA1 replication in a dose-dependent manner, causing more BRCA1 damage in BRCA1-defective breast cancer (HCC1937 - BRCA1 mutant, triple-negative breast cancer) compared to BRCA1-competent adenocarcinoma (MCF-7) at equal doses (21). RAPTA-T exhibits a more noticeable effect with the highly invasive metastatic MDA-MB-231 breast cancer cells compared to non-invasive MCF-7 cells or non-tumorigenic HBL-100 cells derived from healthy (22) due to the reduction in the ability of several metastasis progression steps, such as the ability to migrate, invade and adhere to a new

organ, and the detachment from the primary tumor and the degradation of the extracellular matrix, comparing the behavior of MDA-MB-231 highly invasive breast cancer cells to that of MCF-7 (14). Polymeric micelles containing RAPTA-C inhibit the growth of 2D and 3D cultures of MCF-7 and MD-MB-231 (23). Another example is the Ru-arene complex $[\text{Ru}(\eta^6\text{-}p\text{-cymene})(o\text{-phenylenediamine})\text{Cl}]^+$ (*o*-PDA) that also presents a potent anticancer effect against breast cancer while not affecting the cell growth of human epithelial cells, MCF10A. It was also demonstrated that *o*-PDA inhibited the production of soluble growth factors such as VEGF-A, PDGF-AA, and GM-CSF at the transcriptional levels (24). Also, the hetero multinuclear complex $[\text{Na}_2][\text{RuCl}_4(\text{DMSO-S})(\mu\text{-pyz})]_2\text{PtCl}_2$, AH197 (Ru11/AH197) effect on the motility and DNA electrophoretic mobility was studied in breast cancer (MDA-MB-231) cells and was compared to that of $[\text{K}][\text{RuCl}_4(\text{DMSO-S})(\mu\text{-pyz})]\text{Pt}(\text{DMSO-S})\text{Cl}_2$ (IT127), NAMI-A, and $\text{Na}[trans\text{-RuCl}_4(\text{DMSO})(\text{pyz})]$ (AH403) (25). The Ru_2Pt trinuclear species showed higher inhibitory activity in the order $\text{Ru11} > \text{IT127} > \text{NAMI-A} > \text{AH403}$ (25), while it was shown that the inhibition of cell motility might contribute to the anti-metastatic properties of the complexes (26).

Since RAPTA compounds display promising activity in MCF-7 and MDA-MB-231 breast cancer cells but are not strongly cytotoxic, we hypothesized that RAPTA-like complexes with higher cytotoxicity might offer enhanced activity against breast cancers while potentially retaining their antimetastatic properties. Hence, we report a systematic study assessing the *in vitro* effects of a small library of ruthenium-arene (RAPTA or RAPTA-like) compounds on MCF-7 and MDA-MB-231 breast cancer cell lines and L929 fibroblast, a model non-tumorigenic cell type. For a cationic complex present in the library, *i.e.* $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]^+$, the impact of different counter-anions on the cytotoxicity was explored – an approach rarely explored in inorganic medicinal chemistry beyond changing the counterion for synthetic, purification reasons or to facilitate crystallisation (27-29).

2. Materials and methods

$[\text{Ru}(\eta^6\text{-toluene})\text{Cl}_2]_2$ (30), $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)\text{Cl}_2]$ (31) and **1** (32), **2** (32), **3** (33), **4** (13), **5** (34), **6** (34), **7** (33), **8** (35) and **9** (31) were prepared according to reported procedures and their spectroscopic data are in agreement with those reported. Compounds **10** and **11CA** (CA = counteranion, 7 examples) were prepared by the treatment of $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]$ with PTA or triphenylphosphine in the presence of the appropriate silver or sodium counterion salt, except in the case of **11Cl** which was prepared by resin anion exchange. Compounds **11BF₄** (36) and **11PF₆** (31) were previously reported, but in this work, they were prepared following a different synthetic route. Full experimental details and characterization data are provided in the Supporting Information.

Adherent mouse fibroblast connective tissue cells (L929) and human breast cancer cells (MCF-7 and MDA-MB-231) were obtained from the European Collection of Authenticated Cell Cultures (ECACC), Public Health England, Salisbury, UK and ATCC, Middlesex, UK, respectively. All cell culture reagents i.e., Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin mixture, and trypsin-EDTA 0.25% (v/v) were purchased from Gibco® (Invitrogen Corporation, UK). Deoxyribonucleic acid (calf thymus) and Dimethyl sulfoxide (DMSO) were purchased from Merck® (EMD Millipore Corp, USA). FITC Annexin V Apoptosis Detection Kit with 7-AAD was purchased from BioLegend®. Agarose basic was purchased from PanReac AppliChem®. pBR322 DNA, GeneRuler 1kb Plus DNA ladder, and SYBR™ Safe DNA Gel Stain were purchased from Thermo Fisher Scientific®. Methyl green, DAPI, and propidium iodide 95% were purchased from Sigma-Aldrich®, Ivalua®, and Acros Organics®, respectively.

2.1. Stability in solution and reactivity with model biomolecules

The stability of **11CA** under pseudo-physiological conditions was assessed using ESI-MS and ^1H and ^{31}P NMR spectroscopy. Furthermore, the interaction between **11Cl** and different model biomolecules (amino acids: glutamic acid, histidine and cysteine, and nucleotide, dGMP), was also studied. The catalytic activity of **11Cl** in transfer hydrogenation and glutathione (GSH) oxidation reactions was monitored by ^1H and ^{31}P NMR spectroscopy under previously reported biologically relevant conditions (37).

2.2. Preparation of stock solutions of the compounds

Stock solutions were prepared in supplemented DMEM with 10% DMSO to ensure solubility. Then, necessary dilutions were made using the supplement DMEM, to prepare the range of tested concentrations. Since DMSO was used to solubilize the compounds, a supplemented DMEM + DMSO (at the same percentage as in the sequential dilutions) was considered as a control in the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

2.3. Cell lines and culture conditions

L929, MCF-7, and MDA-MB-231 cell lines were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) of penicillin-streptomycin. The cells were incubated in a humidified chamber at 37 °C under saturated air with a 5% CO₂ atmosphere. Cells were passed at 80% - 90% of confluency with 0.25% (w/v) trypsin and the cell culture media was changed every 2-3 days.

2.4. Cell viability assessment

The compounds were tested on two breast cancer cell lines (MCF-7 and MDA-MB-231) and L929 fibroblasts, used as a reference for the safety evaluation according to ISO 10993-1:2009 "Biological evaluation of medical devices". Cells were seeded on sterile 96-well plates at 50,000 cells per well (L929) and 20,000 cells per well (MCF-7 and MDA-MB-231) in supplemented DMEM medium (100 µL) for 24 h. The culture medium was removed and new media containing different concentrations of the compounds (0 - 1000 µM) was added to the wells (100 µL). Control cells were incubated under the same conditions in the absence of a compound. Cells were incubated for 24 and 72 h under the same conditions described above. Then, the medium was removed, and the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg mL⁻¹) for 3 h. After this time, the medium was removed, and formazan crystals were solubilized with DMSO (100 µL/well). The absorbance in each well was read on a Synergy™ HT Multimode microplate reader (BioTek Instruments; Winooski, VT, USA) at 570 and 630 nm (for background correction), and the cell viability was calculated according to the following equation (Eq. 1):

$$\text{Cell viability (\%)} = \frac{\text{OD}_{570}(\text{sample}) - \text{OD}_{630}(\text{sample})}{\text{OD}_{570}(\text{control}) - \text{OD}_{630}(\text{control})} \times 100 \quad (1)$$

where OD_{570(sample)} and OD_{630(sample)} are the optical densities of the solutions containing the compounds and OD_{570(control)} and OD_{630(control)} are controls without compound.

2.5. Analysis of cell apoptosis

Apoptosis in MCF-7 and MDA-MB-231 cells was determined using FITC Annexin V with a 7-aminoactinomycin D (7-AAD) staining kit (BioLegend®, California, US) according to the manufacturer's instructions. Briefly, cells (120,000 cells per well) were incubated in sterile 24-well plates with the IC₅₀ concentration of the compounds for 24 h. Following incubation, the cells were washed with PBS twice and detached using 0.25% trypsin. After obtaining

cell pellets by centrifugation (300 x g for 5 min), the cells were resuspended in 100 µL of PBS, followed by the addition of 10 µL of FITC Annexin V and 7-AAD, and then incubated for 15 min at room temperature while protected from light. After the incubation period, 200 µL of binding buffer was added to the cell suspension and the samples were analyzed immediately using a BD Accuri C6 flow cytometer with a minimum of 10,000 events. The assays were performed in quadruplicate and the results were analyzed with Accuri C6 analysis software.

2.6. Cell uptake assay

MCF-7 and MDA-MB-231 cell lines were seeded in 6-well plates, ca. 1,200,000 cells, and incubated in supplemented DMEM (2 mL). After 24 h, the IC₅₀ concentrations and 10 times the IC₅₀ concentration were added to each well in duplicate. Controls for each cell line were also added to each 6-well plate. The cells were incubated for 8 h and then washed 3 times with PBS solution. Then, 750 mL of 0.25% trypsin was added and after about 5 minutes in an incubator all the cells had detached from the flask and 1.25 mL of DMEM was added to each well. The cells were centrifuged at 120 xg for 10 min and then digested with 2 mL of concentrated HNO₃ (69%, ROTIPURAN Supra, Roth) in PP digestion vials using a heating block system (DigiPREP Jr. 15ml, 40 Pos, SCP Science). The digestion program comprised 15 min to heat to 100 °C and was then maintained at 100 °C for 60 min. After digestion, the samples were precisely adjusted to 5 mL with ultrapure water. Samples were further diluted 50 times with 2% HNO₃ solution and their Ru content was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using KED mode with He as a collision gas on a Nexlon 350 D ICP-MS instrument (PerkinElmer). Yttrium was added as an internal standard at a concentration of 2 ppb to all the solutions and Ru quantitation was performed using an external calibration curve with standards in the 0.005-50 ppb range. Analysis was performed in triplicate.

2.7. BSA binding assay

BSA (16 mM) in 10 mM Tris-HCl pH 7.6 + 1 mM EDTA solution and varying concentrations of ¹¹¹Cl (0-32 mM) were incubated at 37 °C for 24 h. Afterward, the fluorescence spectra were measured for each solution between 300 and 500 nm (I_{ex} = 280 nm). The intensity at I_{max} (340 nm) was recorded. Quenching parameters were obtained by applying the Stern–Volmer equation (Eq. 2):

$$\frac{F_0}{F} = 1 + K_{sv} [Q] = 1 + k_q \tau_0 [Q] \quad (2)$$

where F_0 and F represent the fluorescence intensities of the DNA–dye complex in the absence and presence of **11Cl**, respectively; K_{sv} is the linear Stern–Volmer quenching constant; $[Q]$ is the molar concentration of the quencher, i.e., metal complex; k_q is the bimolecular quenching rate constant; and τ_0 is the lifetime of BSA fluorescence in the absence of any quencher (6.0 ns (38)). The modified Stern–Volmer plot (Eq. 3) was used to obtain the binding parameters:

$$\log \frac{F_0 - F}{F} = \log K_b + n \log [Q] \quad (3)$$

where, F_0 and F are the fluorescence intensities of BSA before and after metal complex binding; K_b is the binding constant; n is the number of binding sites, and $[Q]$ is the molar concentration of the quencher.

2.8. Competitive DNA binding assay

Emission intensity measurements were performed using a SpectraMax M5e reader at room temperature. A 50 μ M (bp) of ct-DNA was pre-incubated with the groove binding agents (MG and DAPI) (2 μ M) and the intercalating agent propidium iodide (2 μ M) for 30 min at 37 °C, to allow full interaction of the dyes into the ctDNA. Increasing amounts of **11Cl** (0 - 100 μ M) were added to this mixture and the final samples were incubated for 24 h at 37 °C. After incubation, the emission spectra were recorded at excitations of 633, 358, and 535 nm for the measurements of MG, DAPI, and PI, respectively. Quenching parameters were obtained by applying the Stern–Volmer equation (Eq. 2). The apparent binding constants, K_{app} , were calculated according to the competitive binding model described by Tse and Boger (39) using Eq. 4 (40):

$$K_{probe} [probe] = K_{app} c_{50} \quad (4)$$

where K_{probe} is the binding constant of the probe, 9×10^5 and 5×10^6 M^{-1} for MG (41) and DAPI (42), respectively; $[probe]$ is the concentration of probe used, 10 μ M for both MG and DAPI; and c_{50} is the complex concentration when the fluorescence intensity of the probe is 50%. This value is obtained from the plot F_0/F vs $[Q]$ when $F_0/F = 2$

2.9. Interaction of **11Cl** and **11BPh₄** with plasmid DNA by gel electrophoresis

Assays were performed using 1.2% (w/v) agarose gels. A plasmid pBR322 DNA was purchased from Thermofisher Scientific[®] at a concentration of 0.5 µg/µL in phosphate buffer 50 mM (pH 7.4). Stock solutions of the complexes were prepared in DMSO at 1 mM and assays were performed with a total volume of 20 µL. A 20 µL portion containing 0.125 µg/µL of DNA in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was incubated with the compounds at r_i values ranging from 0.05 to 0.5 ($r_i = [\text{complex}]/[\text{no. nucleotide}]$). Samples were incubated at 37 °C for 24 h, after which 2 µL of the TriTrack DNA loading dye buffer was added. The total amount of the sample (20 µL) was loaded in the agarose gel (1.2% w/v), and electrophoresis was carried out for a period of 100 min at 40 V in TAE 1× (Tris-acetate/ EDTA) buffer. After electrophoresis, the gel was immersed in 200 mL of TAE 1x buffer containing 20 µL of a stock solution of SYBR safe for 30 min to stain the DNA. Then, the gel was washed with Mili Q water for 10 min and the stained gel was analyzed with a blue light in a Safe Imager[™] 2.0 (Invitrogen).

2.10. Interaction of **11Cl** with calf thymus DNA by circular dichroism

Calf thymus (ct) DNA (Merck[®]) was used to perform the circular dichroism assay to detect conformational changes in the ctDNA induced by the compounds. CD spectral data was collected on a Chirascan[™] V100 (AppliedPhotophysics) spectropolarimeter equipped with a large area Avalanche photodiodes detector and computer-controlled thermoelectrical cell holder using a 1 cm path length quartz cuvette. CD spectra of ctDNA in the absence and presence of different concentrations of **11Cl** were recorded at wavelengths ranging from 320 to 190 nm under constant nitrogen flush at 25 °C. For each CD spectrum, the number of scans was set to 5, and spectral data were collected at 0.1 ms intervals. Also, methyl green (MG) and 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were analyzed as groove binders and intercalator, respectively, to help identify the binding mode of **11Cl** to DNA. Samples were prepared in 10 mM Tris-HCl buffer (pH 7.6) with 1 mM EDTA. The concentration of ctDNA was fixed at 100 µM (concerning base pairs) and the ctDNA to **11Cl** ratio was varied between 0 and 1. The samples were incubated for 24 h at 37 °C and then analyzed in the CD spectropolarimeter.

2.11. Molecular docking studies

Molecular docking studies were performed using AutoDock 4.2 (43). The crystal structure of a B-DNA dodecamer d(CGCGAATTCGCG)₂, **1BNA** (44), the 12-mer with DAPI bound in the minor groove, **5T4W** (45), and the 12-mer with an acridine-based intercalator, **1G3X** (46), were obtained from the Protein Data Bank (PDB). The structure for **11Cl** was

obtained from the X-ray crystal structure with the counterion and solvent molecules removed to model **11**⁺.

2.12. Statistical analysis

Each cell viability result represents the mean $\pm$ standard deviation (SD) for $n = 4$ replicates in each of 3 independent assays, resulting in 12 independent values. Each apoptosis value determination represents the mean $\pm$ standard deviation of 2 independent assays. The IC₅₀ values were obtained using GraphPad Prism software and ANOVA (analysis of variance) was performed to determine the statistical difference between the IC₅₀ values of different cell lines.

3. Results and discussion

3.1. Synthesis, characterization, and stability in solution of the compounds

A collection of ruthenium(II) arene compounds (Figure 1) was acquired. Compounds **1** (32), **2** (32), **3** (33), **4** (13), **5** (34), **6** (34), **7** (33), **8** (47) and **9** (31) have been reported previously and were prepared according to the published procedures. Compounds **10**, **11NO**₃, **11BF**₄, **11OTf**, **11PF**₆, **11SbF**₆, and **11BPh**₄ were prepared by the treatment of [Ru(η^6 -toluene)(PPh₃)Cl₂] with PTA (**10**) or triphenylphosphine (**11**⁺) in the presence of the appropriate silver or sodium counterion salt. **11Cl** was prepared by resin anion exchange. Compound **10** is similar to the previously reported [Ru(η^6 -*p*-cymene)(PTA)(PPh₃)Cl] (**48**). Compounds **11BF**₄ (**36**) and **11PF**₆ (**31**) had been reported previously and employed as the homogenous catalyst, but in this work, they were prepared following a different synthetic route.

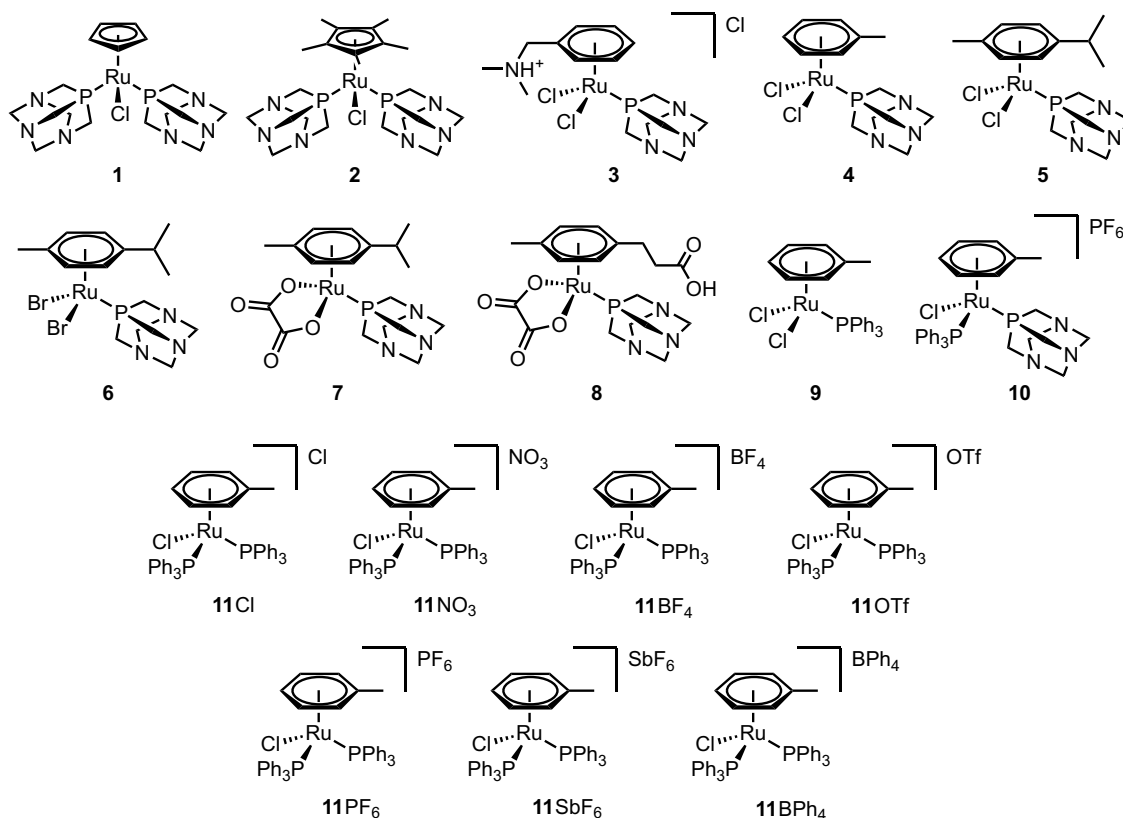


Figure 1. Structures of the compounds used in this study.

³¹P NMR spectroscopy confirmed the successful synthesis of compounds **10** and **11CA**, which show an upfield shift of the triphenylphosphine singlet of [Ru(η⁶-toluene)(PPh₃)Cl₂]. The spectra of the asymmetrical bis-phosphine complex **10** exhibit two doublets of equal intensity with large ²J_{PP} couplings of ca. 54 Hz, confirming the presence of the two phosphines coupling to each other. Chemical shifts of the coordinated PPh₃ ligands in **11CA** range from 22.1 to 22.8 ppm. No major differences were observed in the chemical shifts of the cation **11**⁺ in the ¹H and ¹³C NMR spectra of **11CA**. Additionally, complexes **10** and **11CA** were analyzed by ESI-MS in positive ion mode, which afforded intense peaks with the correct isotopic pattern corresponding to the molecular species at m/z 648 and 753, respectively. Fragmentation of the peak at m/z 753 corresponding to **11**⁺ revealed that a phosphine ligand is lost in preference to the arene or the chloride, followed by the loss of the halide and then arene at higher dissociation energies.

Single crystal X-ray structures of **10**, **11Cl**, **11OTf**, **11PF₆**, **11SbF₆**, and **11BPh₄** were obtained and are shown in Figure 2 (see Supporting Information for crystal growth and data collection and refinement details). All the analyzed complexes have the expected piano-stool-type conformation with the methyl group of the coordinated arene locked in the same plane as the Ru–Cl bond. Furthermore, the length of the Ru–Cl and the Ru–P1/P2 bonds were slightly shorter than other reported phosphine and bisphosphine

ruthenium(II) arene complexes studied as anticancer agents. No significant structural differences were observed in the selected bond lengths and angles of the solid-state structures of the cation in **11CA** (Table S1), highlighting the absence of an effect in the structure due to the changing nature of the counterions.

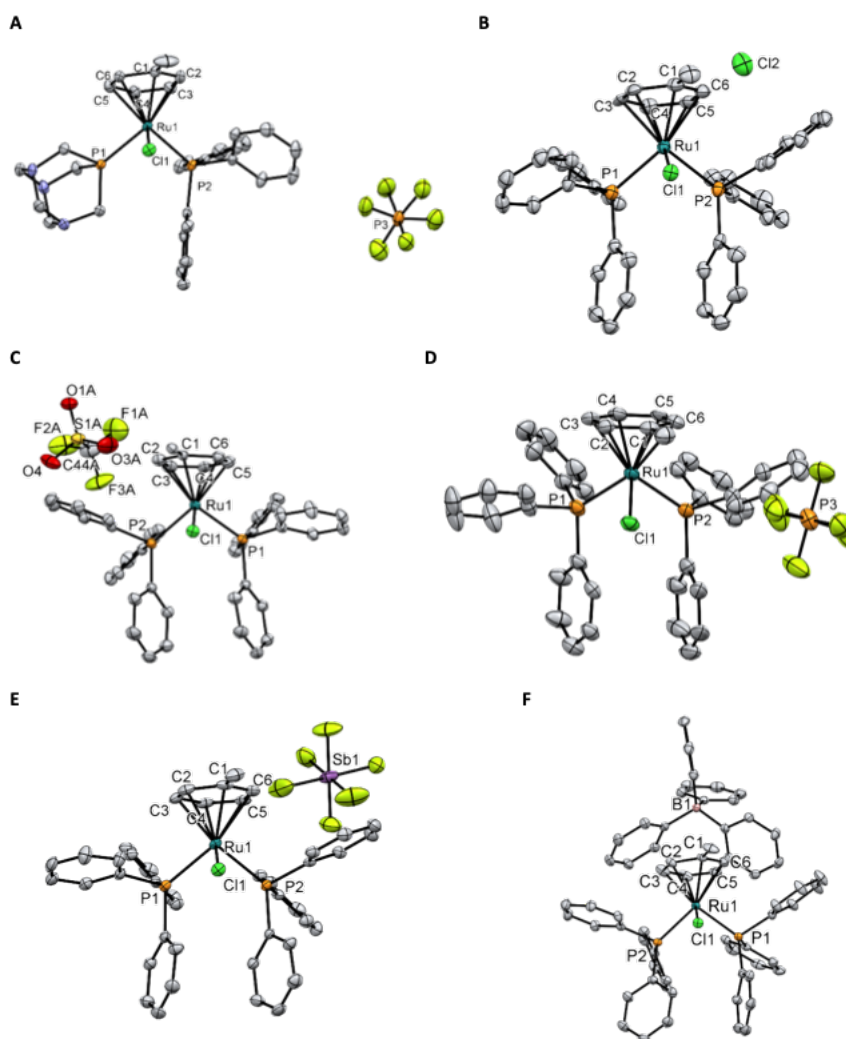


Figure 2. ORTEP diagrams of **10** (A), **11Cl** (B), **11Otf** (C), **11PF₆** (D), **11SbF₆** (E), and **11BPh₄** (F). Thermal ellipsoids are drawn with a 50% probability. Solvates and disorders have been omitted for clarity.

Compounds containing labile metal-halide bonds, such as cisplatin or RAPTA-C, are known to undergo hydrolysis and/or ligand substitution as a key activation hallmark of their therapeutical activity (49). Among other methods, NMR spectroscopy and mass spectrometry are widely employed to study this reaction and elucidate the compounds' fate in a physiological environment. The stability of **11CA** under pseudo-physiological conditions was evaluated by ESI-MS: speciation and the relative intensity of different

ruthenium-containing ions were measured over 72 h. All complexes remained stable at the tested conditions (Figures S4-S10), and no noticeable differences were observed in their behavior as a function of the nature of the counterion. As it displays higher solubility in aqueous media (Table S3), the stability **11Cl** was additionally studied by NMR spectroscopy in neutral conditions. In both cases, no signs of hydrolysis or decomposition were observed (Figures S11-S12). To further prove the inertness of the Ru–Cl bond, **11Cl** and **11BPh₄** were incubated for 72 h in DMSO, a highly coordinating solvent. No changes were observed whatsoever, confirming the overall resistance of **11CA** to solvolysis (Figures S13-S16). The chemical inertness of **11CA** is likely explained by several factors. The steric bulk provided by the two phosphine ligands and the coordinated arene (particularly, the methyl group) might hinder the approach of incoming molecules. Triphenylphosphines and toluene are electron-poor ligands, which might reduce the reactivity of the ruthenium center. Furthermore, the substitution of an X-type ligand by an L-type ligand in cationic complexes resulting in a doubly-charged species is disfavoured in the absence of good counterions. As an example, the second hydrolysis of RAPTA-C is only facilitated after the deprotonation of first-bound water (49). In fact, the deprotonation or anation (OH^-) renders less reactive complexes.

3.2. *In vitro* studies

3.2.1. *Cytotoxicity studies*

The cytotoxicity of the complexes was assessed against human breast cancer cell lines, MCF-7 and MDA-MB-231, and nontumoral adherent mouse fibroblast connective tissue cells (L929) using the MTT assay after 24 h (Table S5) and 72 h (Table 1). The dose-response curves of compounds **10** and **11CA** at 24 h and 72 h are presented in Figure S2.

Table 1 – *In vitro* antiproliferative activities of compounds **1** – **11CA** against breast cancer (MCF-7 and MDA-MB-231), and non-tumoral adherent mouse fibroblast connective tissue (L929) cell lines after 72 h of exposure. See Table S6 in the Supporting Information for the full statistical analysis.
*The SI (selectivity index) was calculated as IC_{50} non-cancerous L929 divided by the average of the IC_{50} of the breast cancers cell lines.

Compound	IC ₅₀ (mM) (average ± standard deviation) 72 h			SI*
	MCF-7	MDA-MB-231	L929	
1	48 ± 10	41 ± 3	58 ± 4	1.3
2	51 ± 6	63 ± 3	64 ± 4	1.1
3	51 ± 4	60 ± 3	84 ± 5	1.5
4	68 ± 7	83 ± 4	79 ± 4	1.0
5	57 ± 5	71 ± 4	84 ± 4	1.3
6	74 ± 7	63 ± 5	82 ± 3	1.2
7	62 ± 4	83 ± 5	104 ± 4	1.4
8	41 ± 5	50 ± 2	50 ± 3	1.1
9	52 ± 7	44 ± 3	70 ± 4	1.5
10	3.1 ± 0.2	2.8 ± 0.2	31 ± 3	10.5
11Cl	0.061 ± 0.003	0.086 ± 0.006	0.87 ± 0.05	11.8
11NO₃	0.078 ± 0.005	0.109 ± 0.009	1.04 ± 0.05	11.1
11BF₄	0.14 ± 0.01	0.19 ± 0.01	1.13 ± 0.05	6.8
11OTf	0.60 ± 0.07	1.4 ± 0.1	1.8 ± 0.2	1.8
11PF₆	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	1.1
11SbF₆	0.08 ± 0.01	0.131 ± 0.009	1.35 ± 0.09	12.8
11BPh₄	0.52 ± 0.06	1.7 ± 0.3	3.9 ± 0.3	3.5

In general, the relationship between cell growth inhibition and the chemical structures indicates that the cytotoxicity of the complexes increases with the increase in lipophilicity (Table S3). Compounds **1** – **9** present cytotoxicity values over 40 μ M and low selectivity indexes over all the tested cell lines. The presence of hydrophobic triphenylphosphine (PPh₃) ligands results in a gradual increase in the toxicity of the complexes. Compound **9**, with only one PPh₃ ligand, is ca. 17 times less cytotoxic for MCF-7 and MDA-MB-231 cells than cationic **10**, bearing a hydrophilic PTA and a hydrophobic PPh₃ ligand. Furthermore, **10** discriminates between non-cancerous and breast cancer cell lines. The cationic

character of the hydrophobic compounds **11CA** improves their anticancer activity: enhancing their cell growth inhibitory rate values to the low micromolar range ($IC_{50} < 5 \mu M$), and even reaching the nanomolar range for **11Cl**, **11NO₃**, and **11SbF₆** (Table 1), as well as increasing their selectivity between non-tumoral and breast cancer cell lines ($SI > 10$, SI = selectivity index). As the sodium salt of BPh_4^- presented the lowest IC_{50} values in all the cell lines and in both incubation times (see Table S7 in Supporting Information), it was expected that **11BPh₄** would also present the highest cytotoxicity in the counterion series (e.g., IC_{50} of **11BPh₄** and **11Cl** for MCF-7 = $0.52 \pm 0.06 \mu M$ vs. $0.061 \pm 0.003 \mu M$). Contrarily, **11Cl** presents the lowest toxicity among all the compounds **11CA**, highlighting that the activity of these compounds is not tied to the intrinsic toxicity of the counterions. **11CA** are more active against breast cancer cell lines than platinum-based chemotherapeutics currently used in the clinic (cisplatin, $28 \pm 6 mM$ (50) and $38.70 \pm 0.03 mM$ (51); carboplatin, $27.3 mM$ (52) and $15.4 \pm 3.3 mM$ (53) for MCF-7 and MDA-MB-231 cells, respectively). Compared to previously reported complexes tested against breast cancers, the most active representatives of the **11CA** series (**11Cl**, **11NO₃** and **11SbF₆**) present higher IC_{50} than the most active ruthenium-based agents (54) and comparable to the most effective metal drugs (55). Furthermore, **11CA** were more cytotoxic than RAPTA-EA, a selective glutathione transferase (GSTP-1) inhibitor, which presented good cytotoxicity profiles against breast cancer cell lines ($20.0 \pm 2.2 mM$ and $10.5 \pm 0.5 mM$ for MCF-7 and MDA-MB-231 cells, respectively) (56).

Overall, cationic ruthenium(II) arene complexes **10** and **11CA** were efficient and selective inhibitors against breast cancer cell lines. In particular, **11Cl**, **11NO₃**, and **11SbF₆** demonstrate the highest antiproliferative activity as well as the highest cell line selectivity. Considering their biological behavior, significant cell growth inhibition rates in the nanomolar range ($IC_{50} < 100 nM$) and selectivity between non-tumorigenic and cancer cell lines (ca. 10-fold), these compounds could have potential applications in drug combination chemotherapy treatments against breast cancers.

3.2.2. Apoptotic effect of compounds tested in breast cancer cell lines

Most anticancer drugs, including metal compounds, induce cell death by apoptosis (57), which is sometimes associated with multidrug-resistance (58). Thus, compounds **10** and **11CA**, which are cytotoxic and selective towards cancer cells, were tested for their ability to induce cell death via apoptosis in the two breast cancer cell lines. The compounds were incubated with MCF-7 and MDA-MB-231 cells at their IC_{50} for 24 h. Afterward, FITC Annexin V with a 7-AAD staining kit was used to determine the apoptosis effect using a caspase assay (Figure 3).

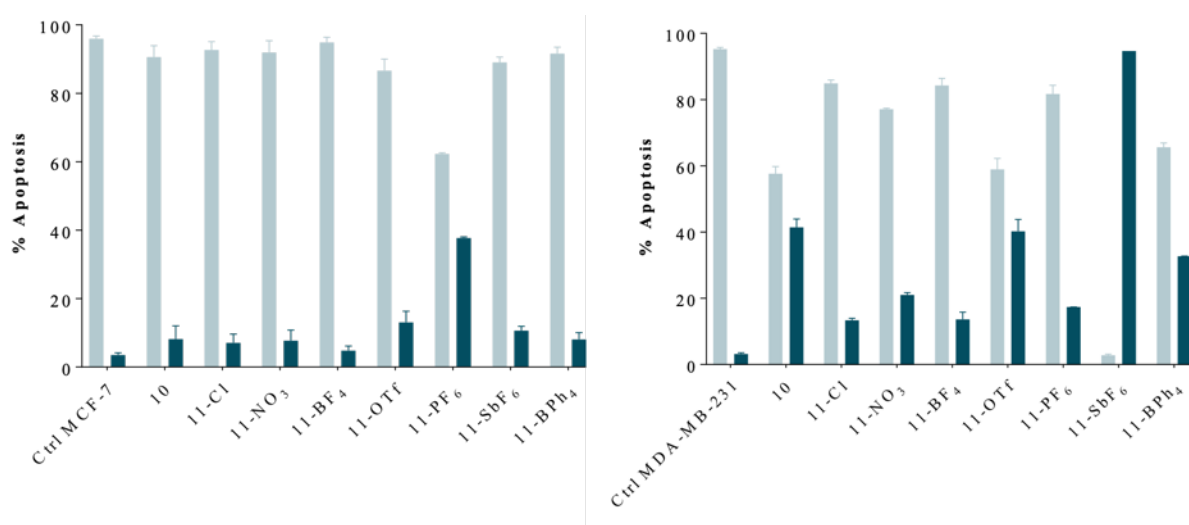


Figure 3. The apoptosis-induction effect of ruthenium-based compounds on breast cancer cell lines. Determination of live and apoptotic cells treated with the selected ruthenium-based compounds for 24 h. **1)** MCF-7 and **2)** MDA-MB-231; ■ Viable cells and ■ Apoptotic cells. The flow cytometry graphs for the tested compounds are provided in Figure S3 and the IC_{50} values at 24 h are provided in Table 5 of the Supplementary Information.

Except for **11PF₆**, the tested compounds induced a slight increase in the number of apoptotic cells of around 10% concerning to the control in the MCF-7 cell line. Induction of apoptosis was more pronounced in the MDA-MB-231 cells, especially for **10**, **11OTf**, **11SbF₆**, and **11BPh₄** which presented apoptosis percentages of 41%, 40%, 95%, and 33%, respectively. Negligible necrosis (< 2%) was observed for all the compounds (Figure S3). However, the induction of apoptosis did not necessarily correlate with the higher cytotoxicity or the selectivity of the complexes either at 24 or 72 hours, indicating that other cell death mechanisms may be involved (59, 60). Furthermore, compounds that are able to induce programmed cell death via alternative mechanisms might be able to overcome apoptosis-related resistance, which suggests that **11CA** could have potential applications in cancers that develop apoptosis resistance (61).

3.2.3. Ruthenium cell uptake assay

To clarify the relationship between lipophilicity, uptake, and cytotoxic activity a cellular uptake assay was performed with **11Cl** and **11BPh₄**, which represent the two extremes in the lipophilic character of the series. **11Cl** is highly hydrophilic and, coincidentally, highly cytotoxic, while **11BPh₄** is highly lipophilic and the least cytotoxic compound in the **11CA** series.

Table 2. *n*-Octanol/water partition coefficient of **11Cl** and **11BPh₄** and ruthenium cellular uptake in MCF-7 and MDA-MB-231 cells after treatment with **11Cl** and **11BPh₄** at 2 different concentrations for 8 h.

Compound	LogP _{ow}	Cell line	Concentration (mM)	[Ru] (ng/10 ⁵ cells)
11Cl	1.48	MCF-7	1	15 ± 7
			10	166 ± 16
		MDA-MB-231	1	13 ± 2
			10	60 ± 21
11BPh₄	> 3.5	MCF-7	1	16 ± 5
			10	25 ± 1
		MDA-MB-231	1	12 ± 4
			10	11 ± 4

Two concentrations (1 and 10 μM) of **11Cl** and **11BPh₄** were tested in MCF-7 and MDA-MB-231 cell lines to evaluate ruthenium cell uptake for 8 h. After this time, the cells were collected and centrifuged. The supernatants were removed, and the cells pellet were then analyzed by ICP-MS. At low concentrations (1 mM), no significant difference in ruthenium cell uptake was observed between **11Cl** or **11BPh₄** or between the two different cell lines (Table 2). However, noticeable differences can be observed at 10 mM. Counterintuitively, the less lipophilic **11Cl** showed higher uptake in both cell lines than its more lipophilic counterpart, **11BPh₄**. This can be attributed to the higher energy of hydration of the **11BPh₄** (Table S4), which leads to lower dissociation of the ionic complex in an aqueous solution (62). This less dissociated neutral complex is less likely to be internalized. Additionally, the higher uptake in MCF-7 cells than in MDA-MB-231 cells also becomes more pronounced, with over a 2-fold difference, likely due to the presence of a higher number of glycolipids in the MCF-7 cell membranes (63). The results suggest that the internalization efficacy of **11CA** has a direct impact on the growth inhibitor rate of the complexes toward the breast cancer cell lines, with **11Cl** being more effective than **11BPh₄**. Furthermore, the higher uptake in MCF-7 cells could explain the higher anticancer activity displayed by the compounds in this cell line compared to MDA-MB-231.

3.3. Interaction of 11CA with relevant biomolecules

Metallo drugs, particularly ruthenium-containing compounds, often interact covalently with proteins and nucleotides, which are their main pharmacological targets (64). To study the possible chemical interaction between **11Cl** and these targets, nucleophilic model biomolecules were employed. The ruthenium compound was incubated with three amino acids: glutamic acid (Glu), histidine (His), and sulfur-bearing cysteine (Cys); and one nucleotide: deoxyguanosine monophosphate (dGMP). No changes in the model biomolecules or the metal complex, or adduct formation were observed during the incubation of **11Cl** with Glu, His, Cys, or dGMP (Figures S17-S28), emphasizing the stability of the complex and ruling out covalent interactions with model biomolecules as responsible for the high toxicity of the compounds. The therapeutic mechanism of other highly stable metal complexes has been previously linked to their intracellular catalysis activity (65). Considering **11Cl**'s chemical inertness, and its similarity to **11PF₆** (which has been previously explored as a homogeneous alkene hydrogenation catalyst (31)), its potential use as a catalytic metallo drug was tested in two model reactions: the oxidation of GSH (66); and the transfer of hydrogenation of NAD⁺ to 1,4-NADH (37). No catalytic or stoichiometric conversion was observed in any of the screened reactions, while the compound remained unchanged (Figures S29-S32). The absence of reactivity of **11Cl** is likely a consequence of the lack of aquation in the complex, a crucial hallmark in metal-based drugs enabling the activation of the compound and further ligand substitution reactions by biomolecules (49, 67). Overall, these results indicate that **11Cl** might engage in non-covalent interactions with biomolecules, rather than through more typical mechanisms associated with metallic drugs.

Various methods can be used to study non-covalent interactions between metal complexes and proteins (62, 68, 69). RAPTA-T (**4**) and other ruthenium(II) arene complexes are able to bind plasma and cancer-related proteins through both covalent and non-covalent interactions (64, 70-72). One of the most common methods to assess these interactions is by observing the fluorescence quenching of amino acids, particularly tryptophan. Therefore, the interaction between **11Cl** and bovine serum albumin (BSA), employed as a model plasma protein, was evaluated using this methodology (Table 3).

Table 3. Protein quenching constant (K_{sv}), bimolecular quenching rate (k_q), binding constant (K_b), and number of binding sites (n) for the interaction of **11Cl** with BSA.

Protein	K_{sv} (10^4 M^{-1})	k_q (10^{12} M^{-1})	K_b (10^5 M^{-1})	n
BSA	1.13 ± 0.08	1.88 ± 0.14	5.6 ± 0.3	1.4

The calculated value for the bimolecular quenching rate due to short-range interactions, k_q , is in the order of $10^{12} \text{ M}^{-1} \text{ s}^{-1}$. This value is higher than the average diffusion-controlled quenching rates of representative biomolecules in an aqueous media ($2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) (73), indicating that the fluorescence quenching was consistent with a mechanism of static quenching resulting from specific interactions between **11Cl** and the protein. The binding of **11Cl** to BSA was moderate compared to the reported binding constants of other ruthenium(II) arene complexes (74, 75). Furthermore, the estimated value of n (~ 1) supports the likelihood of a unimolecular single binding site for **11Cl** in BSA.

However, given the lipophilic nature of **11Cl**, it is highly likely that it targets biomolecules with hydrophobic environments, such as DNA which is the most common cellular target of metal complexes (76). In fact, RAPTA-C (**5**) and related ruthenium(II) arene complexes bind the DNA sequence of BRCA1 (20, 77), a tumor suppressor gene relevant to breast cancers. To gain insights into the interaction between **11Cl** and DNA, a fluorescence-based competitive binding assay was conducted using three DNA probes (Table 4): DAPI (minor groove binder), MG (major groove binder), and PI (intercalator). The binding of these probes to DNA results in an enhancement of the probe's quantum yield and, consequently, a rise in fluorescence intensity. Conversely, if the ruthenium complex competes with or displaces the probes, a decrease in fluorescence is observed. Apparent DNA binding constants, K_{app} , were calculated according to the competitive binding model described by Tse and Boger (39).

Table 4. Protein quenching constant (K_{sv}), bimolecular quenching rate (k_q), c_{50} , and apparent binding constant (K_{app}) of the interaction between **11Cl** and ctDNA from the DAPI and MG competitive binding assay.

Probe	K_{sv} (10^3 M^{-1})	k_q (10^{12} M^{-1})	C_{50} (10^{-4} M)	K_{app} (10^5 M^{-1})
DAPI	8.8 ± 0.6	3.3 ± 0.3	1.1	4.4 ± 0.3
MG	4.8 ± 0.7	0.48 ± 0.07	2.1	0.43 ± 0.04

A decrease in the emission intensity of DAPI and MG was observed as the concentration of **11Cl** increased, without any change in their respective emission wavelengths. In contrast, no linear quenching was seen in the case of PI. These findings suggest that **11Cl** binds to DNA by interacting with either the major or the minor groove, rather than by intercalation. As before, the observed bimolecular quenching rate, k_q , values were higher than the average diffusion-controlled quenching rates of molecules in an aqueous medium (73), implying that the fluorescence quenching was due to specific interactions between

11Cl and DNA. The different binding constant, K_{app} , values calculated from the competitive binding against MG and DAPI suggest that the compound prefers binding to the minor groove. The calculated binding constants values are similar to those reported for other ruthenium(II) arene DNA-binding complexes (75, 77) and ruthenium(II) groove-binding compounds (78, 79), but are lower than those reported for classical groove binding probes ($\sim 10^7 \text{ M}^{-1}$).

The interaction of **11Cl** and **11BPh₄** with plasmid DNA was assessed by visualizing the relaxation of the plasmid using agarose gel electrophoresis (Figure 4).

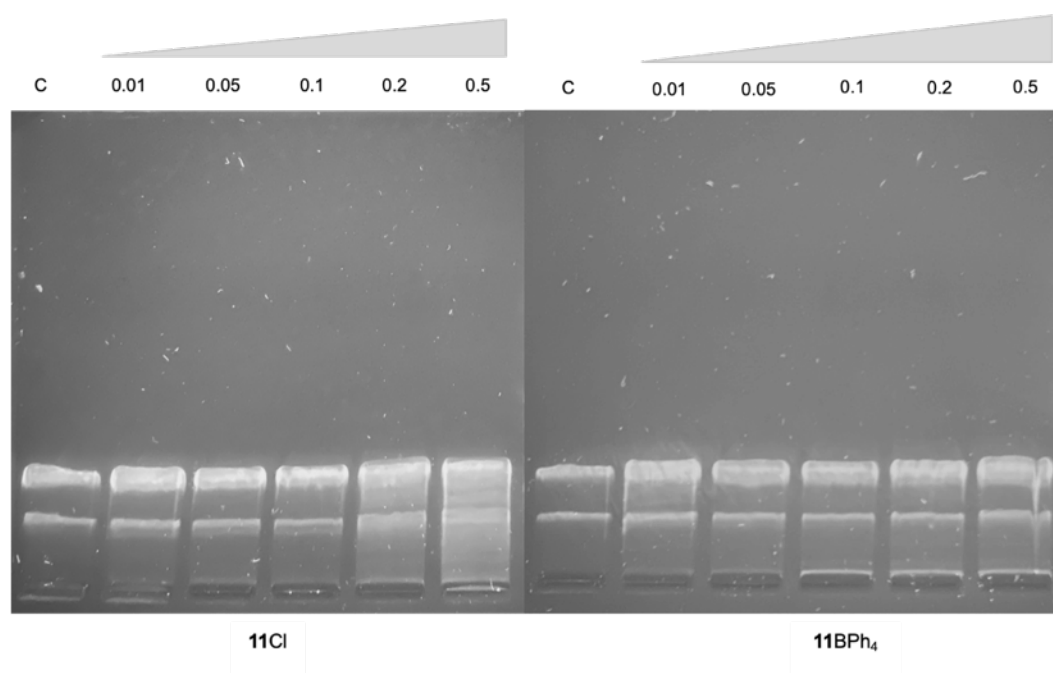


Figure 4. Gel electrophoresis of compounds **11Cl** and **11BPh₄** with pBR322 DNA. C – control; **11Cl** at r_i 0.01; 0.05; 0.1; 0.2 and 0.5 ($r_i = [\text{complex}]/[\text{DNA bp}]$).

Different concentrations of the complexes were incubated with pBR322 DNA at 37 °C for 24 h. Then, these different ratios were loaded in the gel wells and were run for 100 min at 40 V in 1xTAE buffer. The gel was incubated for 30 min in the SyBR safe solution and after being washed for 10 min with MilliQ water, it was analyzed using a blue light obtained from the images in Figure 4. Notably, no precipitation was observed for either compound in any of the studied concentrations, implying that electrostatic neutralization is an unlikely cause of the interaction. **11Cl** did not induce alterations in the supercoiled structure of the plasmid or the stabilization of the linear form, further corroborating that the complex does not interact covalently with DNA. However, at ratios from 0.2, it induced tailing and slight retardation on the bands, which might be an indication of non-covalent interactions. Contrarily, **11BPh₄** did not display this behavior in any of the tested

concentrations. These results could be a consequence of the higher dissociation of **11Cl** in an aqueous solution (Table S3), which results in a ruthenium cation more readily available to interact with DNA. This enhanced interaction is consistent with the higher cytotoxicity and apoptotic behavior of **11Cl**.

Circular dichroism (CD) has been extensively used to identify and interpret drug-DNA interactions with great sensitivity and, in this work, it was used to identify the binding mode of **11Cl**. The CD profile of right-handed B-DNA (its most common form) displays two positive (220 and 268 nm) and two negative (210 and 246 nm) elliptical signals. CD is usually employed to determine the preferred binding mode within the asymmetric DNA environment by observing the changes in these peaks relative to untreated DNA, and by comparing the induced changes to those of other small drugs with known binding mode.

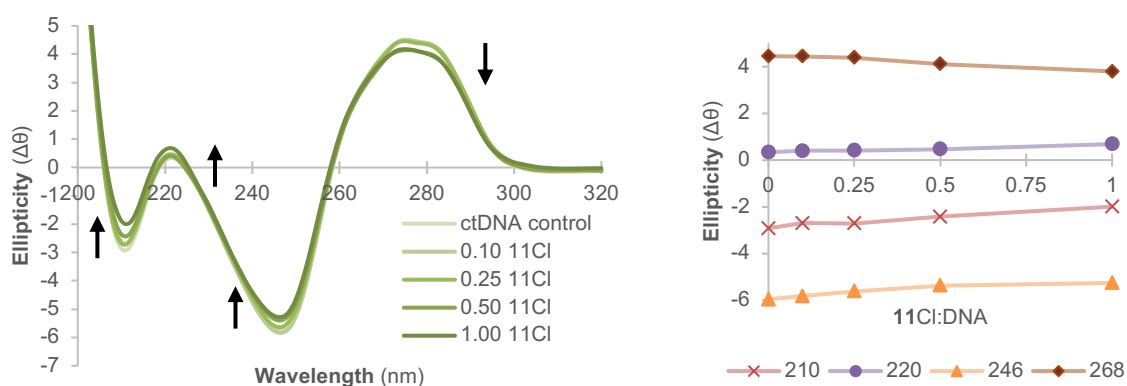


Figure 5. Circular dichroism profile of compound **11Cl** and changes in the intensity of the main bands.

11Cl induced changes in the intensity of the bands of ctDNA (Figure 5), characterized by a decrease in the ellipticity of the bands at 210, 246, and 270 nm, and an increase in the circularity of the band at 220 nm. A small bathochromic (red) shift was also observed for the band at 220 nm, whereas the maximum wavelength remained unchanged for the rest of the bands. These data indicate that **11Cl** caused changes in the circularity of DNA and distortion of its secondary structure. To understand better the binding mode of the complex, we evaluated effects of DAPI (minG), MG (MajG), and PI (Int.) on the circularity of BDNA (Figure S33 and Table S8 in the SI). Comparing the obtained data to that of the tested DNA probes, the results indicate that **11Cl** interacts with ctDNA by binding both the minor and the major groove. This conclusion was further confirmed by studying the induced circular dichroism (ICD) of the DNA probes before and after the addition of **11Cl**. The intensity of the ICD bands of DAPI and MG decreased by 35% and 20%, respectively,

after treatment with **11Cl**, likely due to competitive binding (Figure S34). The difference in quenching between these probes corroborates that **11Cl** has a slight preference for the minor groove. Meanwhile, the ICD band of PI increased after the addition of the ruthenium compound, possibly because of changes in the hydrophobicity of the environment of the probe, resulting from the complex binding to the DNA grooves. Notably, no precipitation was found in the studied ratios, indicating that the neutralization of DNA by electrostatic binding is unlikely.

Molecular docking studies were performed to assess the feasibility and to computationally validate the interaction of **11⁺** (**11CA** without the counterion) with both the major and minor grooves of DNA (Figure 6 and Table 6). Compared to other studies, the resistance of **11CA** to hydrolysis and ligand substitution permits the use of standard biomolecule-ligand docking methodologies, as the role of the metal is mainly structural and does not require precise electronic description (80).

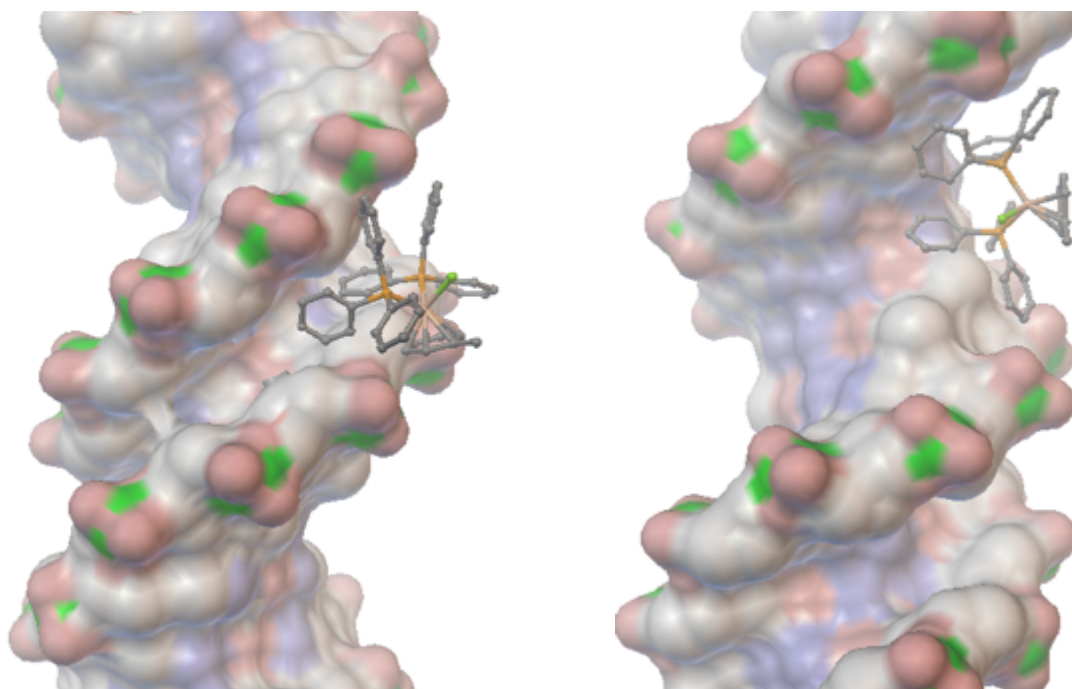


Figure 6. *minG (right) and MajG (left) docked conformations for **11⁺** interacting with 1BNA.*

Table 5. Summary of the docking results between **11⁺** and the different DNA structures, including the number of minor (*minG*) and major (*MajG*) groove poses and the lowest (and average) binding energy of the conformations.

PDB structure	N _{minG} (%)	ΔG _{minG} (kcal/mol)	N _{MajG} (%)	ΔG _{MajG} (kcal/mol)	Other poses	N _{other} (%)	ΔG _{other} (kcal/mol)
1BNA (native)	59	-5.62 (-4.65)	41	-5.38 (-4.77)	0	-	-
5T4W (minor groove)	79	-6.41 (-5.39)	21	-5.68 (-5.12)	0	-	-
1G3X (intercalator)	23	-8.89 (-8.23)	0	-	Int. + minG	76	-8.95 (-8.63)
					Int. + MajG	1	-6.42

The docking results confirm that, under the studied conditions, both the major and the minor grooves are likely binding sites for **11⁺** (Figure 6 and Figure S35). Furthermore, in agreement with the experimental results, minor groove binding is favored, as observed by the larger number of poses and lower binding energies (Table 5). The higher binding energy values obtained from the redocking of DAPI and MG to the different DNA structures (Table S9) compared to the binding energy of **11⁺**, indicate that the binding of the ruthenium complex to DNA is weaker than that of the probes (something also observed in the fluorescence studies). The nature of the interaction of **11⁺** with DNA depends largely on the electrostatic attraction between the ruthenium cation and the DNA phosphate backbone, as well as π -interactions between the aromatic moieties of the complex and the nucleobases.

The performed studies have helped elucidate the possible biological targets of **11CA**. The hydrolysis-resistant complexes interact with biomolecules in non-covalent manners, deviating from the more typical mechanisms associated with metallic drugs. **11Cl** binds both proteins and nucleotides and, as expected from the highly lipophilic character of the compound, it displays a preference for binding the minor groove of DNA.

4. Conclusions

Seventeen synthesized ruthenium(II) arene compounds were tested to evaluate their cytotoxicity *in vitro* against two breast cancer cell lines (MCF-7 and MDA-MB-231) and the non-tumorigenic L929 cell line. A family of cationic complexes, **11CA**, were found to be chemically inert, displaying high stability against aquation, solvolysis, and a small

representative sample of nucleophilic model biomolecules, while retaining high cytotoxicity. Among them, **11Cl** emerged as a promising candidate, presenting inhibitory growth rates in the nanomolar range, and displaying around a 10-fold selectivity for the breast cancer cell lines compared to the healthy ones. Apoptosis assay suggested that **11Cl** might induce cell death by a non-apoptotic mechanism, which could have ramifications in the treatment of apoptosis-resistant cancers. Additionally, it showed significant cellular uptake and water solubility and intermediate partition coefficient values - ideal characteristics for IV injection and systemic administration. The interaction studies confirmed that **11Cl** interacts with biomolecules in a non-covalent fashion with a preference for binding the minor groove of DNA. This work demonstrates the potential for improving the cytotoxicity profile of RAPTA-like compounds, particularly against breast cancers. Further research will be necessary to understand if **11Cl** retains the antimetastatic properties of RAPTA drugs and their lower general toxicity, and to study their potential action in *in vivo* models, which may contribute to the development of new treatments against breast cancer.

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Supporting Information

for

Tailoring the cytotoxicity of ruthenium(II) arene compounds for enhanced activity against breast cancers

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1. Synthesis and characterization of the screened compounds

All organometallic manipulations were carried out under a nitrogen atmosphere using standard Schlenk techniques. CH_2Cl_2 was dried catalytically under nitrogen using a solvent purification system, manufactured by Innovative Technology Inc. All other solvents were dried over molecular sieves (3 Å) and saturated with nitrogen prior to use. $[\text{Ru}(\eta^6\text{-toluene})\text{Cl}_2]_2$ (**1**), $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)\text{Cl}_2]$ (**2**) and complexes **1** (**3**), **2** (**3**), **3** (**4**), **4** (**5**), **5** (**6**), **6** (**6**), **7** (**7**), **8** (**8**), and **9** (**2**) were prepared according to reported procedures and the characterization data were in agreement with those reported. Compounds **11BF₄** (**9**) and **11PF₆** (**2**) have been previously reported, but in this work they have been prepared following a different synthetic route. All commercial products were used as received. Spectra were recorded with a Bruker Avance 400 MHz spectrometer at room temperature, unless otherwise stated. Chemical shifts are given in ppm and coupling constants (J) in Hz.

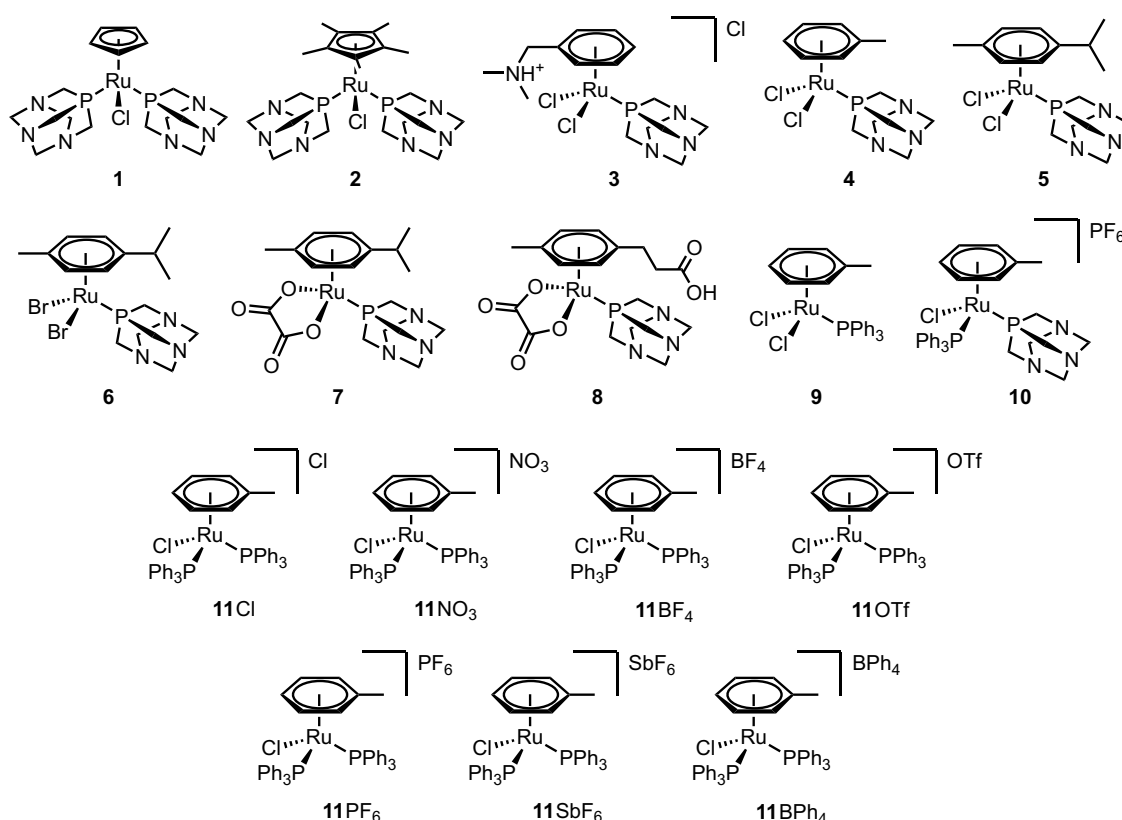
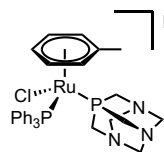


Figure S1. Structures of the studied ruthenium arene compounds.

[Ru(η^6 -toluene)(PPh₃)(PTA)Cl]PF₆ (10**)**



A suspension of [Ru(η^6 -toluene)(PPh₃)Cl₂] (200 mg, 0.38 mmol, 1.0 eq.), 1,3,5-triaza-7-phosphaadamantane (90 mg, 0.57 mmol, 1.5 eq.) and ammonium hexafluorophosphate (99 mg, 0.61 mmol, 1.6 eq.) in a 1:1 mixture of MeOH:CH₂Cl₂ (20 mL) was vigorously stirred at 45°C for 1 h.

The solvent was evaporated, and the crude redissolved in CH₂Cl₂ (30 mL) which was then washed with H₂O (2 × 15 mL), dried over anhydrous Na₂SO₄ and the solvent evaporated. The solid was redissolved in CH₂Cl₂ (5 mL), and the product was precipitated by the addition of pentane, filtered, washed with cold diethyl ether (2 × 5 mL), and dried under vacuum overnight. Light yellow powder (106 mg, 35%). Light orange crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C of pentane into a solution of CH₂Cl₂.

¹H NMR (400 MHz, DMSO-*d*₆) δ : 7.58 (qdd, *J* = 8.6, 5.9, 2.1 Hz, 10H), 7.54 – 7.44 (m, 5H), 6.58 (q, *J* = 5.4 Hz, 1H), 6.29 (d, *J* = 5.9 Hz, 1H), 6.21 (d, *J* = 6.3 Hz, 1H), 5.54 (td, *J* = 5.8, 3.6 Hz, 1H), 4.39 – 4.16 (m, 7H), 4.09 (ddd, *J* = 14.7, 3.6, 1.5 Hz, 3H), 3.74 (ddd, *J* = 14.9, 3.8, 1.6 Hz, 3H), 2.12 (d, *J* = 1.1 Hz, 3H) ppm.

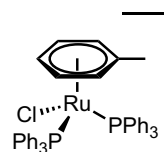
³¹P NMR (162 MHz, DMSO-*d*₆) δ : 37.9 (d, *J* = 52.8 Hz), -34.8 (d, *J* = 52.8 Hz), -139.4 (hept, *J* = 711.9, 711.2, 711.0, 709.8, 706.3 Hz) ppm.

³¹P NMR (162 MHz, Chloroform-*d*) δ : 31.6 (d, *J* = 54.0 Hz), -40.7 (d, *J* = 54.0 Hz) ppm.

¹⁹F NMR (376 MHz, DMSO-*d*₆) δ : -72.2 (d, *J* = 713.0 Hz) ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) *m/z*: [M – PF₆]⁺ Calcd for C₃₁H₃₅ClN₃P₂Ru⁺ 648.1033; Found 648.1014.

[Ru(η^6 -toluene)(PPh₃)₂Cl]Cl (11Cl**)**



75 mg of **11**PF₆ (0.08 mmol) were dissolved in MeOH (50 mL). 5 g of Amberlite® IRA-900 chloride form were added, and the mixture was stirred for 12 h at room temperature. The suspension was filtered and an additional 5 g of Amberlite® IRA-900 chloride form were added, and the mixture was stirred for 12 h more at room temperature. The suspension was filtered again, and the solvent was evaporated. The crude was redissolved in EtOH (5 mL), and the product was filtered after precipitation by the addition of a 2:1 mixture of diethyl ether and hexane, filtered, and dried under vacuum overnight. Counterion exchange was monitored and confirmed by the absence of PF₆ signals in the ³¹P and ¹⁹F NMR spectra and by the appearance of a signal in the ³⁵Cl NMR spectrum (bound chloride was not observed due to the quadrupolar moment of the nuclei). Dark orange crystalline powder (133 mg, 71 %). Light orange crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C

of pentane into a solution of CH_2Cl_2 .

^1H NMR (400 MHz, Chloroform-*d*) δ : 7.45 – 7.33 (m, 18H), 7.26 (t, J = 7.5 Hz, 12H), 5.90 (bs, 2H), 5.84 (d, J = 5.6 Hz, 1H), 4.65 (d, J = 5.5 Hz, 2H), 2.07 (s, 3H) ppm.

^{31}P NMR (162 MHz, Chloroform-*d*) δ : 22.8 ppm.

^{13}C NMR (101 MHz, Chloroform-*d*) δ : 134.19 (t, J = 4.6 Hz), 134.15 (d, J = 48.0 Hz), 130.9, 128.6 (t, J = 5.1 Hz), 122.9 (t, J = 2.8 Hz), 99.3, 94.5 (t, J = 4.9 Hz), 84.9, 19.5 ppm.

^1H NMR (400 MHz, Methanol-*d*₄) δ : 7.52 – 7.38 (m, 18H), 7.29 (td, J = 7.5, 6.6, 3.7 Hz, 12H), 5.89 (tt, J = 5.8, 2.0 Hz, 2H), 5.02 (d, J = 6.1 Hz, 2H), 4.63 (t, J = 5.6 Hz, 1H), 2.15 (s, 3H) ppm.

^{31}P NMR (162 MHz, Methanol-*d*₄) δ : 22.1 ppm.

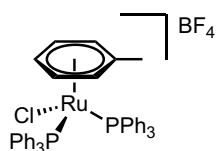
^{35}Cl NMR (39 MHz, Methanol-*d*₄) δ : -31 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z : $[\text{M} - \text{Cl}]^+$ Calcd for $\text{C}_{43}\text{H}_{38}\text{ClP}_2\text{Ru}^+$ 753.1175; Found 753.1155.

General Procedure for the synthesis of bis-triphenylphosphine ruthenium(II)-toluene cationic compounds (11CA)

A suspension of $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}_2]$ (100 mg, 0.19 mmol, 1.0 eq.), triphenylphosphine (55 mg, 0.21 mmol, 1.1 eq.) and the corresponding silver salt of the counterion (0.23 mmol, 1.2 eq.) in a 1:1 mixture of MeOH: CH_2Cl_2 (15 mL) was vigorously stirred at 45°C for 6 h while protected from light. The mixture was filtered through celite to remove the formed silver chloride salt and washed with MeOH (5 mL) and CH_2Cl_2 (5 mL). The solvent was evaporated, and the crude redissolved in CH_2Cl_2 (5 mL). Finally, the product was precipitated by the addition of pentane, filtered, washed with cold diethyl ether (2×5 mL), and dried under vacuum overnight.

$[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]\text{BF}_4$ (**11BF₄**)



Light green powder (137 mg, 86%)

^1H NMR (400 MHz, Chloroform-*d*) δ : 7.44 – 7.34 (m, 18H), 7.31 – 7.22 (m, 12H), 5.80 (tt, J = 5.9, 2.0 Hz, 2H), 5.37 (t, J = 5.7 Hz, 1H), 4.63 (d, J = 6.0 Hz, 2H), 2.07 (s, 3H) ppm.

^{31}P NMR (162 MHz, Chloroform-*d*) δ : 22.4 ppm.

^{19}F NMR (376 MHz, Chloroform-*d*) δ : -152.61, -152.67 ppm.

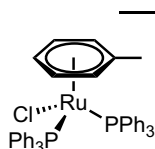
^{11}B NMR (128 MHz, Chloroform-*d*) δ : -0.9 ppm.

^{13}C NMR (101 MHz, Chloroform-*d*) δ : 133.99 (d, J = 48.3 Hz), 133.98 (t, J = 4.6 Hz), 130.9, 128.5 (t, J = 5.0 Hz), 122.7 (t, J = 1.5 Hz), 98.7, 94.4 (t, J = 4.9 Hz), 83.7, 19.3

ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M - BF_4]^{+}$ Calcd for $C_{43}H_{38}ClP_2Ru^{+}$ 753.1175; Found 753.1154.

$[Ru(\eta^6\text{-toluene})(PPh_3)_2Cl]OTf$ (**11OTf**)



Orange crystalline powder (92 mg, 54 %). Intense yellow crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C of pentane into a solution of CH_2Cl_2 .

1H NMR (400 MHz, Chloroform-*d*) δ : 7.39 (qd, J = 7.0, 3.6 Hz, 18H), 7.30 – 7.21 (m, 12H), 5.80 (ddt, J = 5.9, 4.1, 2.1 Hz, 2H), 5.32 – 5.25 (m, 1H), 4.67 (d, J = 6.0 Hz, 2H), 2.08 (s, 3H) ppm.

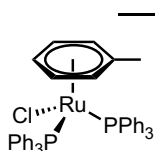
^{31}P NMR (162 MHz, Chloroform-*d*) δ : 22.4 ppm.

^{19}F NMR (376 MHz, Chloroform-*d*) δ : -78.0 ppm.

^{13}C NMR (101 MHz, Chloroform-*d*) δ : 134.1 (d, J = 48.1 Hz), 134.1 (t, J = 4.6 Hz), 131.0, 128.6 (t, J = 5.0 Hz), 122.8 (t, J = 2.8 Hz), 121.0 (q, J = 321.5 Hz), 98.7, 94.6 (t, J = 5.0 Hz), 83.9, 19.4 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M - OTf]^{+}$ Calcd for $C_{43}H_{38}ClP_2Ru^{+}$ 753.1175; Found 753.1155.

$[Ru(\eta^6\text{-toluene})(PPh_3)_2Cl]PF_6$ (**11PF₆**)



Dark yellow powder (151 mg, 89 %). Orange crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C of pentane into a solution of CH_2Cl_2 .

1H NMR (400 MHz, Chloroform-*d*) δ : 7.44 – 7.35 (m, 18H), 7.30 – 7.21 (m, 12H), 5.75 (tt, J = 5.8, 2.1 Hz, 2H), 5.07 (t, J = 5.7 Hz, 1H), 4.67 (d, J = 6.0 Hz, 2H), 2.08 (s, 3H) ppm.

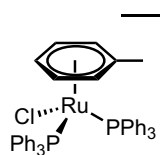
^{31}P NMR (162 MHz, Chloroform-*d*) δ : 22.2, -135.6, -133.0, -144.4, -148.8, -153.2 ppm.

^{19}F NMR (376 MHz, Chloroform-*d*) δ : -71.9, -73.8 ppm.

^{13}C NMR (101 MHz, Chloroform-*d*) δ : 134.0 (t, J = 4.6 Hz), 131.1, 128.6 (t, J = 5.1 Hz), 98.5, 94.7, 94.7, 94.6, 83.78, 19.4 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M - PF_6]^{+}$ Calcd for $C_{43}H_{38}ClP_2Ru^{+}$ 753.1175; Found 753.1155.

[Ru(η^6 -toluene)(PPh₃)₂Cl]SbF₆ (11SbF₆**)**



Light red crystalline powder (133 mg, 71 %). Intense yellow crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C of pentane into a solution of CH₂Cl₂.

¹H NMR (400 MHz, Chloroform-*d*) δ : 7.40 (ddd, *J* = 9.6, 7.0, 3.3 Hz, 18H), 7.30 – 7.21 (m, 12H), 5.74 (tt, *J* = 5.9, 2.1 Hz, 2H), 4.71 (d, *J* = 6.0 Hz, 2H), 2.10 (s, 3H) ppm.

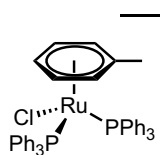
³¹P NMR (162 MHz, Chloroform-*d*) δ : 22.1 ppm.

¹⁹F NMR (376 MHz, Chloroform-*d*) δ : -106.27 – -139.07 (m) ppm.

¹³C NMR (101 MHz, Chloroform-*d*) δ : 134.03 (d, *J* = 48.3 Hz), 134.01 (t, *J* = 4.6 Hz), 131.1, 128.6 (t, *J* = 5.1 Hz), 122.7 (t, *J* = 2.7 Hz), 98.2, 94.8 (t, *J* = 5.0 Hz), 83.5, 19.3 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) *m/z*: [M – SbF₆]⁺ Calcd for C₄₃H₃₈ClP₂Ru⁺ 753.1175; Found 753.1159.

[Ru(η^6 -toluene)(PPh₃)₂Cl]BPh₄ (11BPh₄**)**



Light orange crystalline needles (158 mg, 78 %). Light Orange crystals suitable for X-ray diffraction were obtained by slow diffusion at 4°C of pentane into a solution of CH₂Cl₂.

¹H NMR (400 MHz, Chloroform-*d*) δ : 7.39 (t, *J* = 7.4 Hz, 8H), 7.34 – 7.26 (m, 12H), 7.24 – 7.13 (m, 18H), 6.88 (t, *J* = 7.3 Hz, 8H), 6.79 (td, *J* = 6.9, 6.5, 1.5 Hz, 4H), 5.05 (m, 2H), 4.43 (d, *J* = 6.0 Hz, 2H), 3.69 (t, *J* = 5.7 Hz, 1H), 1.98 (s, 3H) ppm.

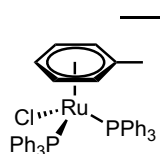
³¹P NMR (162 MHz, Chloroform-*d*) δ : 22.4 ppm.

¹¹B NMR (128 MHz, Chloroform-*d*) δ : -6.8 ppm.

¹³C NMR (101 MHz, Chloroform-*d*) δ : 134.9, 134.2 (t, *J* = 4.6 Hz), 134.2 (d, *J* = 50.8 Hz), 131.0, 128.7 (t, *J* = 5.1 Hz), 128.5, 122.9 (t, *J* = 1.3 Hz), 119.6, 115.9, 99.3, 94.5, 85.5, 19.5 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) *m/z*: [M – BPh₄]⁺ Calcd for C₄₃H₃₈ClP₂Ru⁺ 753.1175; Found 753.1153.

[Ru(η^6 -toluene)(PPh₃)₂Cl]NO₃ (11NO₃**)**



Using a slightly modified procedure, a suspension of [Ru(η^6 -toluene)(PPh₃)Cl₂] (100 mg, 0.19 mmol, 1.0 eq.), triphenylphosphine (55 mg, 0.21 mmol, 1.1 eq.) and NaNO₃ (19 mg, 0.23 mmol, 1.2 eq.) in CH₂Cl₂ (15 mL) was vigorously stirred at room temperature for 24 h.

The mixture was filtered through celite to remove the formed sodium chloride salt and

washed with CH₂Cl₂ (10 mL). The solution was concentrated (5 mL), and the product was precipitated by the addition of pentane, filtered, washed with cold diethyl ether (2 × 5 mL), and, finally, dried under vacuum overnight. Light yellow cream powder (66 mg, 43%).

¹H NMR (400 MHz, Chloroform-*d*) δ : 7.33 – 7.27 (m, 18H), 7.17 (d, *J* = 7.5 Hz, 14H), 5.78 (s, 2H), 5.58 (t, *J* = 5.4 Hz, 1H), 4.54 (d, *J* = 5.8 Hz, 2H), 1.97 (s, 3H) ppm.

³¹P NMR (162 MHz, Chloroform-*d*) δ : 22.6 ppm.

¹³C NMR (101 MHz, Chloroform-*d*) δ : 134.2 (t, *J* = 4.6 Hz), 131.0, 128.6 (t, *J* = 5.1 Hz), 99.0, 94.5, 94.5, 94.4, 89.1, 19.5 ppm.

¹³C NMR (101 MHz, Chloroform-*d*) δ : 134.17 (t, *J* = 4.6 Hz), 134.15 (d, *J* = 46.4 Hz), 131.0, 128.6 (t, *J* = 5.1 Hz), 119.6, 99.0, 94.5 (t, *J* = 4.0 Hz), 89.1, 19.5 ppm.

HRMS (nanochip-ESI/LTQ-Orbitrap) *m/z*: [M – NO₃]⁺ Calcd for C₄₃H₃₈ClP₂Ru⁺ 753.1175; Found 753.1156.

Single clear light orange or intense yellow crystals of **10**, **11Cl**, **11OTf**, **11PF₆**, **11SbF₆** and **11BPh₄** were obtained and measured. Suitable crystals with dimensions 0.09 × 0.08 × 0.05 mm³ (**10**), 0.14 × 0.12 × 0.08 mm³ (**11Cl**), 0.12 × 0.09 × 0.06 mm³ (**11OTf**), 0.51 × 0.09 × 0.06 mm³ (**11PF₆**), 0.19 × 0.12 × 0.06 mm³ (**11SbF₆**) and 0.10 × 0.03 × 0.03 mm³ (**11BPh₄**) were selected and mounted on a XtaLAB Synergy R, DW system, HyPix-Arc 150 diffractometer or on a SuperNova, Dual, Cu at home/near, AtlasS2 diffractometer. The crystals were kept at a steady *T* = 140.00(10) K during data collection. The structures were solved with the **ShelXT** (Sheldrick, 2015) solution program using dual methods and by using **Olex2** 1.5 (Dolomanov et al., 2009) as the graphical interface. The models were refined with **ShelXL** 2018/3 (Sheldrick, 2015) using full matrix least squares minimization on *F*².

Table S1 - Selected bond lengths (Å) and angles (°) for **11Cl**, **11BF₄**^a, **11OTf**, **11PF₆**, **11SbF₆** and **11BPh₄**. ^a Extracted from reference (9).

	11Cl	11BF₄ ^a	11OTf	11PF₆	11SbF₆	11BPh₄
Ru1-Cl1	2.391(2)	2.389(3)	2.4069(4)	2.396(1)	2.4020(9)	2.3913(6)
Ru1-P1	2.362(2)	2.399(3)	2.3759(4)	2.3643(14)	2.379(1)	2.3632(6)
Ru1-P2	2.375(1)	2.384(3)	2.3730(4)	2.3668(13)	2.373(1)	2.3803(6)
Ru1-C1	2.327(6)	2.257(4)	2.3326(18)	2.321(5)	2.332(4)	2.321(2)
Ru1-C2	2.278(5)	2.247(4)	2.2932(19)	2.267(6)	2.260(4)	2.266(2)
Ru1-C3	2.242(6)	2.219(4)	2.2169(18)	2.256(5)	2.225(4)	2.260(2)
Ru1-C4	2.222(7)	2.214(5)	2.2191(17)	2.215(5)	2.219(3)	2.243(3)
Ru1-C5	2.215(7)	2.305(5)	2.2311(17)	2.223(5)	2.212(4)	2.227(3)
Ru1-C6	2.282(7)	2.347(4)	2.2709(17)	2.307(5)	2.285(4)	2.302(2)

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Ru1-C_{avg}	2.26(4)	2.26(5)	2.26(5)	2.26(4)	2.26(5)	2.27(4)
Cl1-Ru1-P1	89.76(6)	85.3(l)	91.682(14)	89.78(4)	91.94(3)	87.68(2)
Cl1-Ru1-P2	85.60(5)	89.3(l)	86.740(14)	89.06(5)	86.10(3)	88.86(2)
P1-Ru1-P2	98.35(5)	100.3(1)	97.894(14)	97.05(5)	97.44(3)	98.34(2)

Table S2 - Crystallographic data for **10**, **11Cl**, **11OTf**, **11PF₆**, **11SbF₆**, and **11BPh₄**.

Compound	10	11Cl	11OTf	11PF₆	11SbF₆	11BPh₄
Formula	C ₃₁ H ₃₅ ClF ₆ N ₃ P ₃ Ru	C _{43.5} H ₄₃ Cl ₃ O ₂ P ₂ Ru	C ₄₄ H ₃₈ ClF ₃ O ₃ P ₂ RuS	C ₄₄ H ₄₀ Cl ₃ F ₆ P ₃ Ru	C ₄₃ H ₃₈ ClF ₆ P ₂ RuSb	C ₆₇ H ₅₈ BClP ₂ Ru
D_{calc.}/ g cm⁻³	1.653	1.507	1.558	1.508	1.721	1.363
m/mm⁻¹	6.739	6.335	5.705	6.174	1.319	3.798
Formula Weight	793.05	867.14	902.26	983.09	988.94	1072.40
Colour	clear light orange	clear light orange	clear intense yellow	clear intense yellow	clear intense orange	clear light orange
Shape	prism-shaped	irregular-shaped	prism-shaped	needle-shaped	prism-shaped	prism-shaped
Size/mm³	0.09×0.08×0.05	0.14×0.12×0.08	0.12×0.09×0.06	0.51×0.09×0.06	0.19×0.12×0.06	0.10×0.03×0.03
T/K	140.00(10)	140.00(10)	139.99(10)	140.00(10)	140.00(10)	140.00(10)
Crystal System	orthorhombic	triclinic	monoclinic	orthorhombic	monoclinic	monoclinic
Flack Parameter	0.143(7)			-0.014(4)		-0.0220(16)
Space Group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> -1	<i>P</i> 2 ₁ / <i>c</i>	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ / <i>c</i>	<i>P</i> 2 ₁
a/Å	9.30092(8)	10.8812(5)	12.47399(8)	15.10287(19)	12.3243(4)	10.47706(6)
b/Å	10.67827(8)	11.0631(5)	10.42115(6)	16.6186(2)	10.3143(3)	16.55786(10)
c/Å	32.0935(2)	16.8995(8)	30.03284(18)	17.2511(3)	30.4650(9)	15.24231(9)
a/°	90	74.198(4)	90	90	90	90
b/°	90	78.406(4)	99.8863(6)	90	99.752(3)	98.9266(5)
g/°	90	82.343(4)	90	90	90	90
V/Å³	3187.46(4)	1910.88(15)	3846.10(4)	4329.83(10)	3816.6(2)	2612.17(3)
Z	4	2	4	4	4	2
Z'	1	1	1	1	1	1
Wavelength/Å	1.54184	1.54184	1.54184	1.54184	0.71073	1.54184
Radiation type	Cu K _α	Cu K _α	Cu K _α	Cu K _α	Mo K _α	Cu K _α
Q_{min}/°	2.754	2.759	2.987	3.693	2.749	2.935
Q_{max}/°	76.220	75.557	76.592	72.474	29.599	76.069
Measured Refl's.	33963	12093	40600	19149	28482	59800
Indep't Refl's	6575	12093	7930	8383	9225	10685
Refl's I≥2σ(I)	6374	10369	7431	7948	6469	10430
R_{int}	0.0282	.	0.0176	0.0279	0.0827	0.0268

Parameters	463	492	571	543	553	650
Restraints	240	29	250	61	288	1
Largest Peak/e Å⁻³	0.638	1.950	0.669	1.071	0.945	0.239
Deepest Hole/e Å⁻³	-0.346	-1.801	-0.381	-0.510	-1.274	-0.398
GooF	1.038	1.054	1.028	1.049	0.989	1.044
wR₂ (all data)	0.0517	0.2149	0.0583	0.1025	0.0947	0.0428
wR₂	0.0513	0.2007	0.0574	0.0998	0.0821	0.0426
R₁ (all data)	0.0226	0.0825	0.0245	0.0404	0.0835	0.0190
R₁	0.0215	0.0725	0.0226	0.0376	0.0503	0.0181
CCDC number	2201998	2210246	2193598	2210241	2210245	2202259

2. Solubility and partition coefficient (Log Pow) of the 11CA

0.5 mg of each compound **11CA** were dispersed in 0.5 mL of MilliQ water, and, after sonication, the mixtures were stirred at 25°C for 1 hour. The saturated solutions were filtered to remove any insoluble solids, dissolved in 0.5 mL of MilliQ water, and analyzed using ICP-MS. Samples were submitted to acidic digestion with 2 mL of concentrated HNO₃ (69%, ROTIPURAN Supra, Roth) in PP digestion vials using a heating block system (DigiPREP Jr. 15ml, 40 Pos, SCP Science). The digestion program was the following: 15 min to heat to 100°C and keep at 100°C for 60 min. After the digestion, the sample volumes were precisely adjusted to 5 mL with MilliQ water. Samples were further diluted 30 times with 2% HNO₃ solution and their Ru content was analyzed by ICP-MS using standard mode on Nexlon 350 D ICP-MS instrument (PerkinElmer). Yttrium was added as an internal standard at a concentration of 2 ppb to all the solution and Ru quantitation was performed using an external calibration curve with standards in the 0.05-50 ppb range. All measurements were performed in triplicates.

The *n*-octanol-water partition ratio is the most common way of identifying the lipophilicity of a compound. This assay was performed following the Shake-flask method (10). To a 0.1 mM of **11CA** in a water-saturated *n*-octanol solution was added *n*-octanol-saturated MilliQ water in different organic: aqueous ratios (1:1; 1:2 and 2:1). The mixture was shaken for 2 minutes, and after that, the solution was centrifuged to separate the organic and aqueous phase. Aliquots of each phase were taken and analyzed separately by ICP-MS. Samples were submitted to acidic digestion with 2 mL of concentrated HNO₃ (69%, ROTIPURAN Supra, Roth) in PP digestion vials using a heating block system (DigiPREP Jr. 15ml, 40 Pos, SCP Science). The digestion program was the following: 15 min to heat up to 100°C and keep at 100°C for 60 min. After the digestion, the sample volumes were precisely adjusted to 5 mL with MilliQ water. Samples were further diluted 30 times with 2% HNO₃ solution and their Ru content was analyzed by ICP-MS using standard mode on Nexlon 350 D ICP-MS instrument (PerkinElmer). Yttrium was added as an internal standard at a concentration of 2 ppb to all the solution and Ru quantitation was performed using an external calibration curve with standards in the 0.05-50 ppb range. The partition coefficient was calculated as $P_{ow} = [Ru]_{org_f} / ([Ru]_{org_i} - [Ru]_{org_f})$, where $[Ru]_{org_i}$ and $[Ru]_{org_f}$ are the concentrations of ruthenium in the organic phase before and after mixing with the aqueous phase, respectively. All measurements were performed in triplicates.

Table S3 - Solubility of the **11CA** in water at 25 °C and *n*-octanol/water partition coefficient. * below limit of detection = 5 ppt.

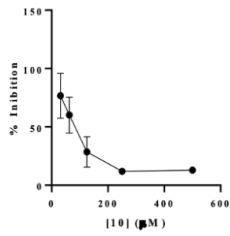
	Solubility (µg/mL)	Solubility (µM)	LogP _{ow}
1	40 x10 ³ (4)	77 x10 ³	-1.85 (11)
2	25 x10 ³ (4)	43 x10 ³	
3	> 10 x10 ³ (12)	> 20 x10 ³	
4			-1.5 (13)
5	~10 x10 ³ (14)	~22 x10 ³	> -1.8 (13)
6			
7	> 48 x10 ³	> 100 x10 ³ (7)	
8		> 25 x10 ³ (15)	-1.57 (8)
9	34 ± 4	65 ± 8	
10	36 ± 7	40 ± 8	
11Cl	416 ± 70	528 ± 88	1.48 ± 0.29
11NO₃	218 ± 34	268 ± 42	1.57 ± 0.29
11BF₄	4 ± 1	5 ± 2	1.60 ± 0.20
11OTf	1.0 ± 0.1	1.1 ± 0.1	1.75 ± 0.18
11PF₆	0.6 ± 0.2	0.6 ± 0.2	1.91 ± 0.23
11SbF₆	below LOD *	below LOD *	2.01 ± 0.07
11BPh₄	below LOD *	below LOD *	> 3.5

Table S4 - Molar Gibbs energies of hydration of selected counteranions calculated using the method described in reference (16). The radius, *r*; width of hydration shell, Δr ; number of water molecules in this shell, *n*; the neutral contribution, $\Delta G_{neutral}$; and the electrostatic contribution, ΔG_{el1+2}

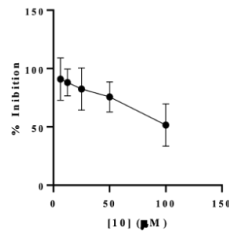
$[11]CA \xrightleftharpoons{\Delta G_{hyd}} [11]^+ + CA^-$						
	<i>r</i> (nm)	<i>n</i>	Δr (nm)	$\Delta G_{neutral}$ (KJ/mol)	ΔG_{el1+2} (KJ/mol)	ΔG_{hyd}^{*calc} (KJ/mol)
Cl⁻	0.168	2.1	0.050	+60	-331	-291
NO₃⁻	0.200	1.8	0.034	+72	-293	-245
BF₄⁻	0.211	1.7	0.029	+76	-281	-231
OTf⁻	0.230	1.6	0.024	+84	-263	-206
PF₆⁻	0.242	1.5	0.021	+90	-252	-191
SbF₆⁻	0.252	1.4	0.018	+95	-243	-178
BPh₄⁻	0.421	0.9	0.004	+217	-150	+67

3. Cytotoxicity studies

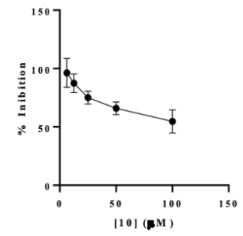
Compound 10 L929 – 24 h



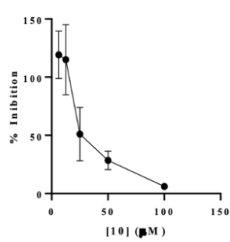
Compound 10 MCF-7 – 24 h



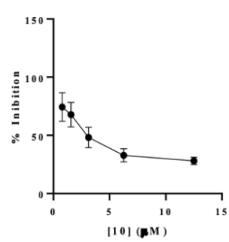
Compound 10 MDA-MB-231 – 24 h



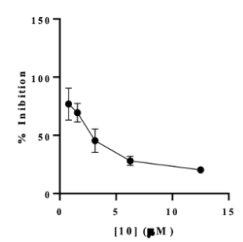
Compound 10 L929 – 72 h



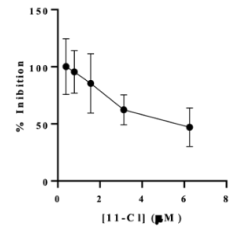
Compound 10 MCF-7 – 72 h



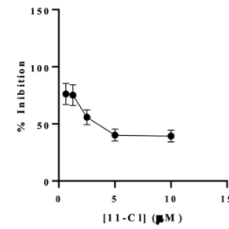
Compound 10 MDA-MB-231 – 72 h



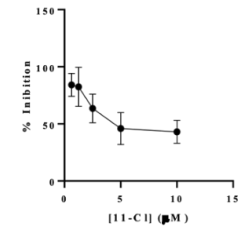
Compound 11-Cl L929 – 24 h



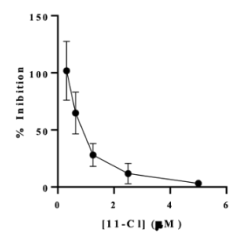
Compound 11-Cl MCF-7 – 24 h



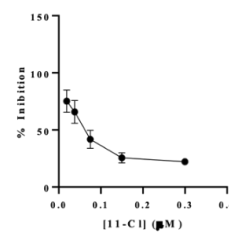
Compound 11-Cl MDA-MB-231 – 24 h



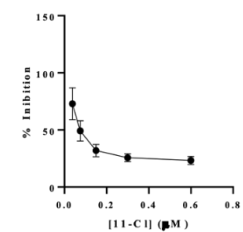
Compound 11-Cl L929 – 72 h



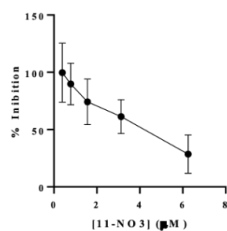
Compound 11-Cl MCF-7 – 72 h



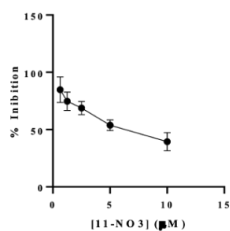
Compound 11-Cl MDA-MB-231 – 72 h



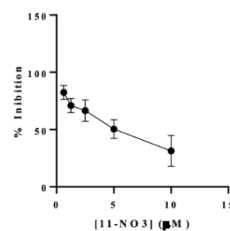
Compound 11-NO₃ L929 – 24 h



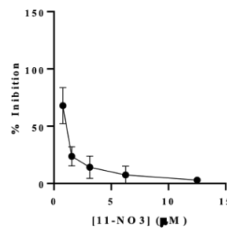
Compound 11-NO₃ MCF-7 – 24 h



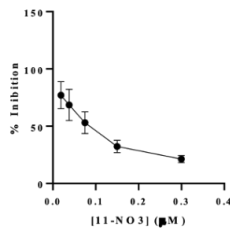
Compound 11-NO₃ MDA-MB-231 – 24 h



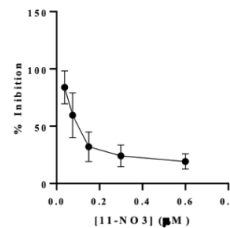
Compound 11-NO₃ L929 – 72 h



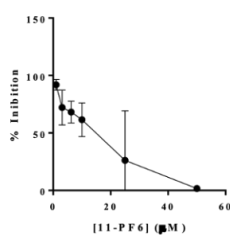
Compound 11-NO₃ MCF-7 – 72 h



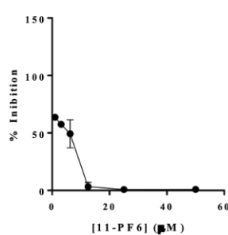
Compound 11-NO₃ MDA-MB-231 – 72 h



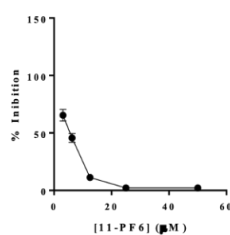
Compound 11-PF₆ L929 – 24 h



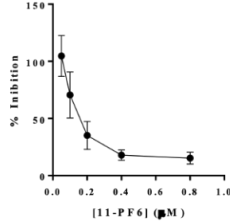
Compound 11-PF₆ MCF-7 – 24 h



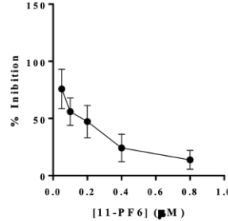
Compound 11-PF₆ MDA-MB-231 – 24 h



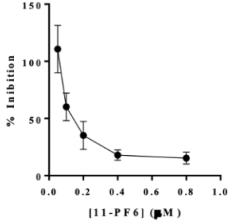
Compound 11-PF₆ L929 – 72 h



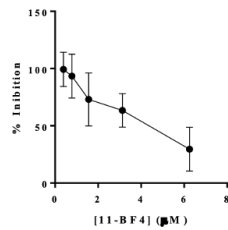
Compound 11-PF₆ MCF-7 – 72 h



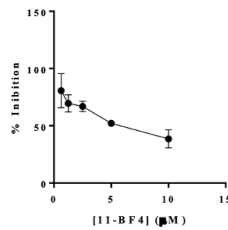
Compound 11-PF₆ MDA-MB-231 – 72 h



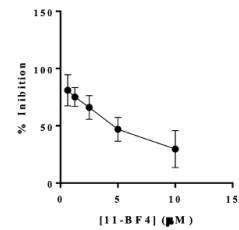
Compound 11-BF₄ L929 – 24 h



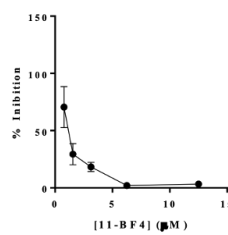
Compound 11-BF₄ MCF-7 – 24 h



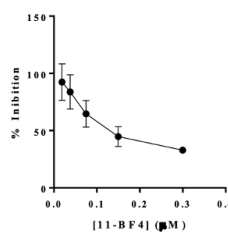
Compound 11-BF₄ MDA-MB-231 – 24 h



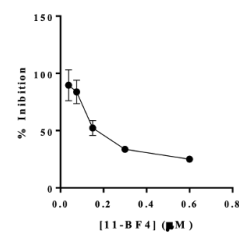
Compound 11-BF₄ L929 – 72 h



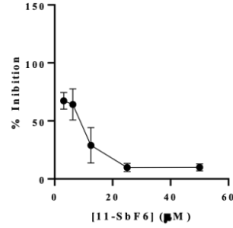
Compound 11-BF₄ MCF-7 – 72 h



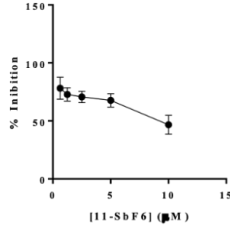
Compound 11-BF₄ MDA-MB-231 – 72 h



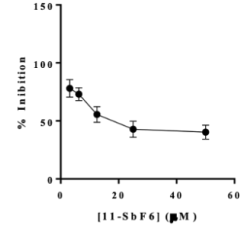
Compound 11-SbF₆ L929 – 24 h



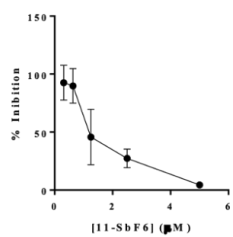
Compound 11-SbF₆ MCF-7 – 24 h



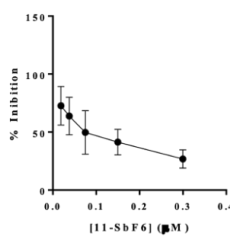
Compound 11-SbF₆ MDA-MB-231 – 24 h



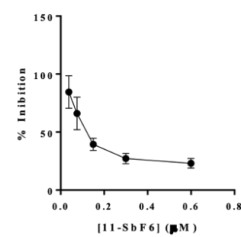
Compound 11-SbF₆ L929 – 72 h



Compound 11-SbF₆ MCF-7 – 72 h



Compound 11-SbF₆ MDA-MB-231 – 72 h



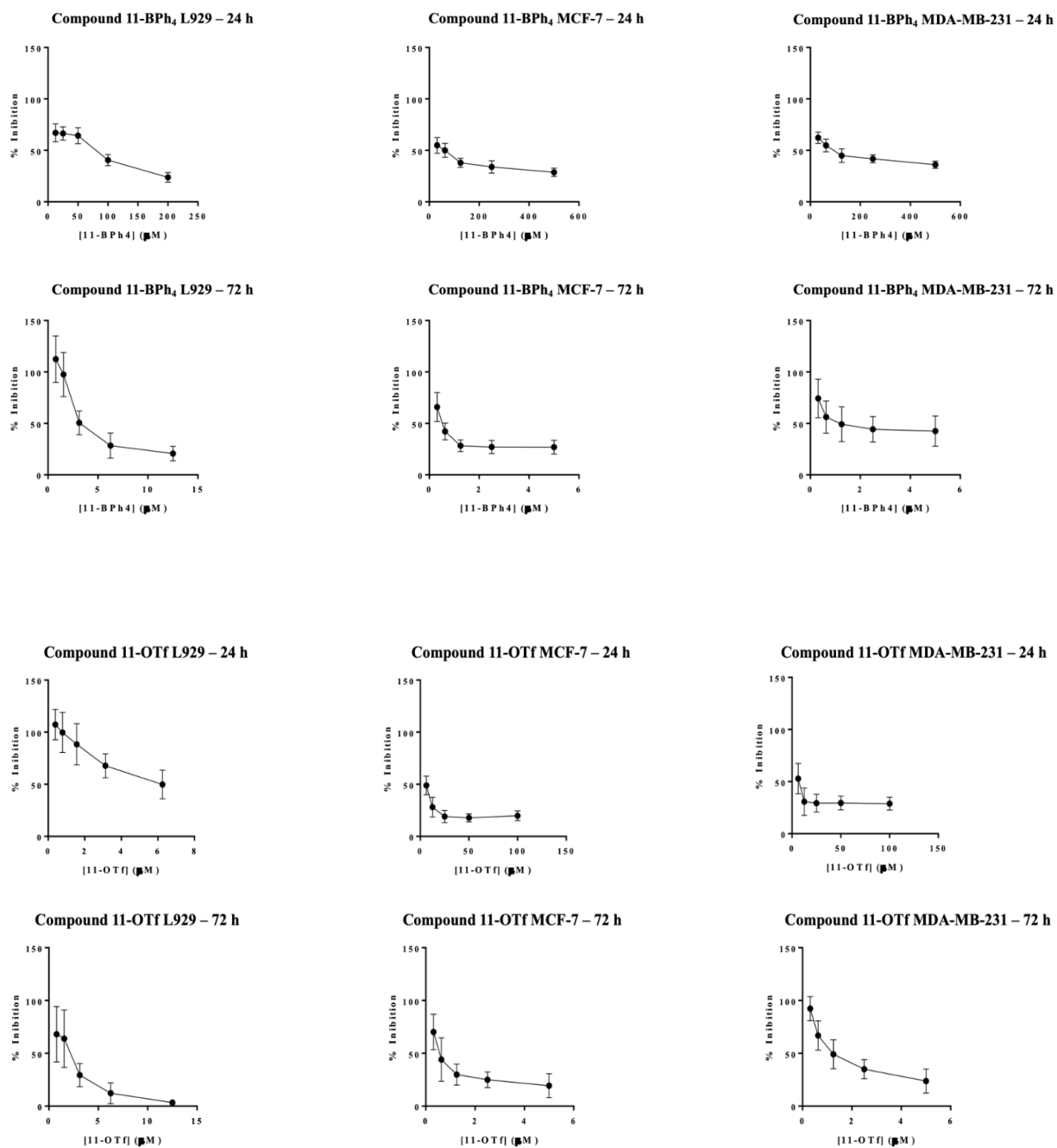


Figure S2. Dose-response curves of **10** and **11CA** over an exposure of 24 h and 72 h.

Table S5 – In-vitro antiproliferative activities of compounds **1** – **11CA** against breast cancer (MCF-7 and MDA-MB-231), and non-tumoral adherent mouse fibroblast connective tissue (L929) cell lines after 24 h of exposure. See Table S2 for the full statistical analysis. *The SI (selectivity index) was calculated as IC_{50} non-cancerous L929 divided by the average of the IC_{50} of the breast cancers cell lines.

Compound	IC_{50} (μ M) (average $\pm$ standard deviation) 24 h			SI*
	MCF-7	MDA-MB-231	L929	
1	275 $\pm$ 12	236 $\pm$ 7	511 $\pm$ 31	2.0
2	403 $\pm$ 9	286 $\pm$ 7	537 $\pm$ 29	1.6
3	463 $\pm$ 19	357 $\pm$ 7	749 $\pm$ 45	1.8
4	474 $\pm$ 19	384 $\pm$ 16	723 $\pm$ 29	1.7
5	526 $\pm$ 19	497 $\pm$ 12	612 $\pm$ 30	1.2
6	376 $\pm$ 15	388 $\pm$ 12	670 $\pm$ 60	1.8
7	407 $\pm$ 17	513 $\pm$ 16	570 $\pm$ 18	1.2
8	213 $\pm$ 8	284 $\pm$ 7	572 $\pm$ 33	2.3
9	304 $\pm$ 19	164 $\pm$ 2	544 $\pm$ 12	2.3
10	128 $\pm$ 30	113 $\pm$ 14	74 $\pm$ 5	0.6
11Cl	4.0 $\pm$ 0.3	5.5 $\pm$ 0.6	5.3 $\pm$ 0.7	1.1
11NO₃	5.9 $\pm$ 0.5	4.6 $\pm$ 0.3	3.7 $\pm$ 0.4	0.7
11BF₄	5.4 $\pm$ 0.6	4.3 $\pm$ 0.4	3.8 $\pm$ 0.4	0.8
11OTf	4 $\pm$ 1	4 $\pm$ 2	5.8 $\pm$ 0.7	1.5
11PF₆	3.0 $\pm$ 0.3	5.0 $\pm$ 0.1	10 $\pm$ 1	2.5
11SbF₆	11 $\pm$ 2	21 $\pm$ 2	7.0 $\pm$ 0.4	0.4
11BPh₄	51 $\pm$ 5	100 $\pm$ 8	59 $\pm$ 5	0.8

Table S6 – Statistical significance of the IC_{50} values between L929, MCF-7, and MDA-MB-231 cell lines at 24 h and 72 h.

Compounds	Statistical significance at 24 h			Statistical significance 72 h		
	L929 / MCF-7	L929 / MDA-MB-231	MCF-7 / MDA-MB-231	L929 / MCF-7	L929 / MDA-MB-231	MCF-7 / MDA-MB-231
1	****	****	****	****	***	****
2	****	****	****	****	****	∅
3	****	****	****	****	****	****
4	****	****	****	****	****	∅
5	****	**	****	****	****	****
6	****	∅	****	****	****	****
7	****	****	****	****	****	****
8	****	****	****	****	****	∅
9	****	****	****	****	****	****
10	****	∅	****	****	∅	****
11Cl	∅	∅	∅	****	∅	****
11NO₃	∅	∅	∅	∅	∅	∅
11BF₄	∅	∅	∅	****	∅	****
11OTf	∅	∅	∅	****	****	****
11PF₆	****	∅	****	∅	∅	∅
11SbF₆	***	****	****	****	∅	****
11BPh₄	****	****	****	****	****	****

∅, no statistical difference; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

Table S7 - IC_{50} values of the sodium counteranion salts tested in the selected cell lines over an exposure of 24 h and 72 h.

Compounds	IC_{50} in L929 cell line Mean $\pm$ SD*		IC_{50} in MCF-7 cell line Mean $\pm$ SD*		IC_{50} in MDA-MB-231 cell line Mean $\pm$ SD*	
	24 h	72h	24 h	72h	24 h	72h
NaCl	782 $\pm$ 55	274 $\pm$ 31	> 2000	280 $\pm$ 33	> 2000	398 $\pm$ 38
NaNO₃	> 1000	331 $\pm$ 57	> 2000	262 $\pm$ 38	> 2000	334 $\pm$ 23
NaBF₄	923 $\pm$ 77	386 $\pm$ 49	> 2000	343 $\pm$ 39	> 2000	347 $\pm$ 19
NaOTf	> 1000	159 $\pm$ 20	> 2000	279 $\pm$ 30	> 2000	354 $\pm$ 27
NaPF₆	759 $\pm$ 77	297 $\pm$ 24	> 2000	311 $\pm$ 27	> 1000	329 $\pm$ 17
NaSbF₆	693 $\pm$ 53	180 $\pm$ 22	> 1000	234 $\pm$ 24	689 $\pm$ 61	236 $\pm$ 13
NaBPh₄	186 $\pm$ 11	55 $\pm$ 4	217 $\pm$ 44	102 $\pm$ 14	441 $\pm$ 61	149 $\pm$ 13

4. Apoptosis effect of compounds tested in breast cancer cell lines

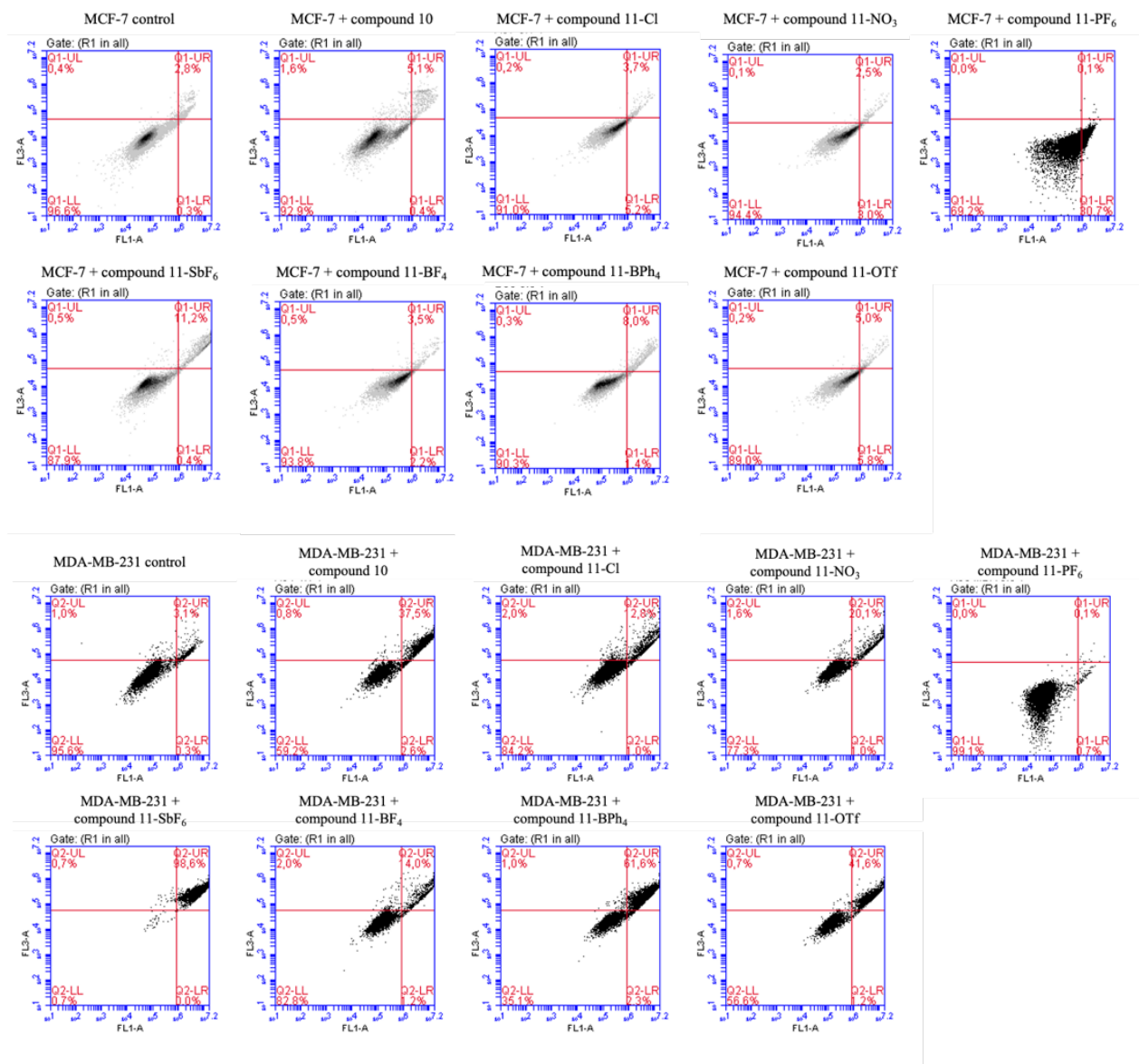


Figure S3 - Apoptosis profiles of **11CA** in MCF-7 and MDA-MB-231 cell lines after 24 h. Dot-plot graphs illustrated the viable cells (the lower left quadrant), early-phase apoptotic cells (the lower right quadrant), late-phase apoptotic or dead cells (the upper right quadrant), and the necrotic cells (the upper left quadrant).

5. Stability in solution and reactivity with model biomolecules

Stability studies were performed on **11CA** over 72 h under at 37 °C. The stability of the complexes **11CA** was studied via ESI-MS. The complexes were dissolved in DMSO and the solution were diluted with milliQ to reach 0.1% DMSO in water (final concentration, 10 μM). Aliquots were taken and injected into the spectrometer without any further sample dilution or preparation. The stability of **11Cl** was also monitored over 72 h via ¹H and ³¹P NMR spectroscopy. The complex was fully dissolved in d₆-DMSO, diluted with D₂O to

reach 10% d_6 -DMSO in D_2O (1 mM final concentration) and, finally, the pD was adjusted to 7.2 ± 0.1 (neutral conditions) and 6.4 ± 0.1 (acidic conditions). Furthermore, the stability of **11Cl** and **11BPh₄** was also assessed in 100% d_6 -DMSO as a highly coordinating solvent.

The interaction between **11Cl** and different model biomolecules (*aminoacids*: glutamic acid, histidine and cysteine; *nucleobase*, dGMP) was studied over 72 h under pseudo-physiological conditions at 37 °C. The complex and the different nucleophiles in a 1:3 ratio were fully dissolved in d_6 -DMSO, diluted with D_2O to reach 10% DMSO in D_2O and, finally, the pD was adjusted to 7.2 ± 0.1 . The interaction with Cys was studied 20% D_2O in $MeOH-d_4$, to avoid the oxidation of the thiol caused by d_6 -DMSO (17) The interaction was monitored via 1H and ^{31}P NMR spectroscopy at different times and by ESI-MS after 72 h.

The catalytic activity of **11Cl** in transfer hydrogenation ($NAD^+/NADH$) and glutathione oxidation reactions (GSH/GSSG) at 37 °C was monitored by 1H and ^{31}P NMR spectroscopy over 8 hours. Complex **11Cl** was dissolved in $MeOH-d_4/D_2O$ (5:1 v/v, 1.4 mM). In a typical experiment, 3 mL of a solution containing complex **11Cl** (0.44 mM) and either NAD^+ (0.88 mM, 2 eq.) and sodium formate (11.02 mM, 25 eq.), for transfer hydrogenation; or reduced glutathione (1.32 mM, 3 eq.), for oxidation, were added to a 10-mm NMR tube. The pD was adjusted to 7.2 ± 0.1 . 1H NMR spectra and ^{31}P NMR spectra were recorded at 310 K every 300 s for 8 h.

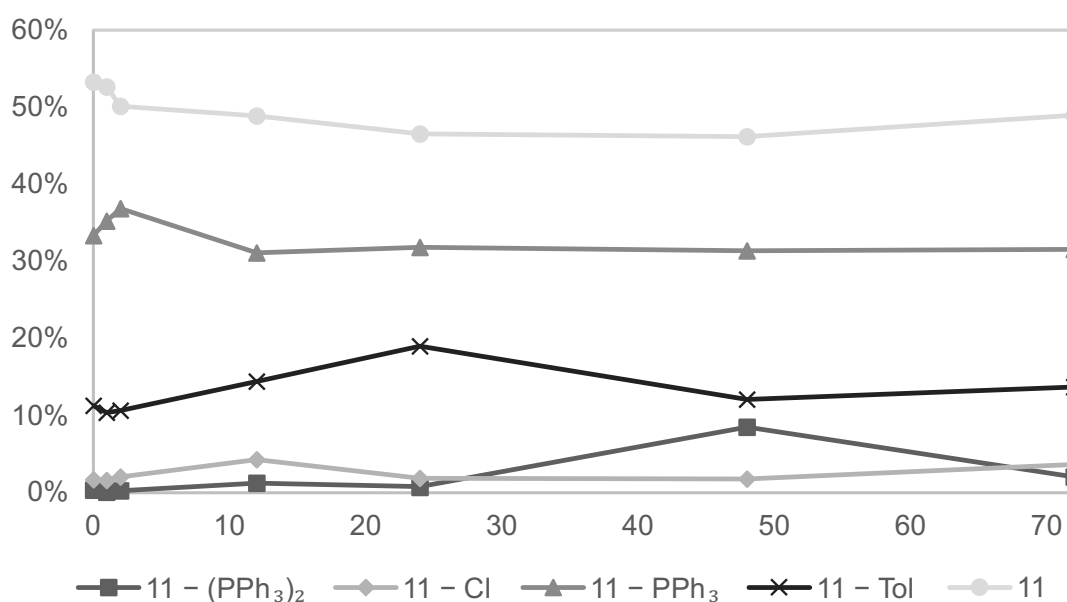


Figure S4 - Stability of **11Cl** in pseudo-physiological conditions monitored by ESI-MS.

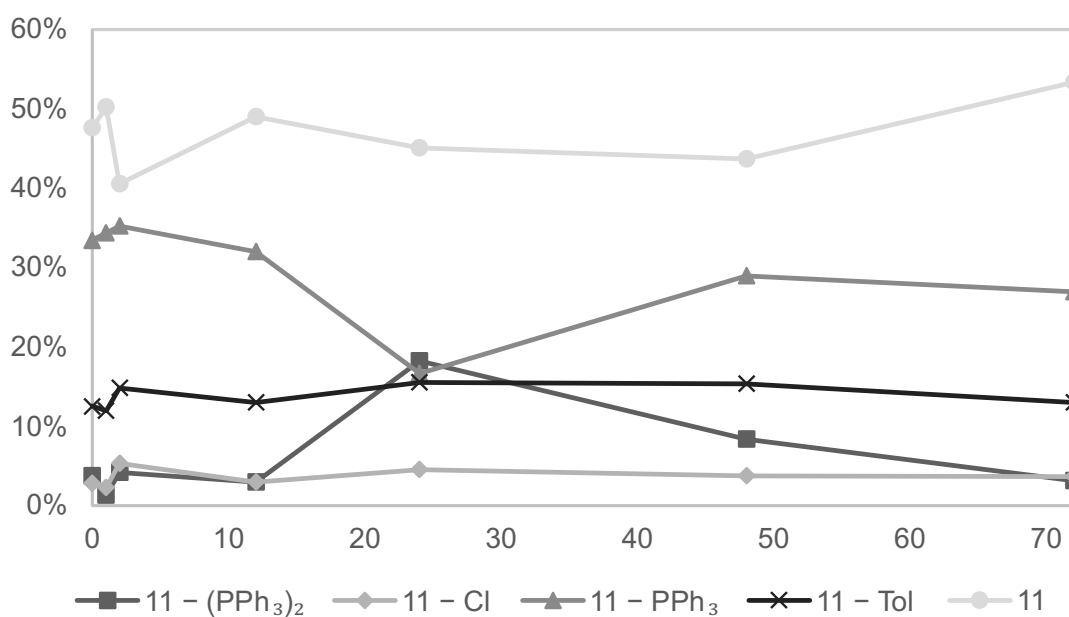


Figure S5 - Stability of 11NO₃ in pseudo-physiological conditions monitored by ESI-MS.

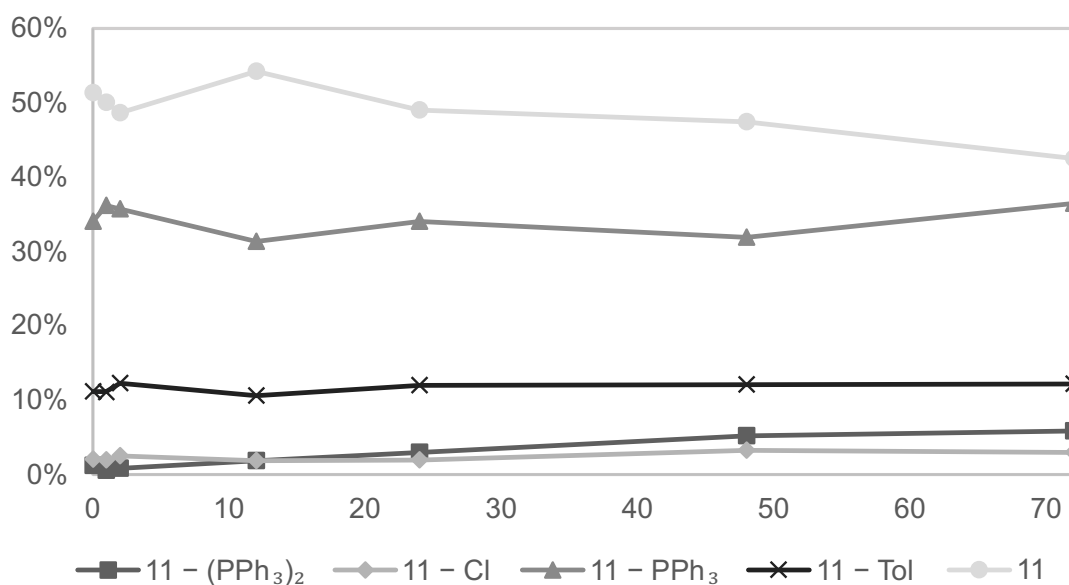


Figure S6 - Stability of 11BF₄ in pseudo-physiological conditions monitored by ESI-MS.

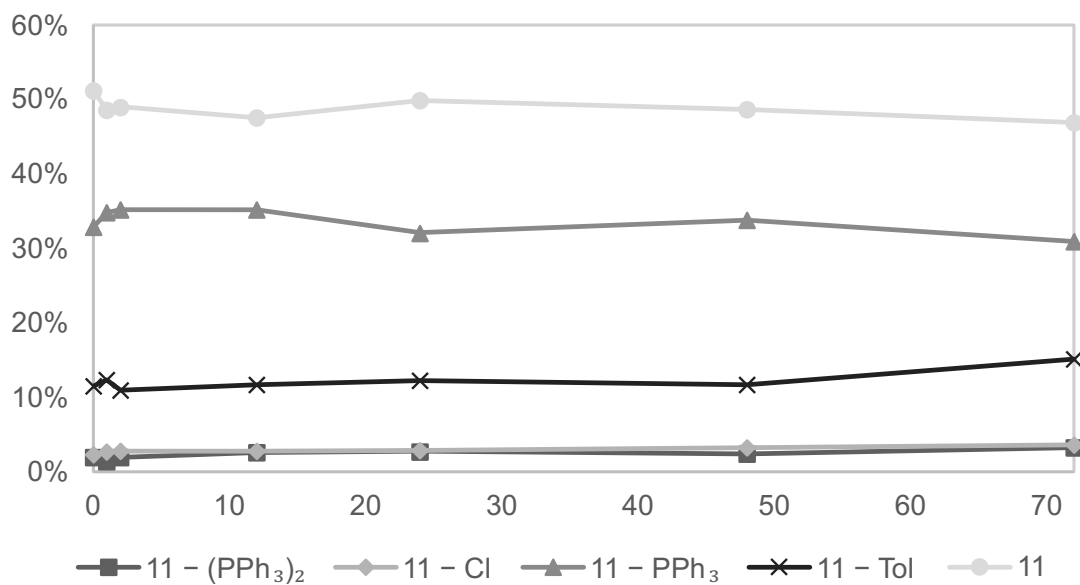


Figure S7 - Stability of **11OTf** in pseudo-physiological conditions monitored by ESI-MS.

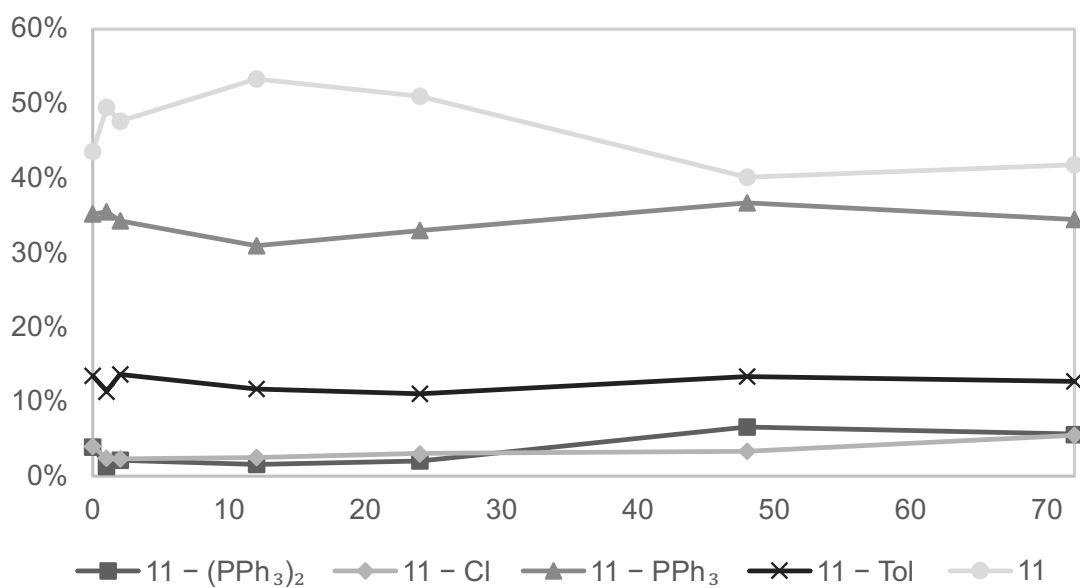


Figure S8 - Stability of **11PF₆** in pseudo-physiological conditions monitored by ESI-MS.

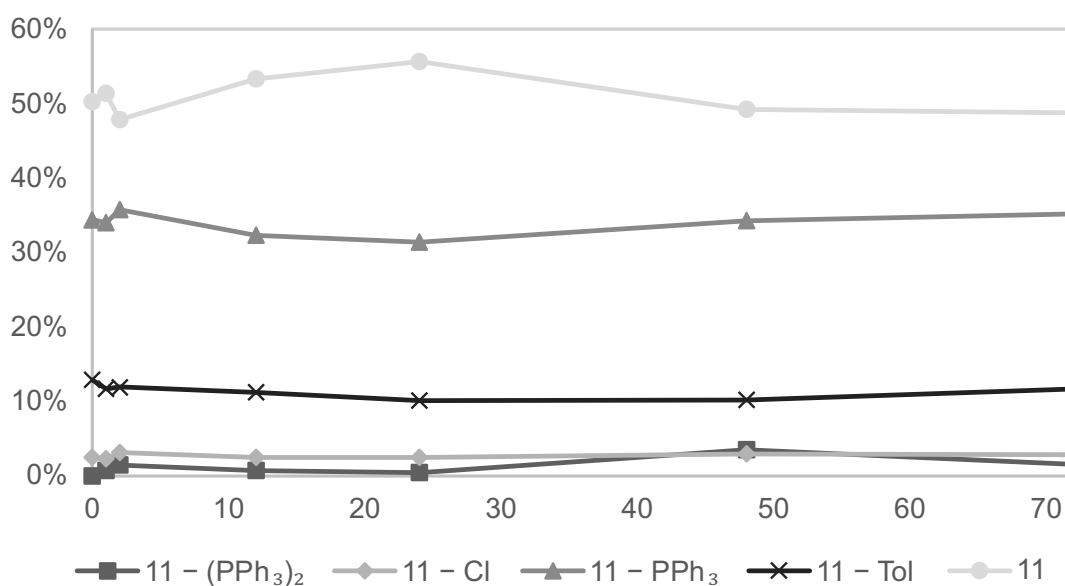


Figure S9 - Stability of **11SbF₆** in pseudo-physiological conditions monitored by ESI-MS.

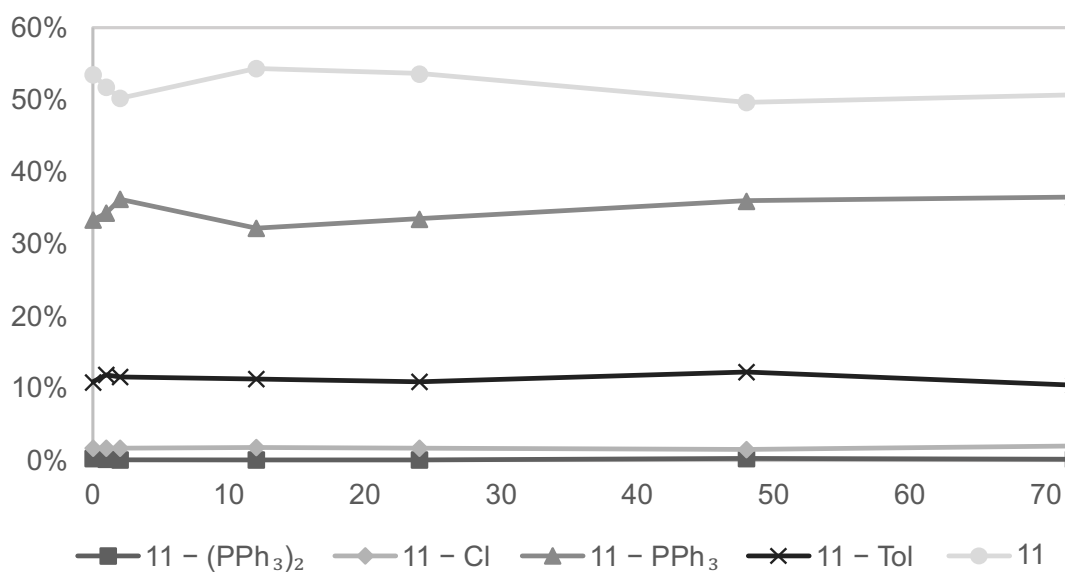


Figure S10 - Stability of **11BPh₄** in pseudo-physiological conditions monitored by ESI-MS.

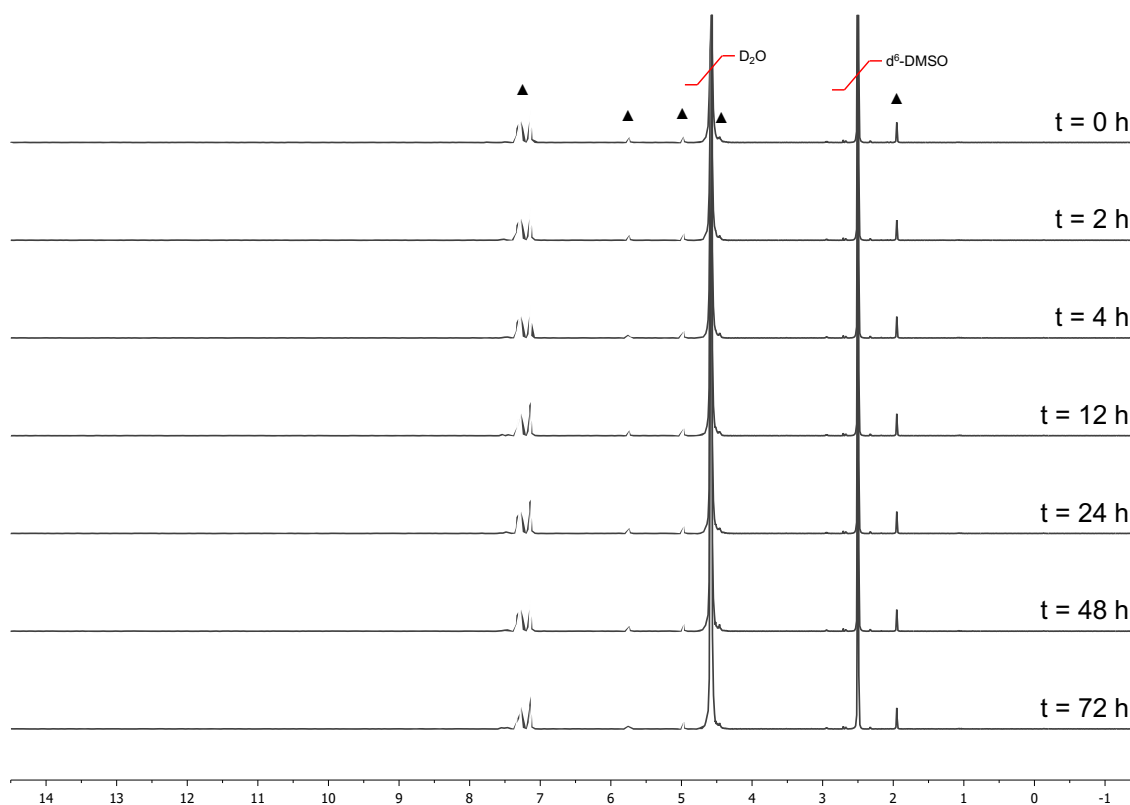


Figure S11 - Stability of ^{11}Cl (▲) in pseudo-physiological conditions at $p\text{D} = 7.2 \pm 0.2$ monitored by ^1H NMR.

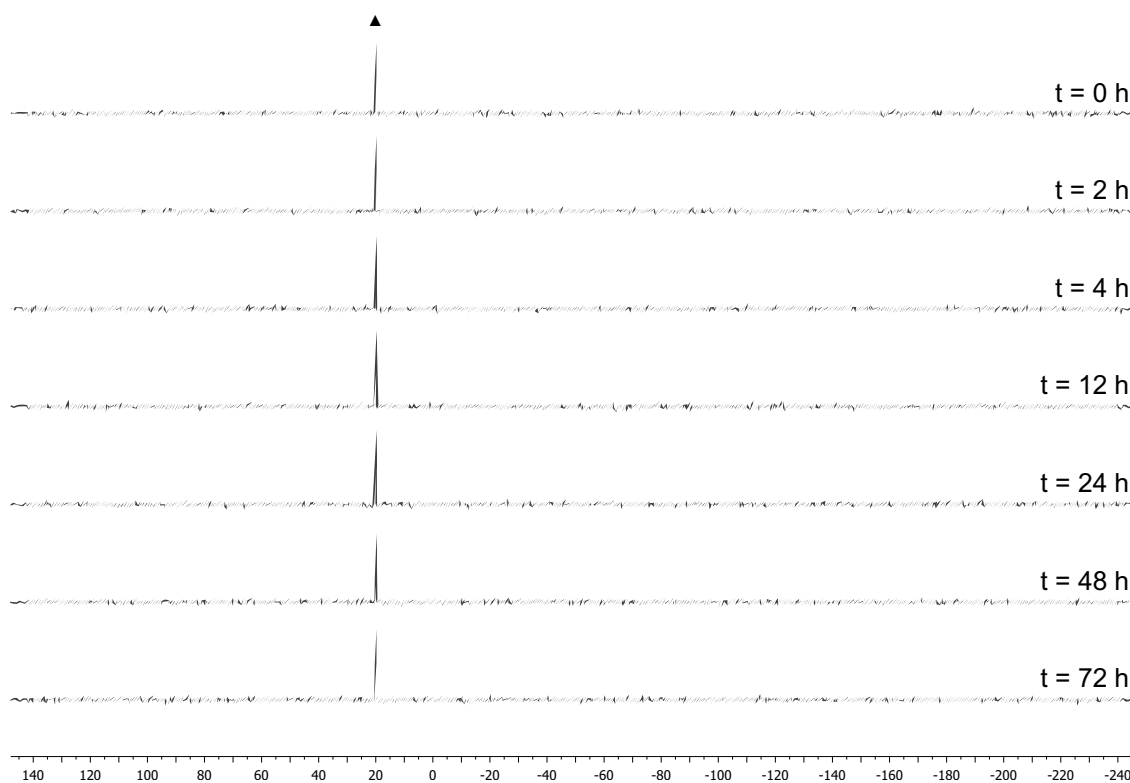


Figure S12 - Stability of ^{11}Cl (▲) in pseudo-physiological conditions at $p\text{D} = 7.2 \pm 0.2$ monitored by ^{31}P NMR.

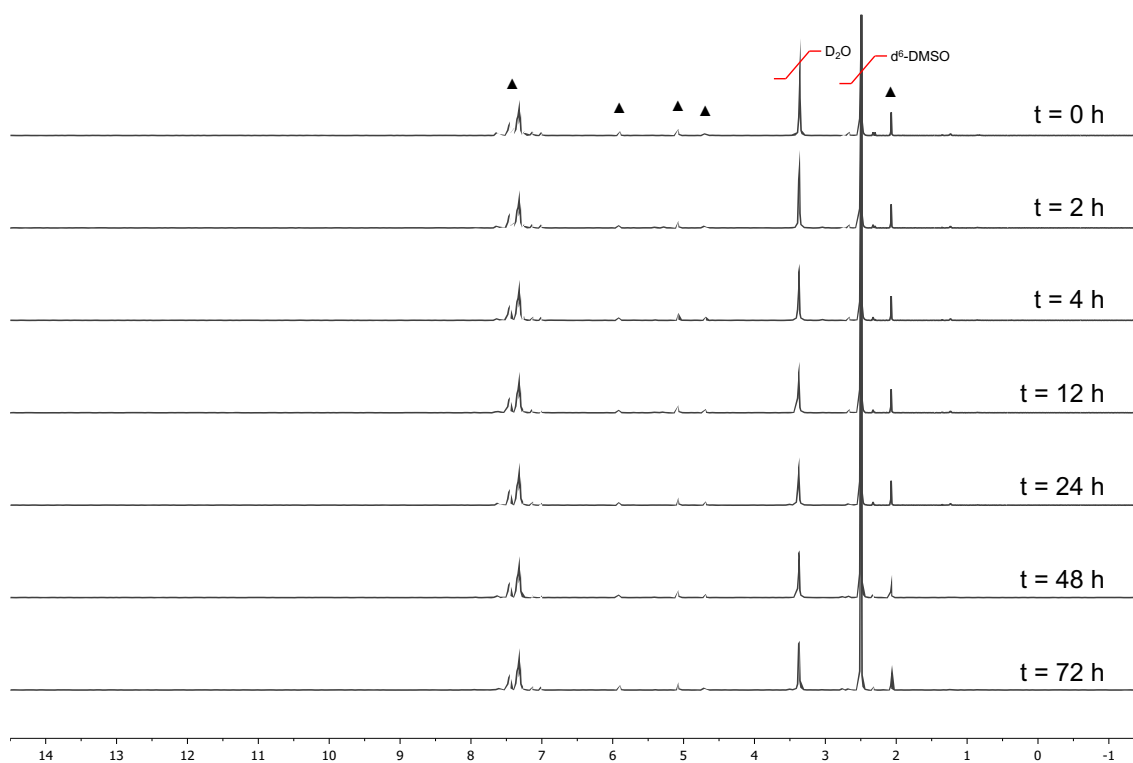


Figure S13 - Stability of ^{11}Cl (▲) in d_6 -DMSO monitored by ^1H NMR.

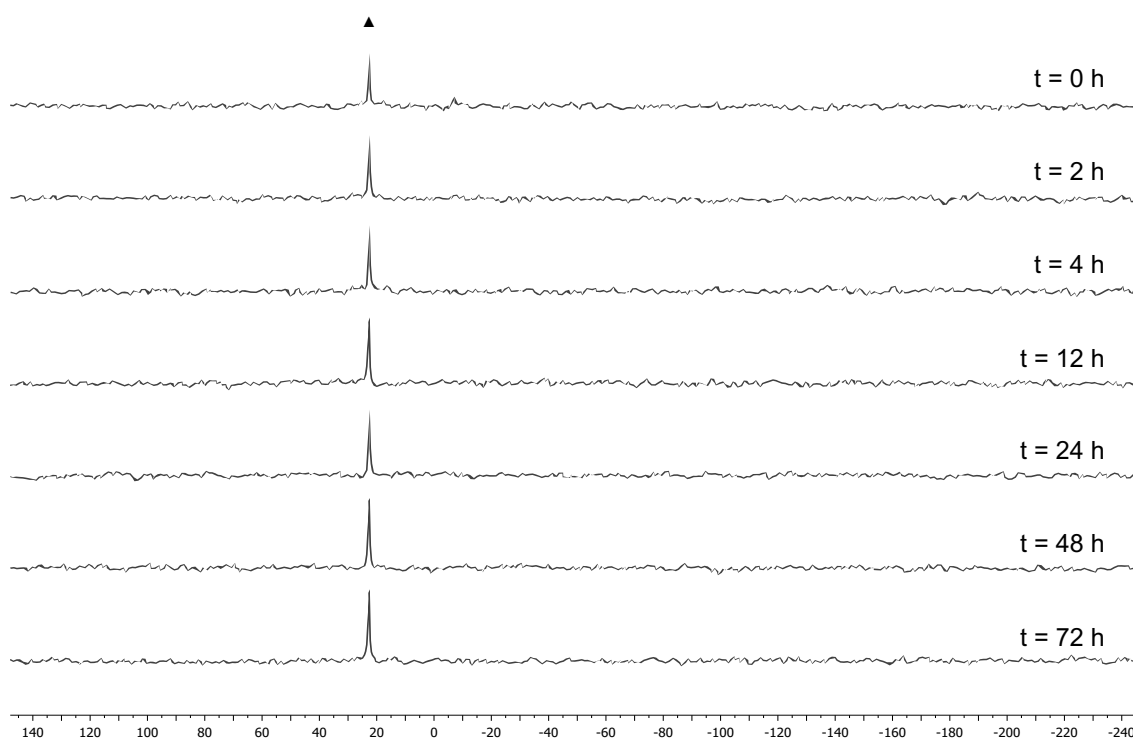


Figure S14 - Stability of ^{11}Cl (▲) in d_6 -DMSO monitored by ^{31}P NMR.

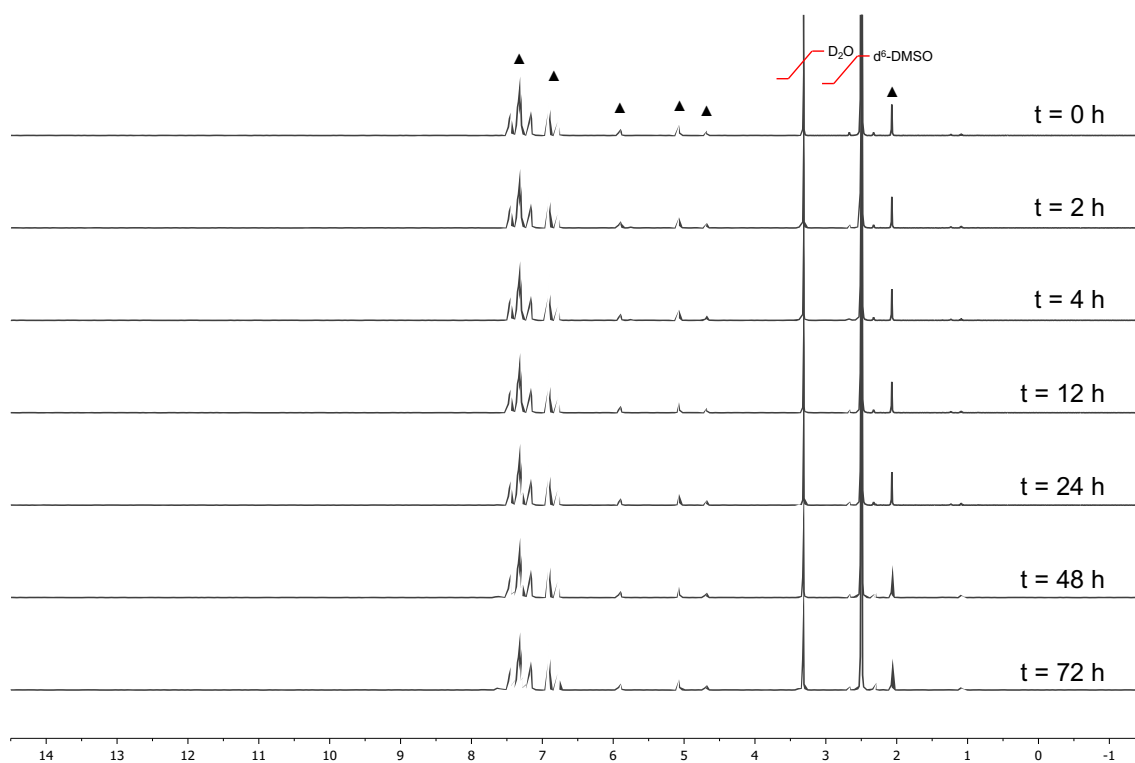


Figure S15 - Stability of 11BPh_4 ($\blacktriangle$) in $\text{d}^6\text{-DMSO}$ monitored by ^1H NMR.

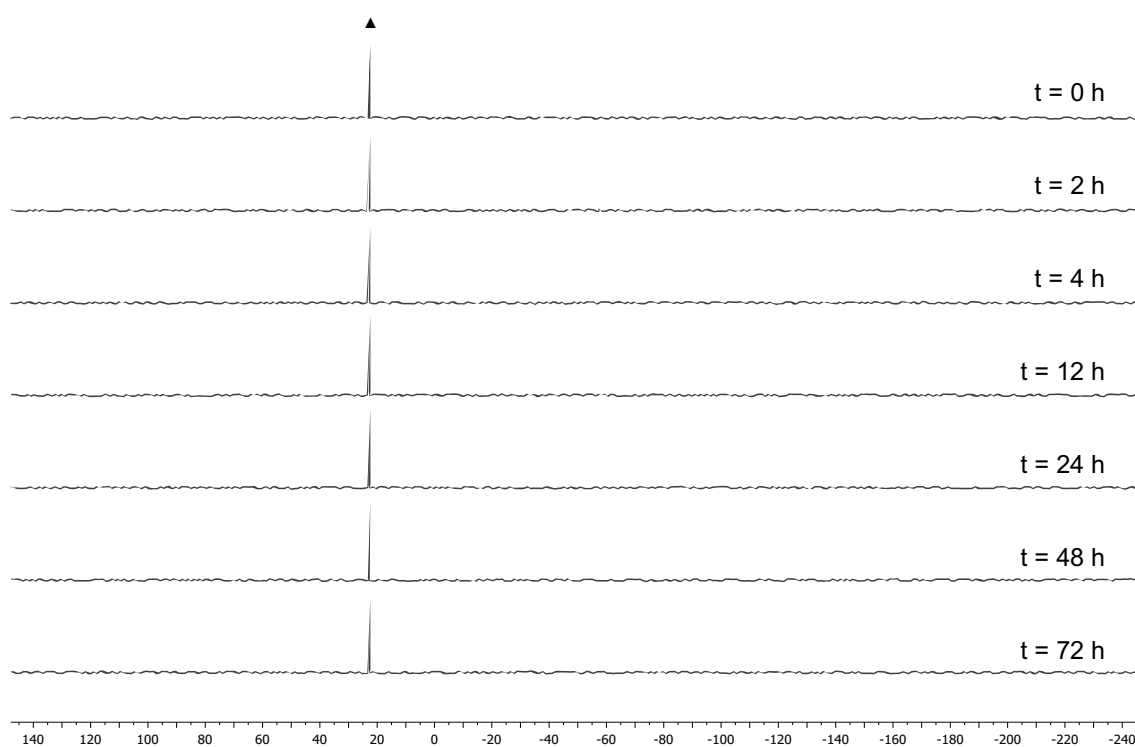


Figure S16 - Stability of 11BPh_4 ($\blacktriangle$) in $\text{d}^6\text{-DMSO}$ monitored by ^{31}P NMR.

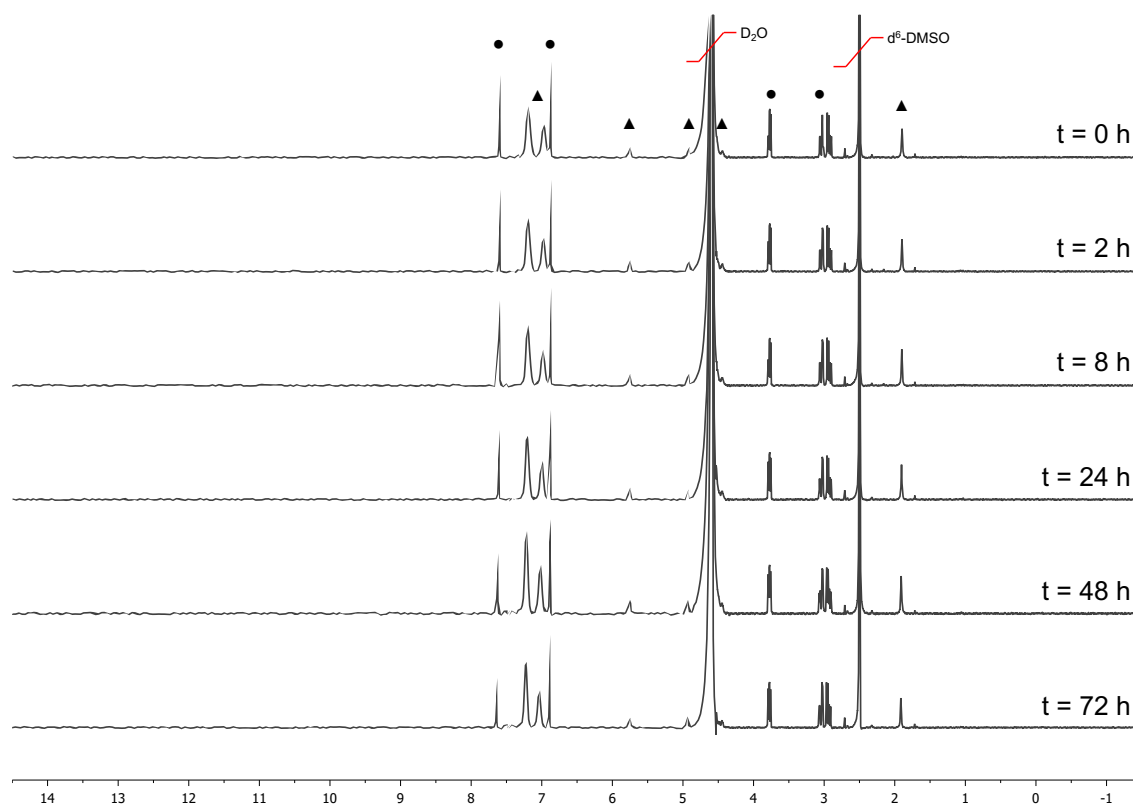


Figure S17 - Interaction of ^{11}Cl (▲) with His (●) in pseudo-physiological conditions monitored by ^1H NMR.

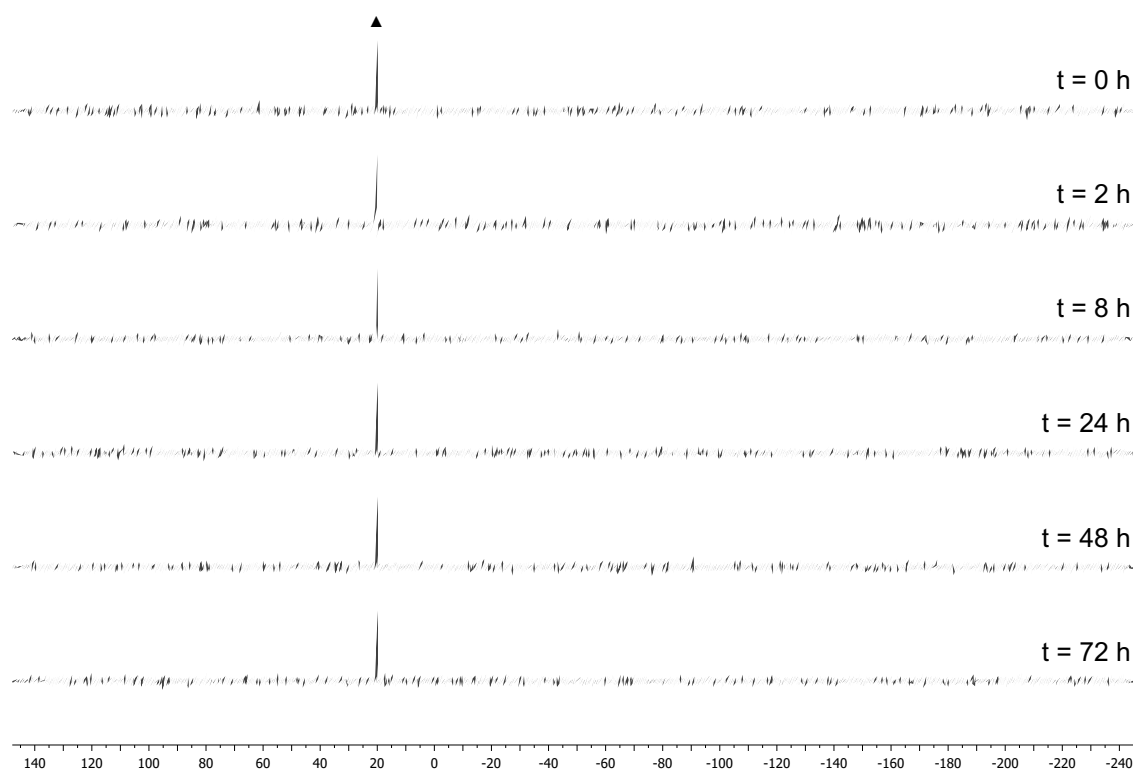


Figure S18 - Interaction of ^{11}Cl (▲) with His (●) in pseudo-physiological conditions monitored by ^{31}P NMR.

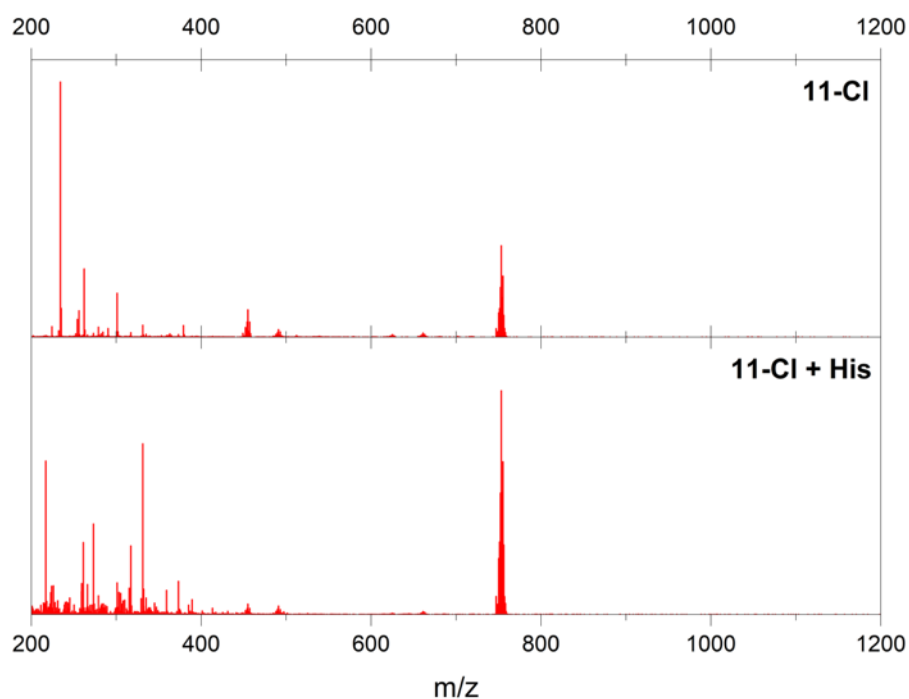


Figure S19 - ESI-MS spectra of **11Cl** and of its interaction with His in pseudo-physiological conditions after 72 hours.

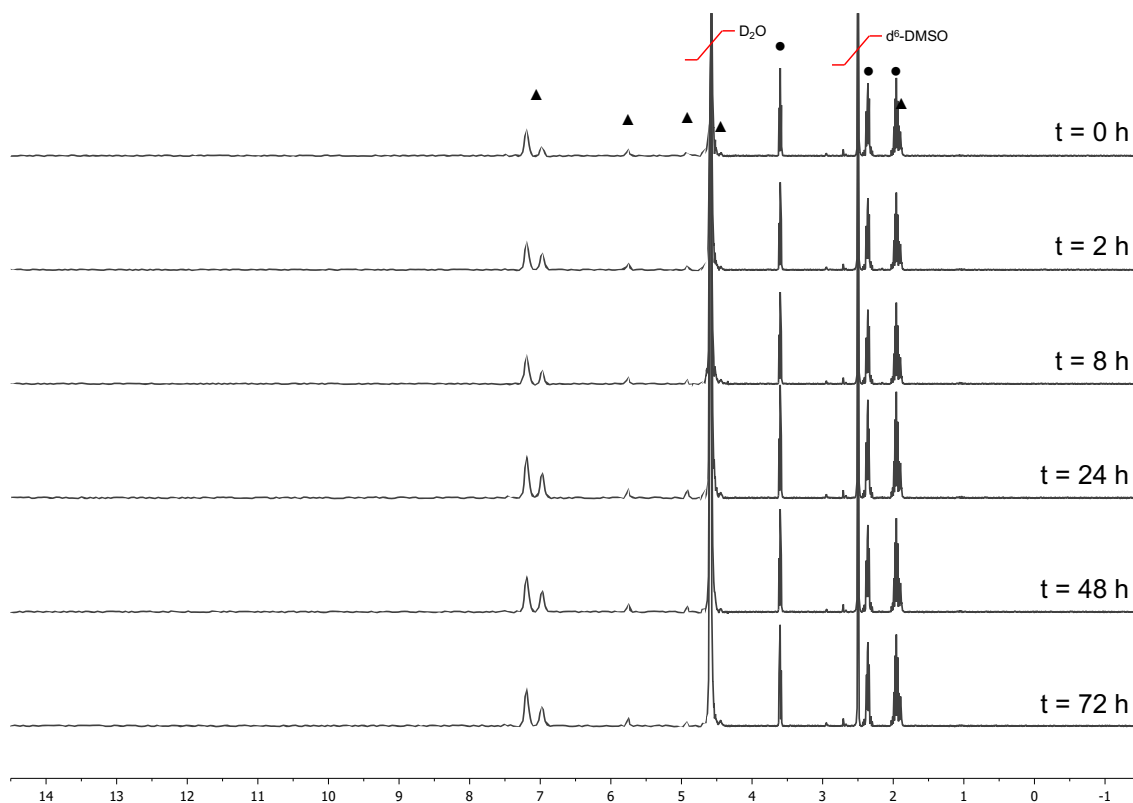


Figure S20 - Interaction of **11Cl** ($\blacktriangle$) with Glu ($\bullet$) in pseudo-physiological conditions monitored by ^1H NMR.

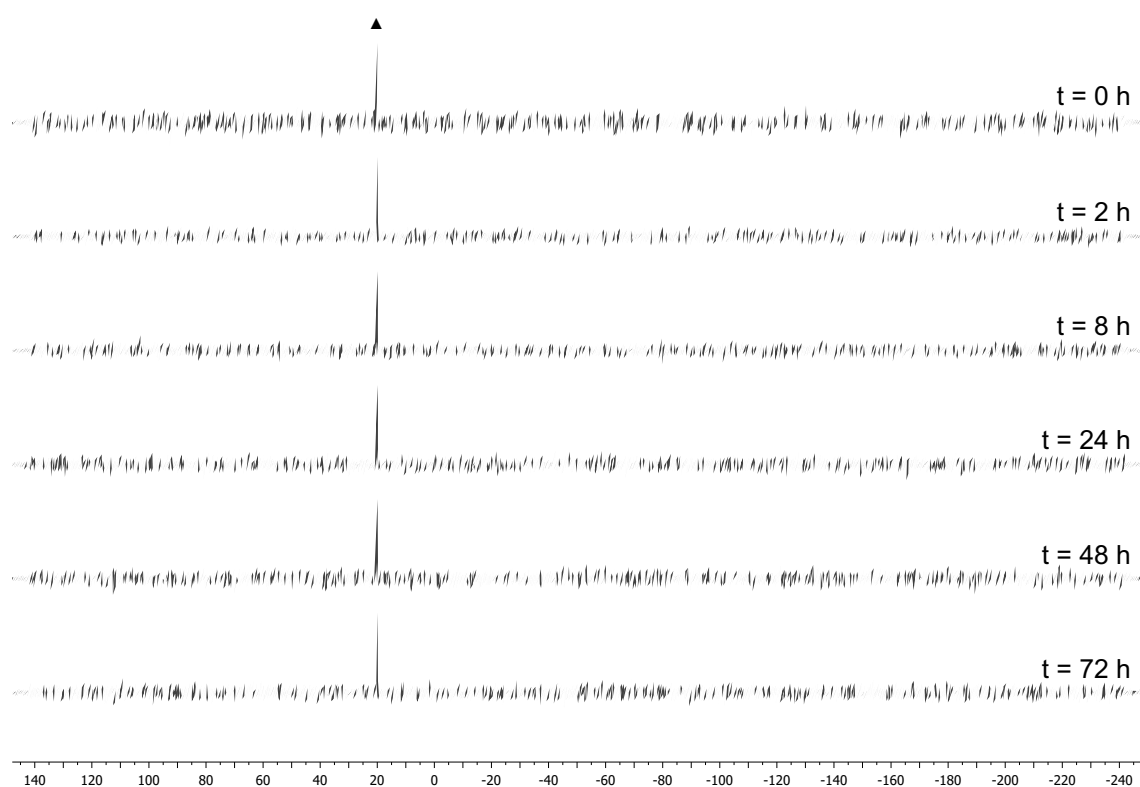


Figure S21 - Interaction of **11Cl** (▲) with **Glu** (●) in pseudo-physiological conditions monitored by ^{31}P NMR.

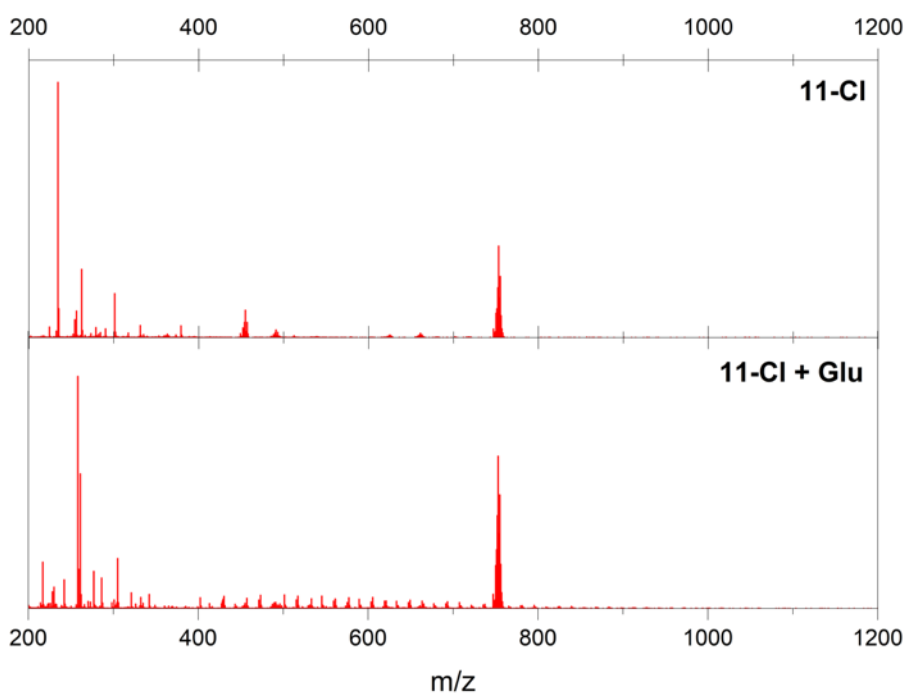


Figure S22 - ESI-MS spectra of **11Cl** and of its interaction with **Glu** in pseudo-physiological conditions after 72 hours.

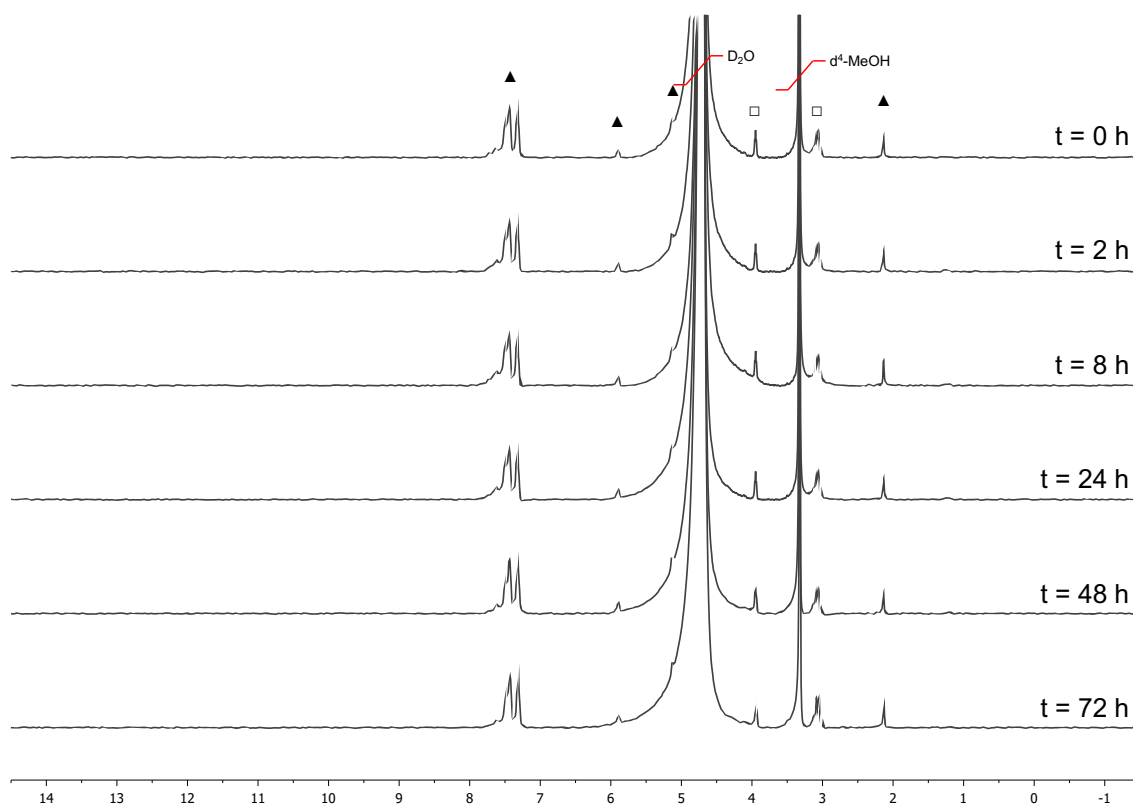


Figure S23 - Interaction of **11Cl** (▲) with Cys (●) in pseudo-physiological conditions monitored by ^1H NMR.

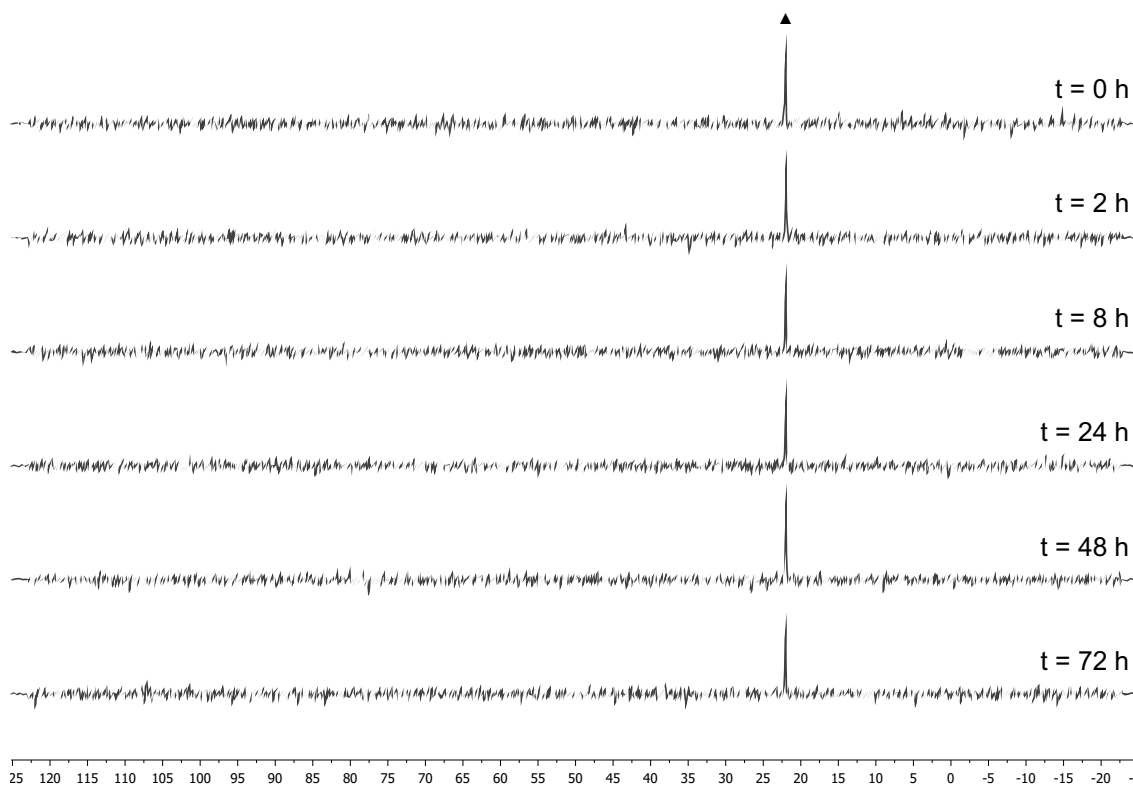


Figure S24 - Interaction of **11Cl** (▲) with Cys (●) in pseudo-physiological conditions monitored by ^{31}P NMR.

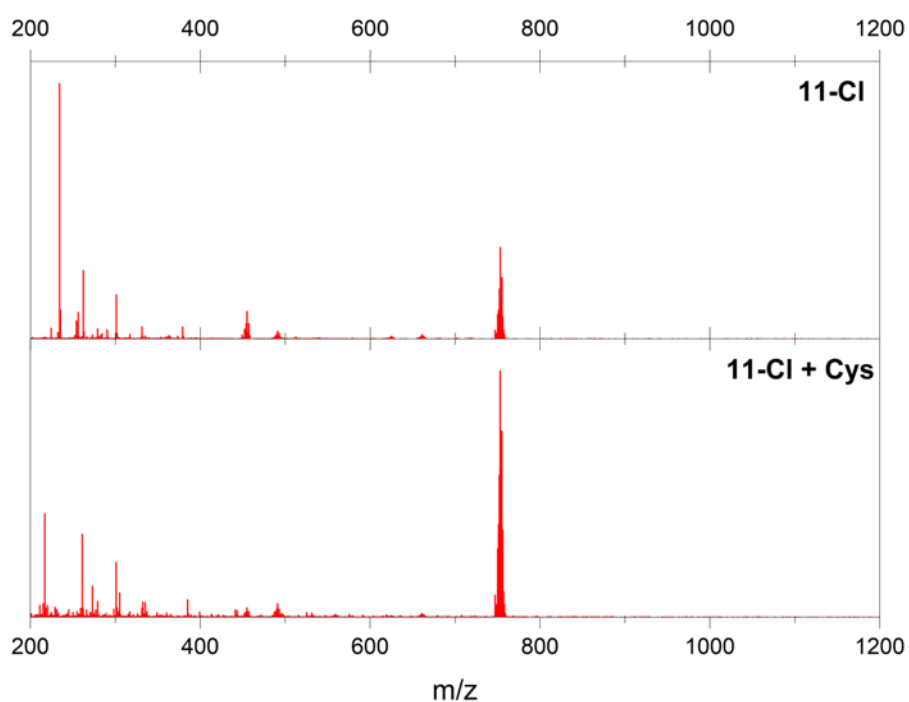


Figure S25 - ESI-MS spectra of **11Cl** and of its interaction with Cys in pseudo-physiological conditions after 72 hours.

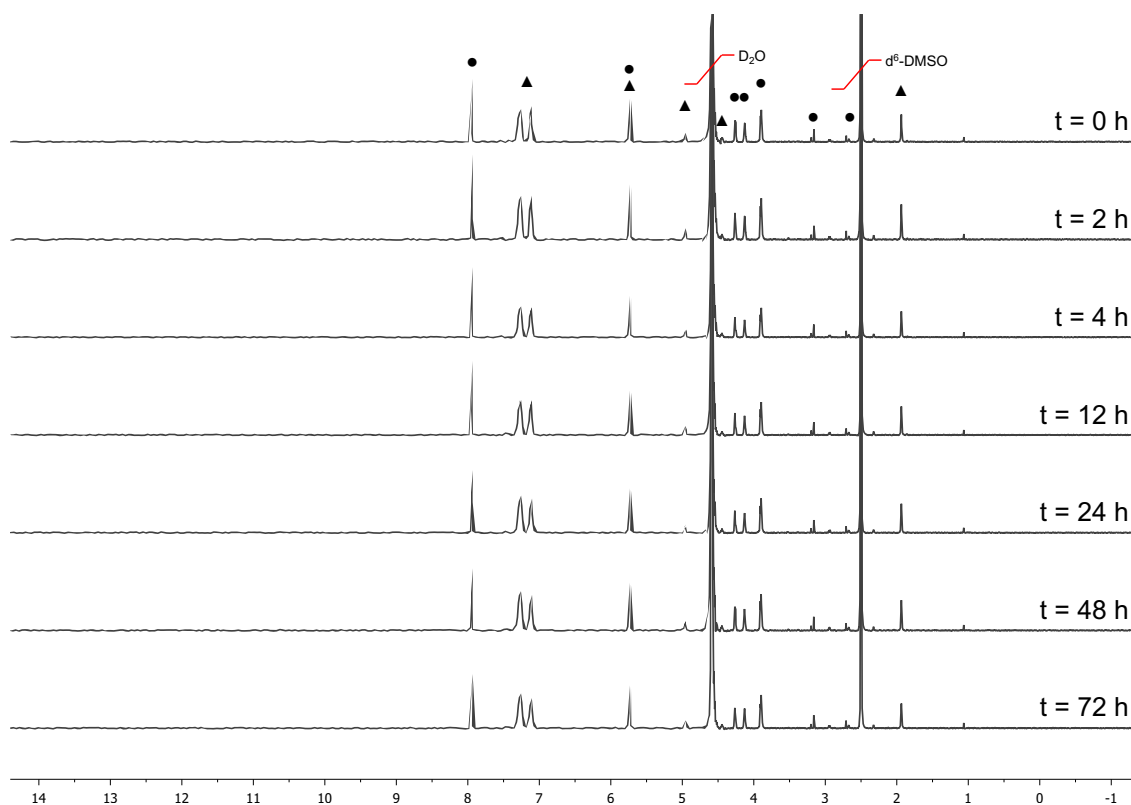


Figure S26 - Interaction of **11Cl** (▲) with dGMP (●) in pseudo-physiological conditions monitored by ^1H NMR.

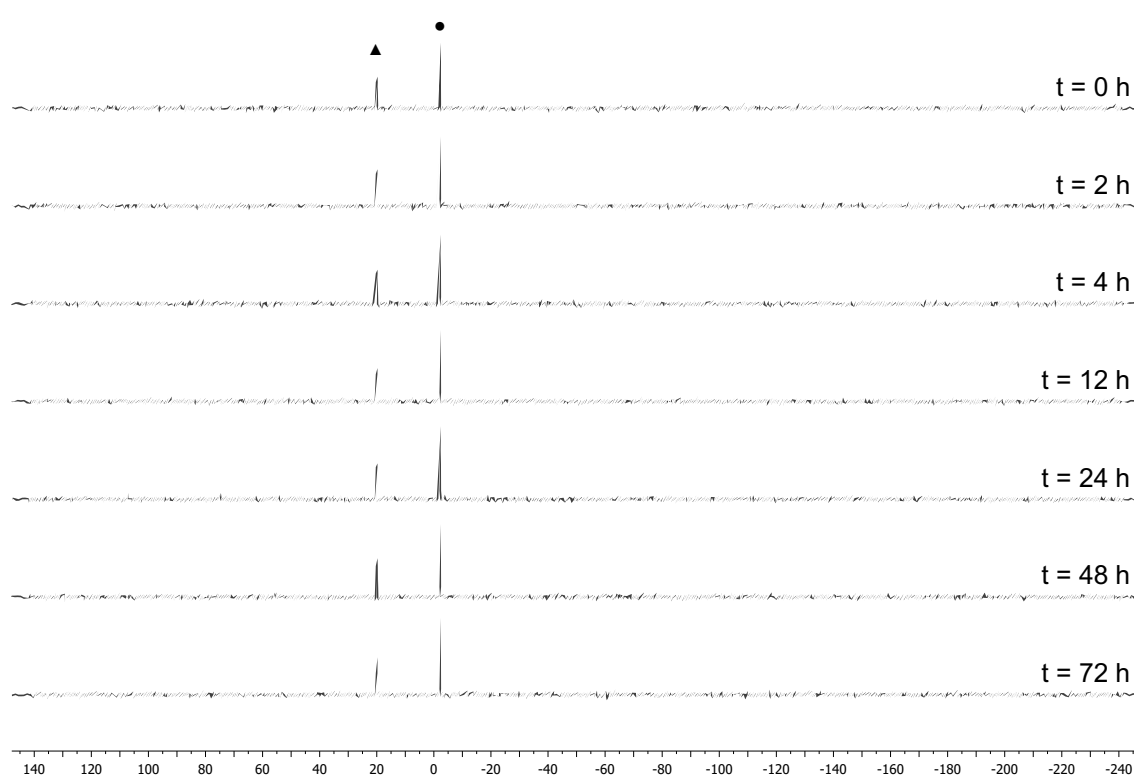


Figure S27 - Interaction of **11Cl** (▲) with dGMP (●) in pseudo-physiological conditions monitored by ^{31}P NMR.

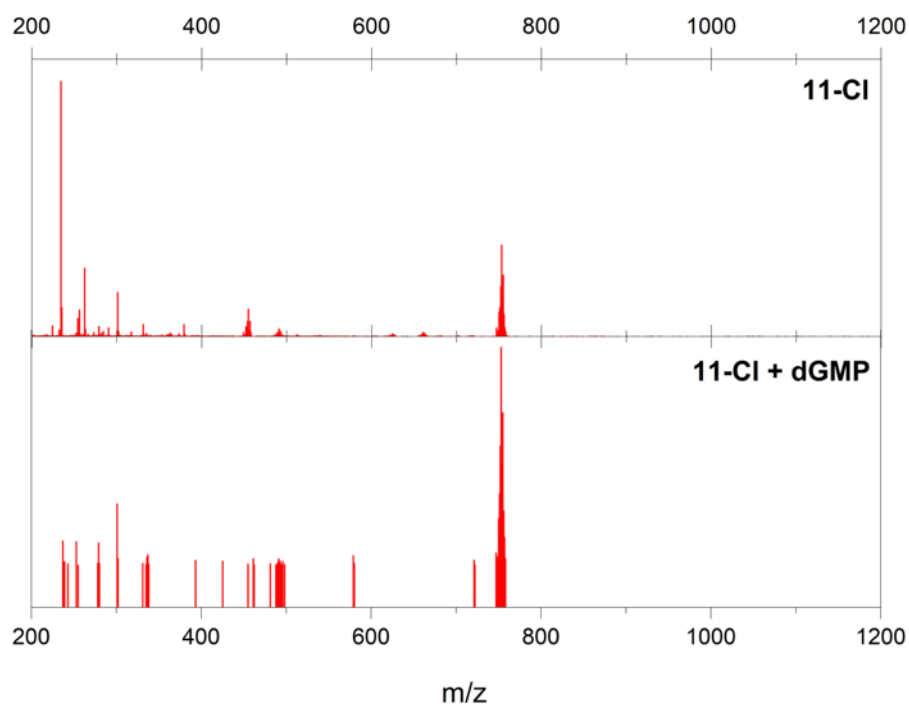


Figure S28 - ESI-MS spectra of **11Cl** and of its interaction with dGMP in pseudo-physiological conditions after 72 hours.

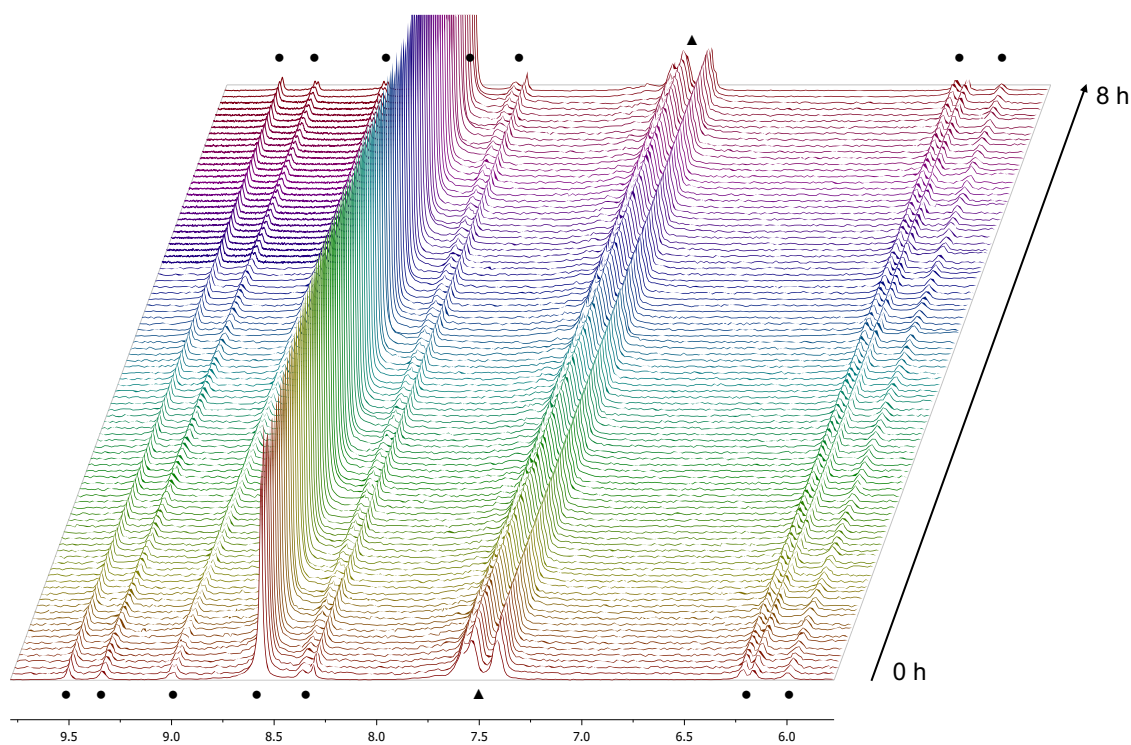


Figure S29 - ^1H NMR monitoring of the NAD^+/NADH (●) transfer hydrogenation by 11Cl (▲).

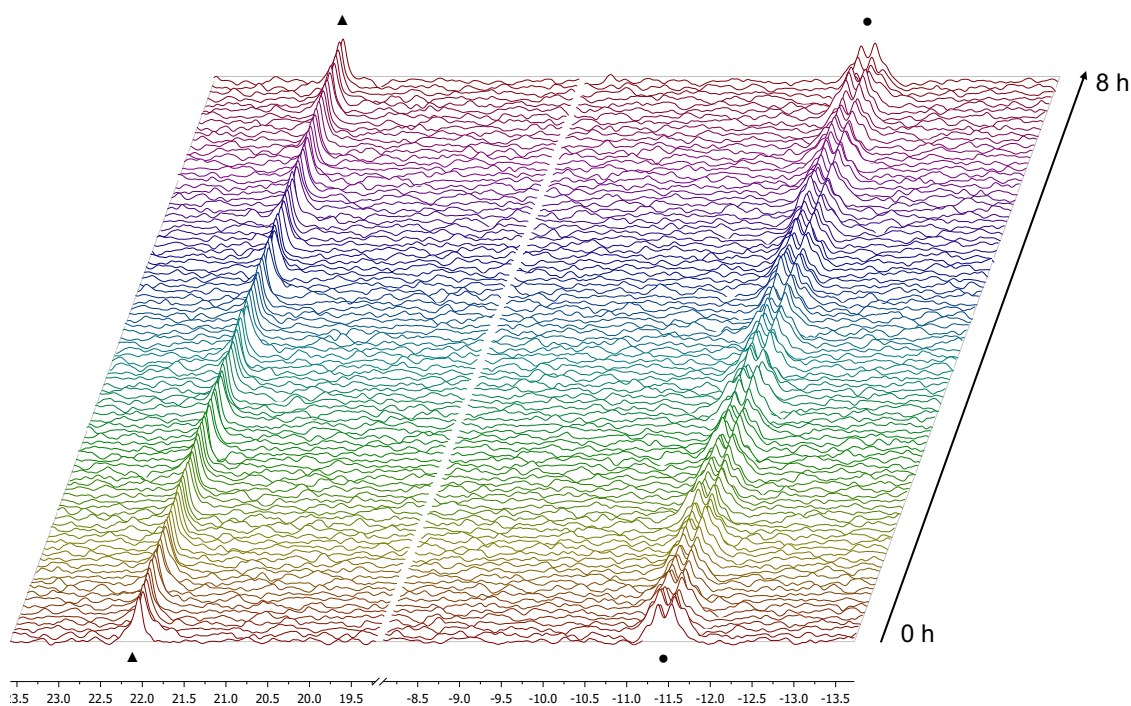


Figure S30 - ^{31}P NMR monitoring of the NAD^+/NADH (●) transfer hydrogenation by 11Cl (▲).

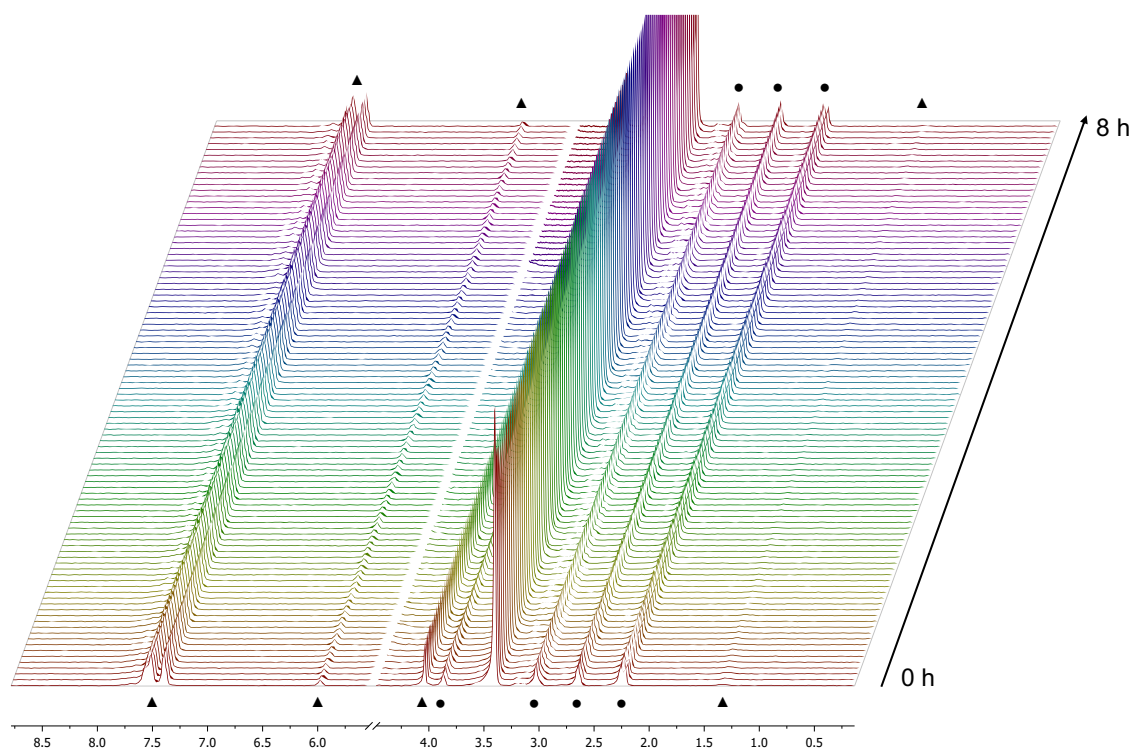


Figure S31 - ^1H NMR monitoring of the GSH (●) oxidation by 11Cl (▲).

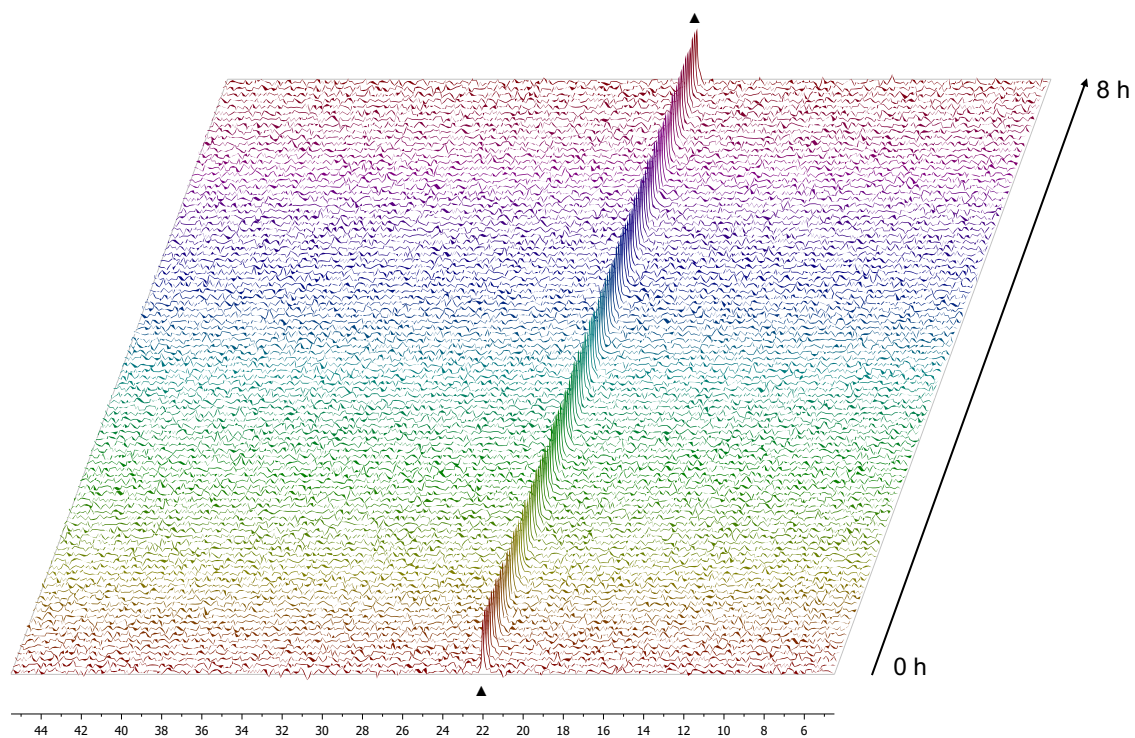


Figure S32 - ^{31}P NMR monitoring of the GSH (●) oxidation by 11Cl (▲).

6. Circular dichroism

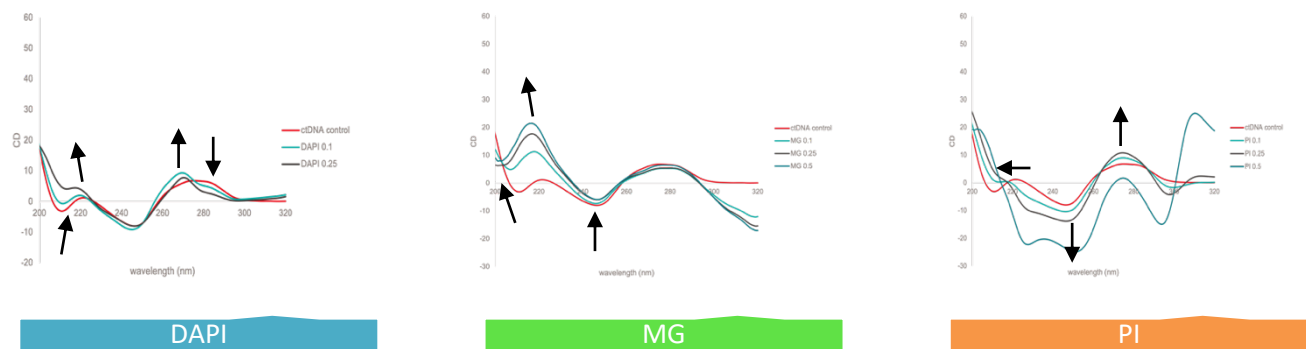


Figure S33 - Circular dichroism spectra of dsDNA incubated with DAPI, MG and PI.

Table S8 - Summary of the CD band changes on dsDNA induced by DAPI, MG, and PI.

Probe	210 nm	220 nm <i>Hydrogen bonding</i>	246 nm <i>Helicity</i>	268–280 nm <i>Base-pair stacking interactions</i>
DAPI <i>Minor groove</i>	Hypochromic, $\Delta \downarrow$ Red shift, $\lambda \uparrow$	Hyperchromic, $\Delta \uparrow$ Blue shift, $\lambda \downarrow$	Unchanged Unchanged	268 nm – Hyperchromic, $\Delta \uparrow$ 280 nm – Hypochromic, $\Delta \downarrow$ Unchanged
MG <i>Major groove</i>	Hypochromic, $\Delta \downarrow$ Blue shift, $\lambda \downarrow$	Hyperchromic, $\Delta \uparrow$ Blue shift, $\lambda \downarrow$	Hypochromic, $\Delta \downarrow$ Unchanged	Unchanged Unchanged
PI <i>Intercalation</i>		Unchanged Blue shift, $\lambda \downarrow$	Hyperchromic, $\Delta \uparrow$ Unchanged	Hyperchromic, $\Delta \uparrow$ Unchanged

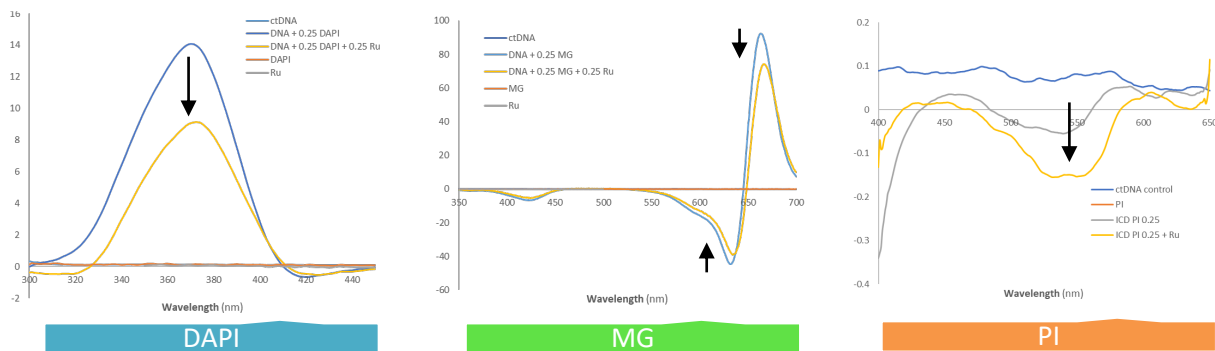


Figure S34 - Induced circular dichroism spectra of dsDNA incubated with DAPI, MG, and PI.

7. Molecular docking

The docking files were prepared using AutoDock Tools. After removing the solvent molecules, cocrystallized ligands, and cofactors, all the hydrogens atoms were added to DNA, then non-polar hydrogen atoms were merged and Gasteiger charges were calculated. The free torsion tree was assigned, and the ruthenium atom parameters were added to the generated PDBQT file. A grid box was created with $96 \times 96 \times 96$ points and a resolution of 0.375 Å (which is roughly a quarter of the length of a carbon-carbon single bond), in order to include the entire DNA fragment. Standard AutoDock force fields were used to calculate the energies and the best conformation was optimized by applying a Lamarckian Genetic Algorithm (LGA). With an initial population size of 300 and several evaluations of 2500000, the GA was allowed to run up to 27000 generations to find one best individual. Each experiment was set to 100 GA runs. The gene mutation and crossover rate were fixed at 2% and 80%, respectively. Clustering was performed to analyze the convergence of the simulations. The resulting poses were visualized with Visual Molecular Dynamics (VMD) (18) (University of Illinois, USA).

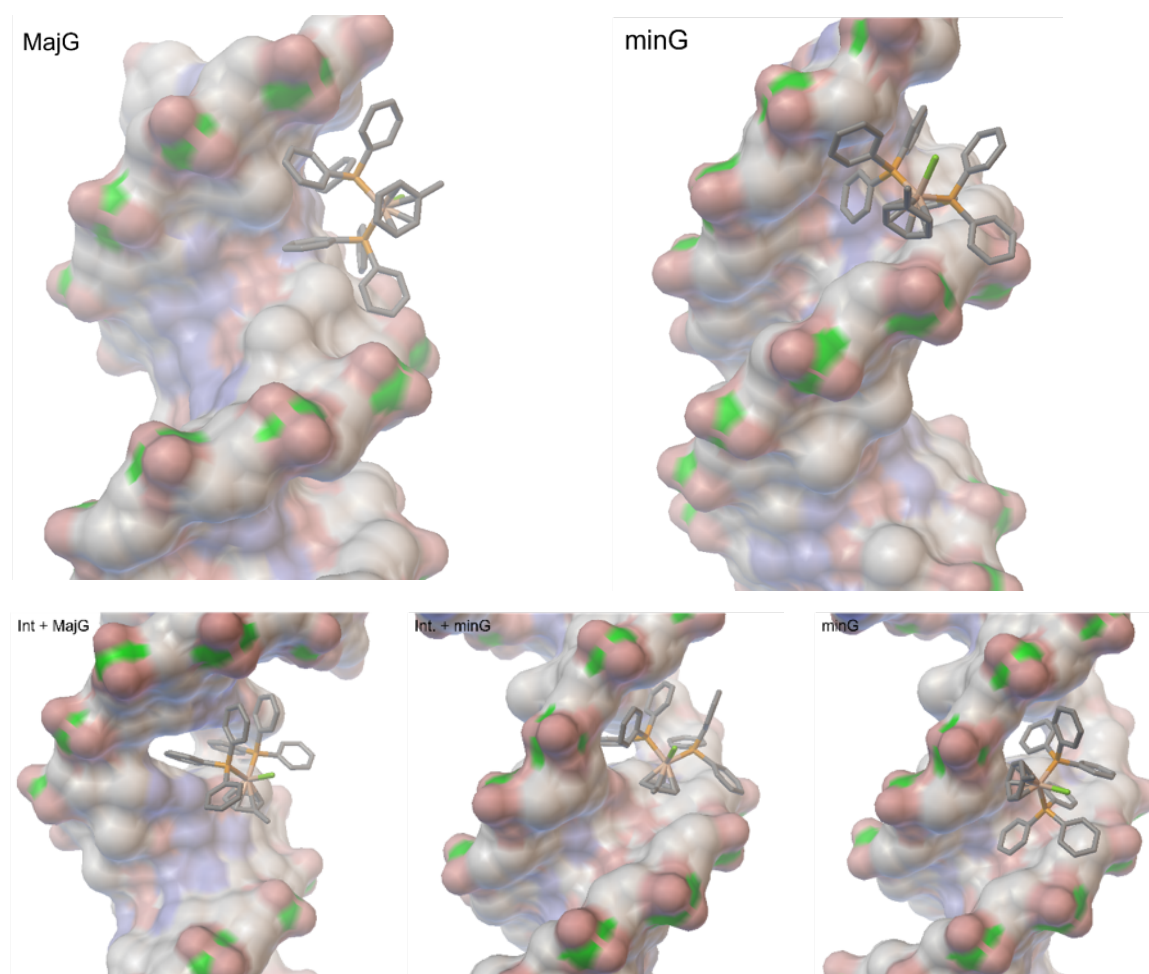


Figure S35 - Docked conformations for **11⁺** interacting with 5T4W, minor groove, (top); and 1G3X. intercalator, (bottom).

Table S9 - Binding energy for DAPI and MG for the different DNA structures.

PDB structure	1BNA	5T4W	1G3X
DAPI	−9.49 kcal/mol	−9.61 kcal/mol	−9.95 kcal/mol
MG	−9.29 kcal/mol	−9.27 kcal/mol	−10.57 kcal/mol

8. NMR spectra of the synthesized compounds

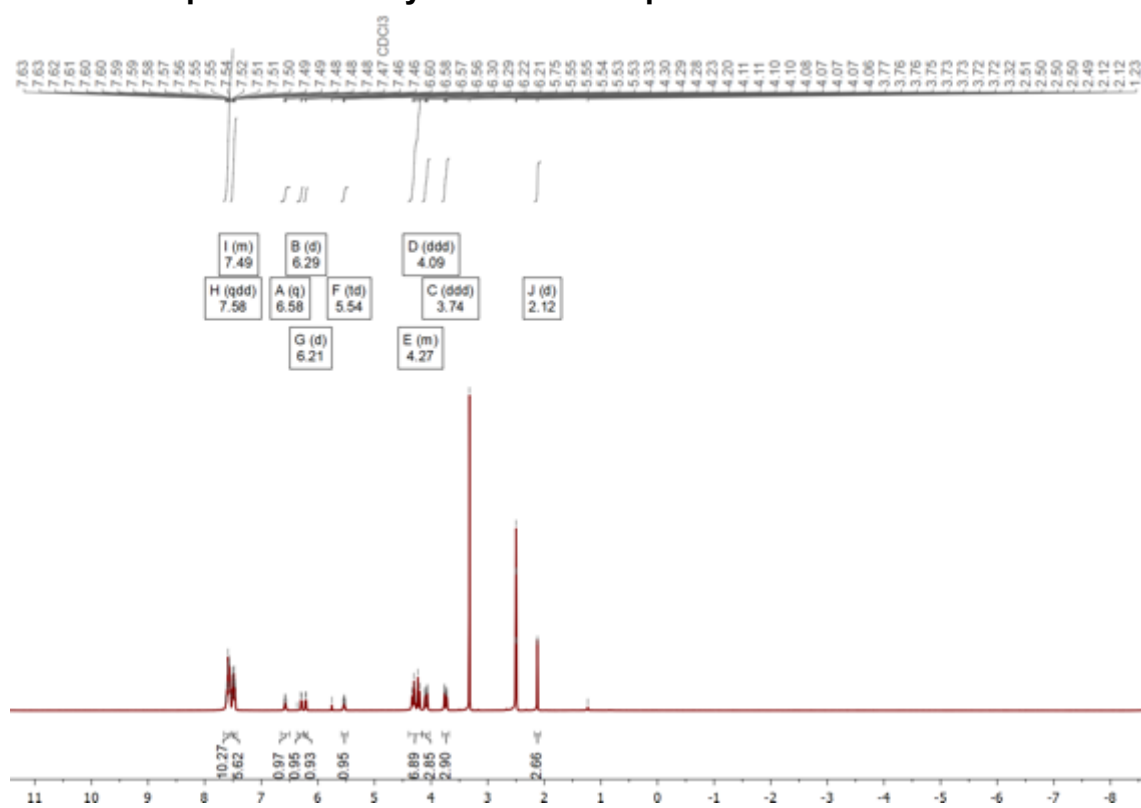


Figure S36 - ¹H NMR spectra of **10**.

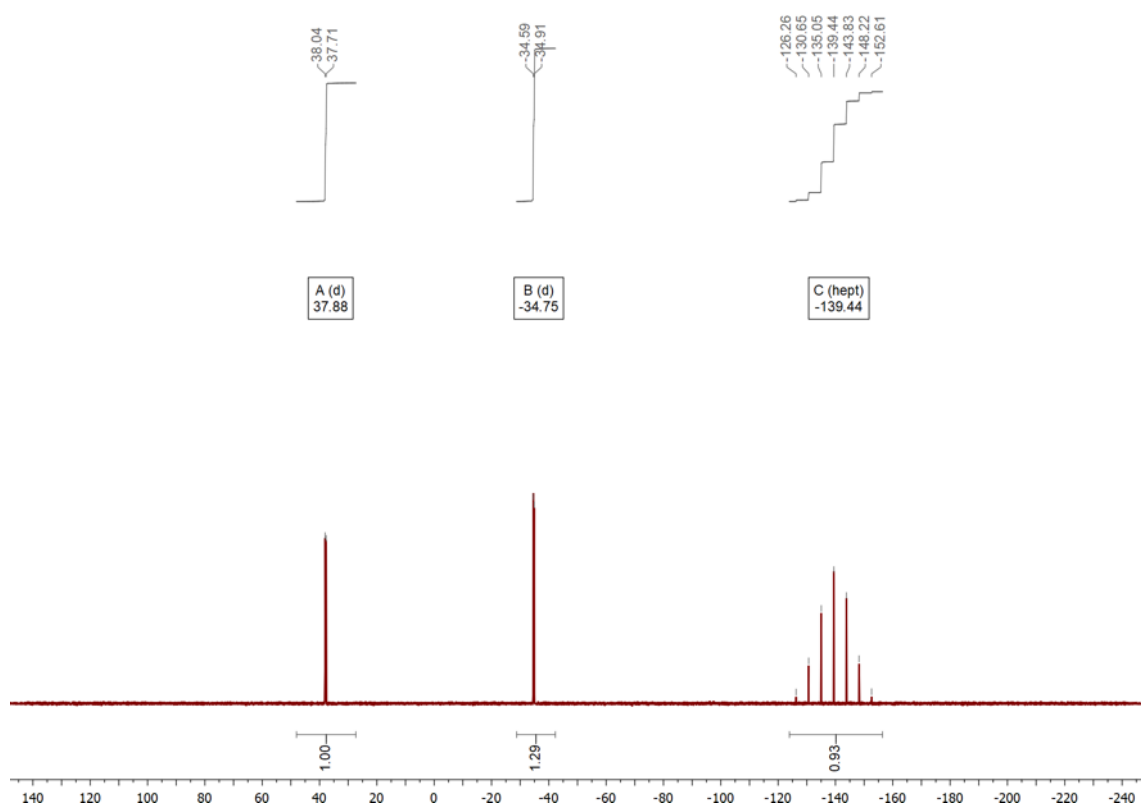


Figure S37 - ³¹P NMR spectra of **10**.

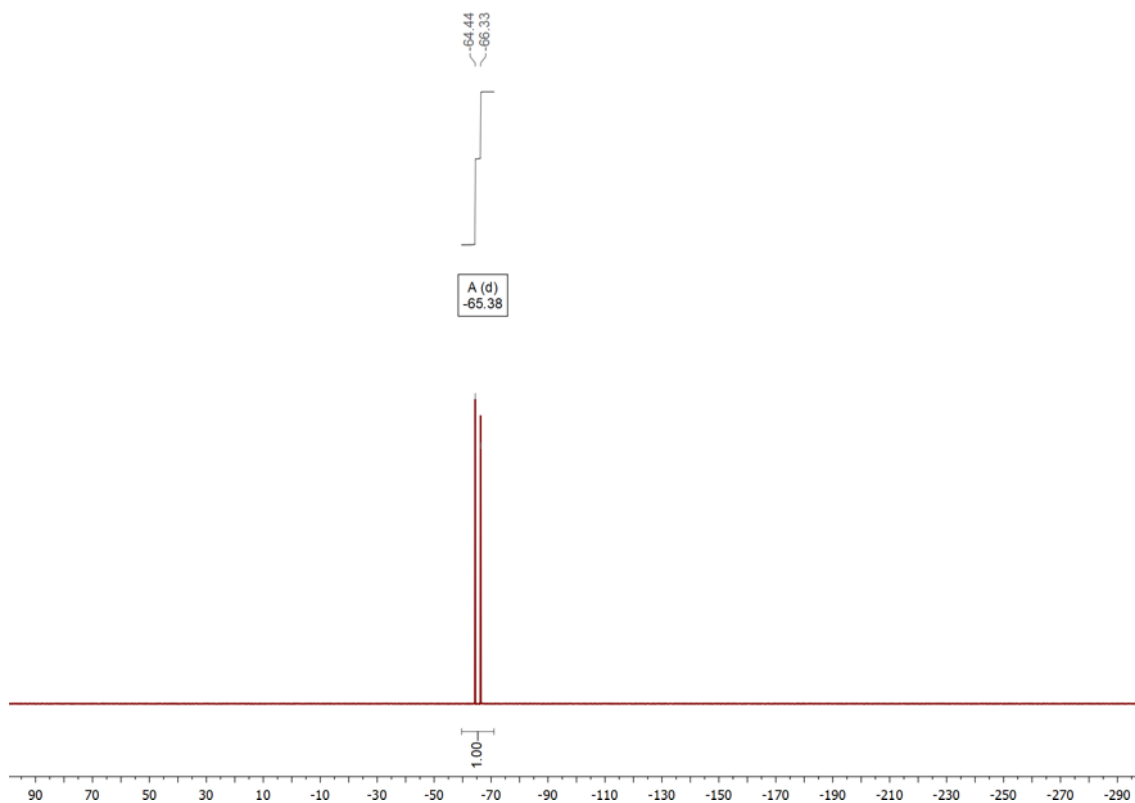


Figure S38 - ^{19}F NMR spectra of **10**. **Figure S41**. ^{13}C NMR spectra of **10**.

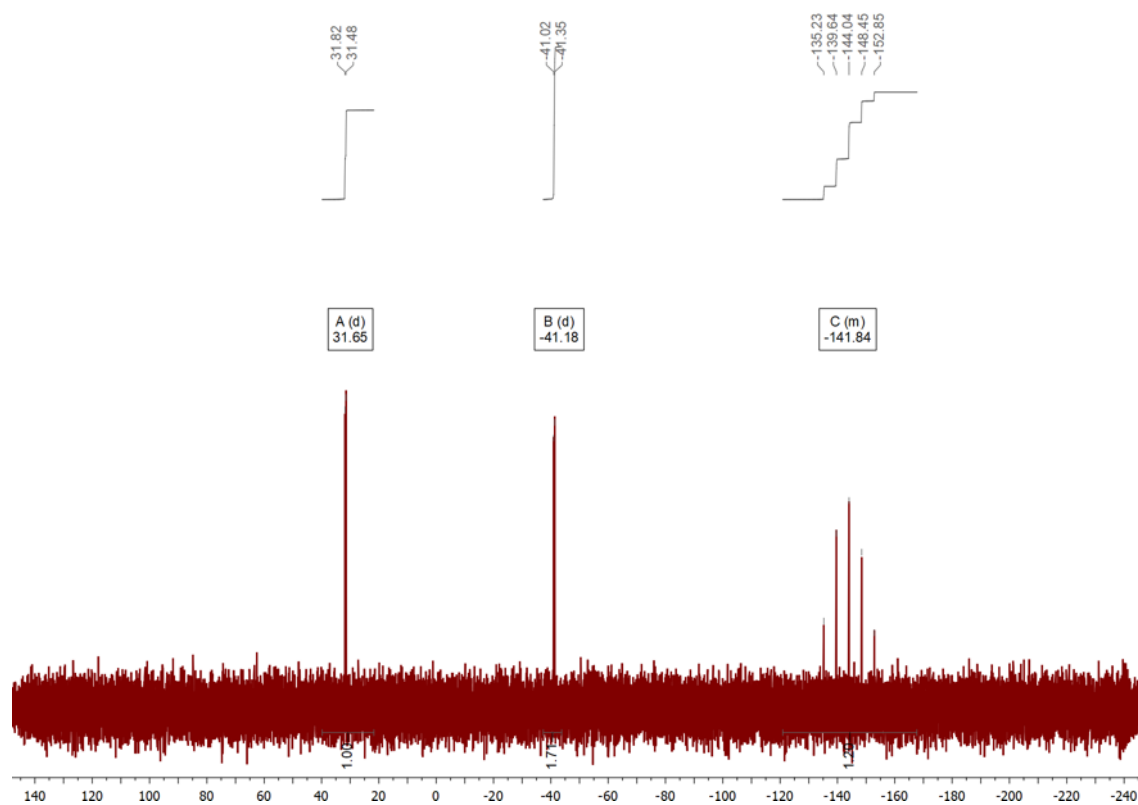


Figure S39 - ^{31}P NMR spectra of **10** in chloroform- d .

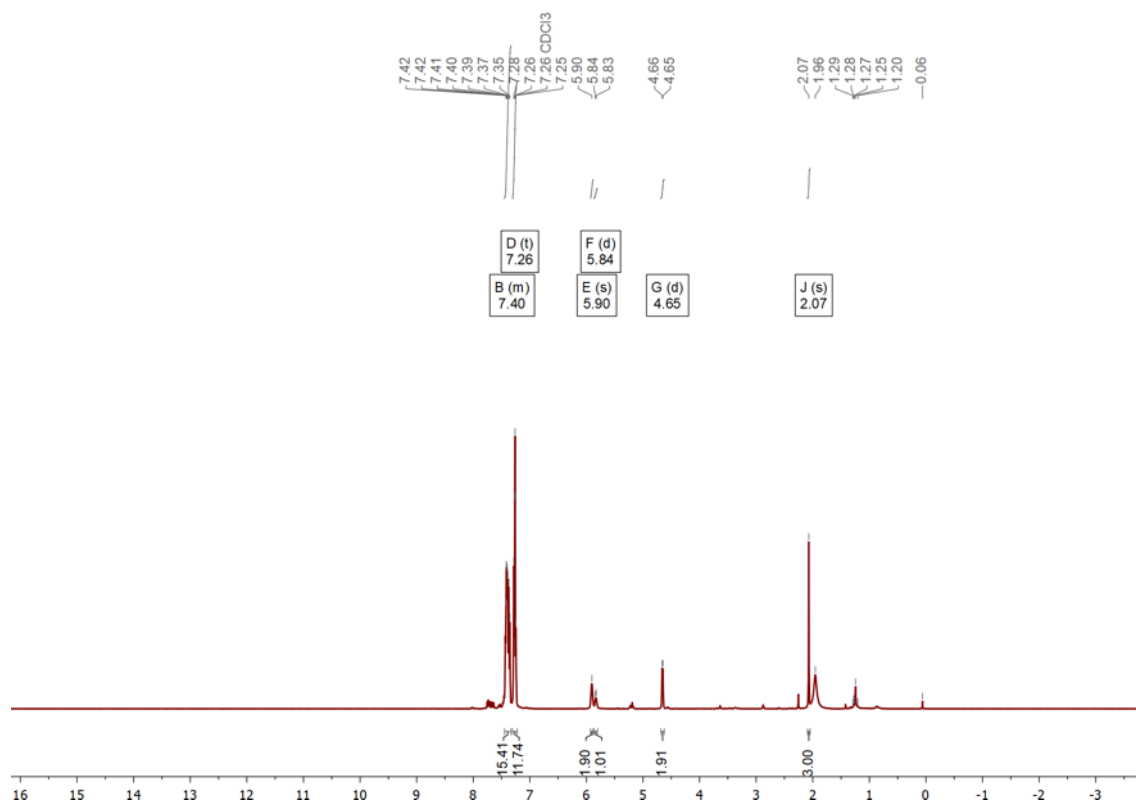


Figure S40 - ¹H NMR spectra of **11Cl**.

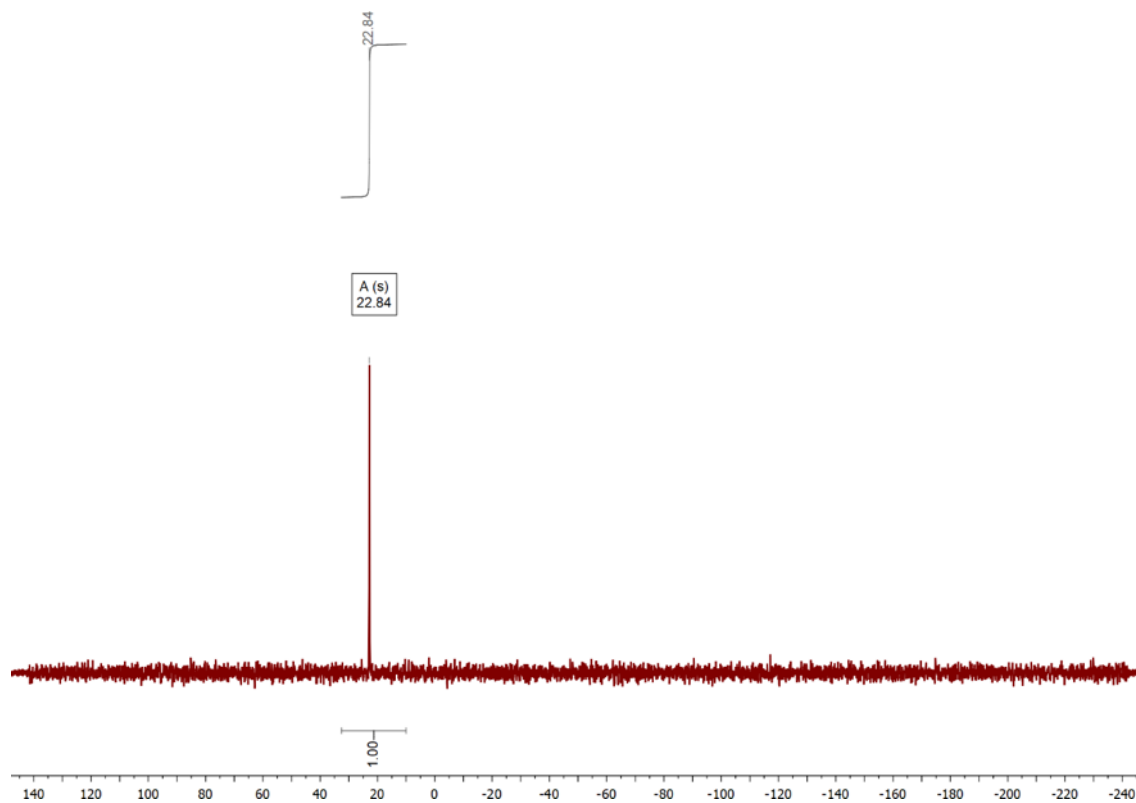


Figure S41 - ³¹P NMR spectra of **11Cl**.

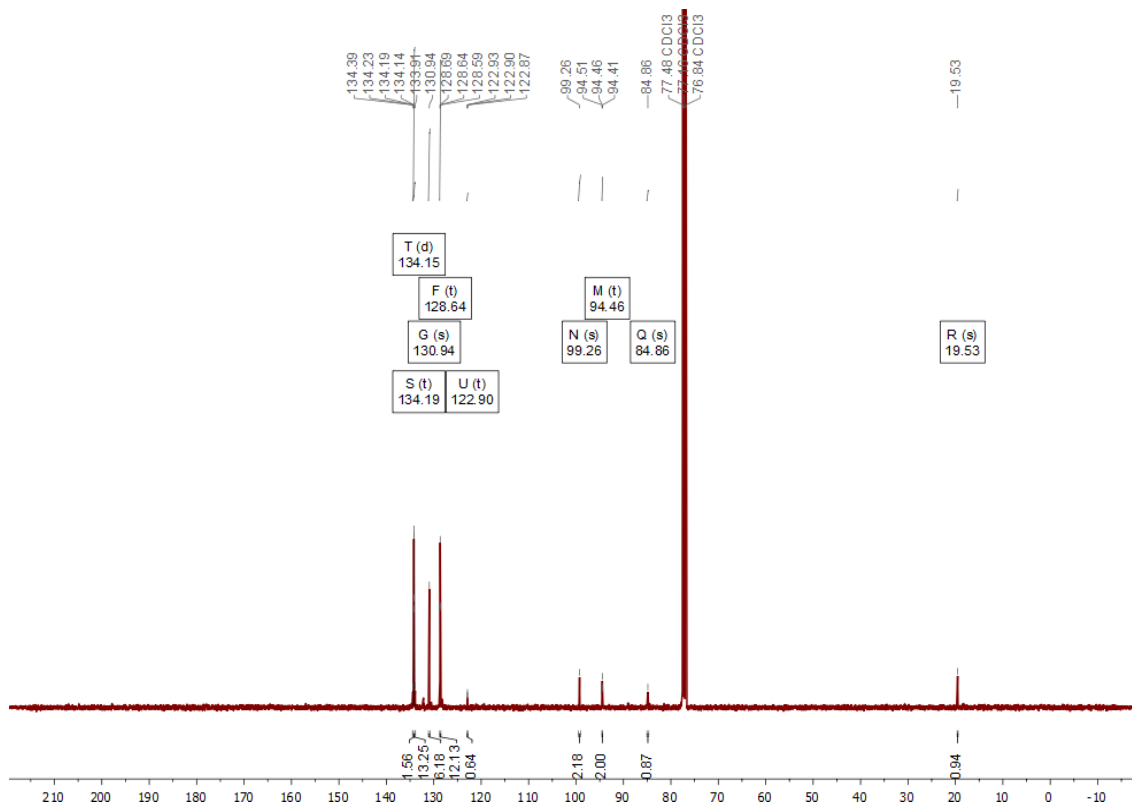


Figure S42 - ^{13}C NMR spectra of 11Cl.

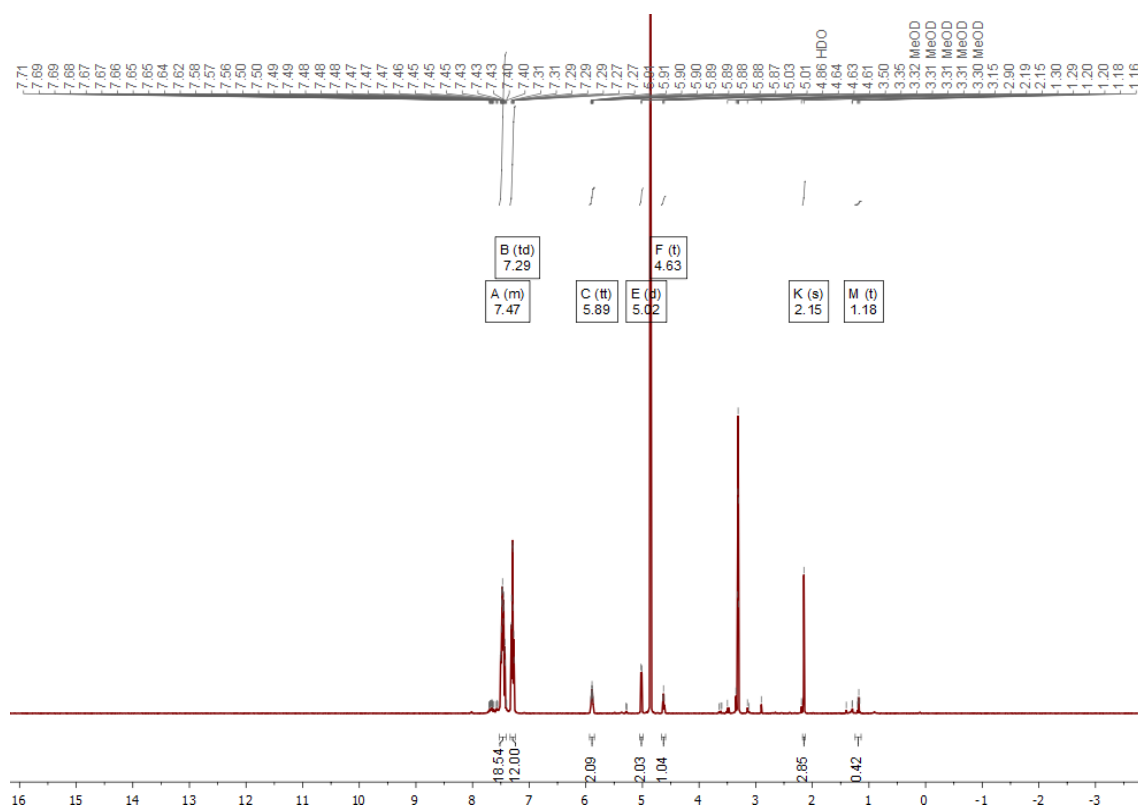


Figure S43 - ^1H NMR spectra of **11Cl** in methanol- d_4 .

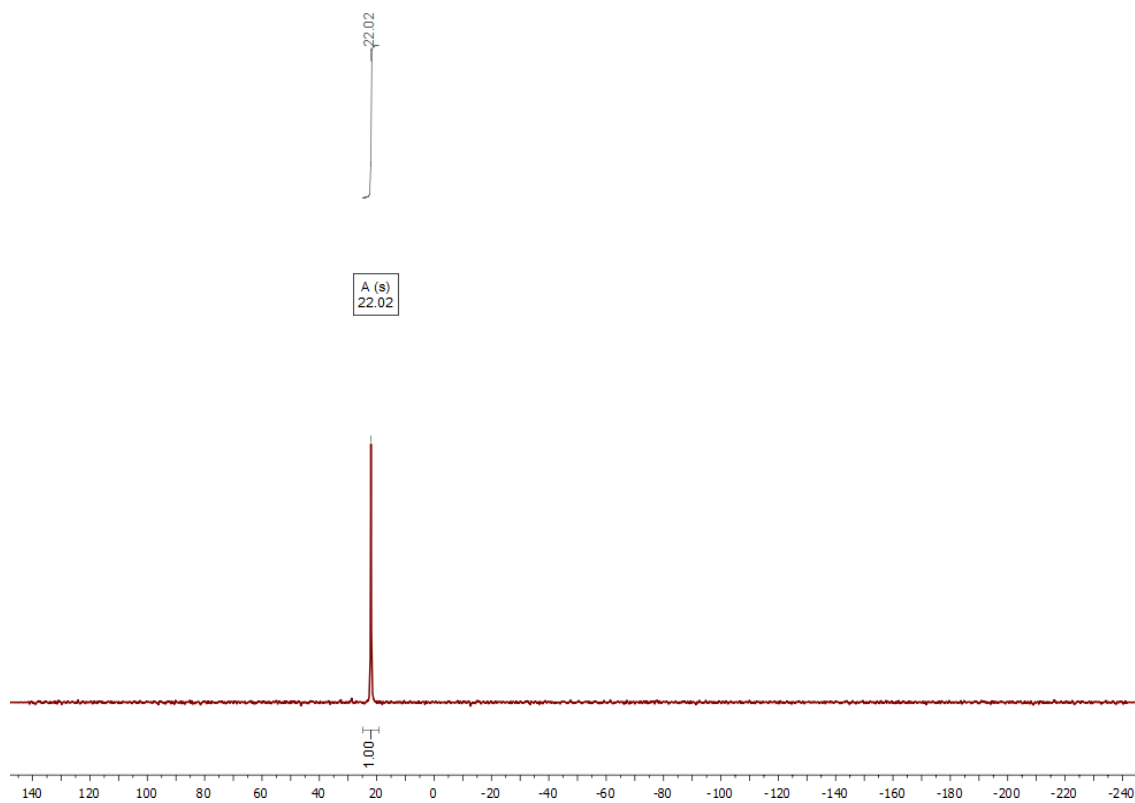


Figure S44 - ^{31}P NMR spectra of **11Cl** in methanol- d_4 .

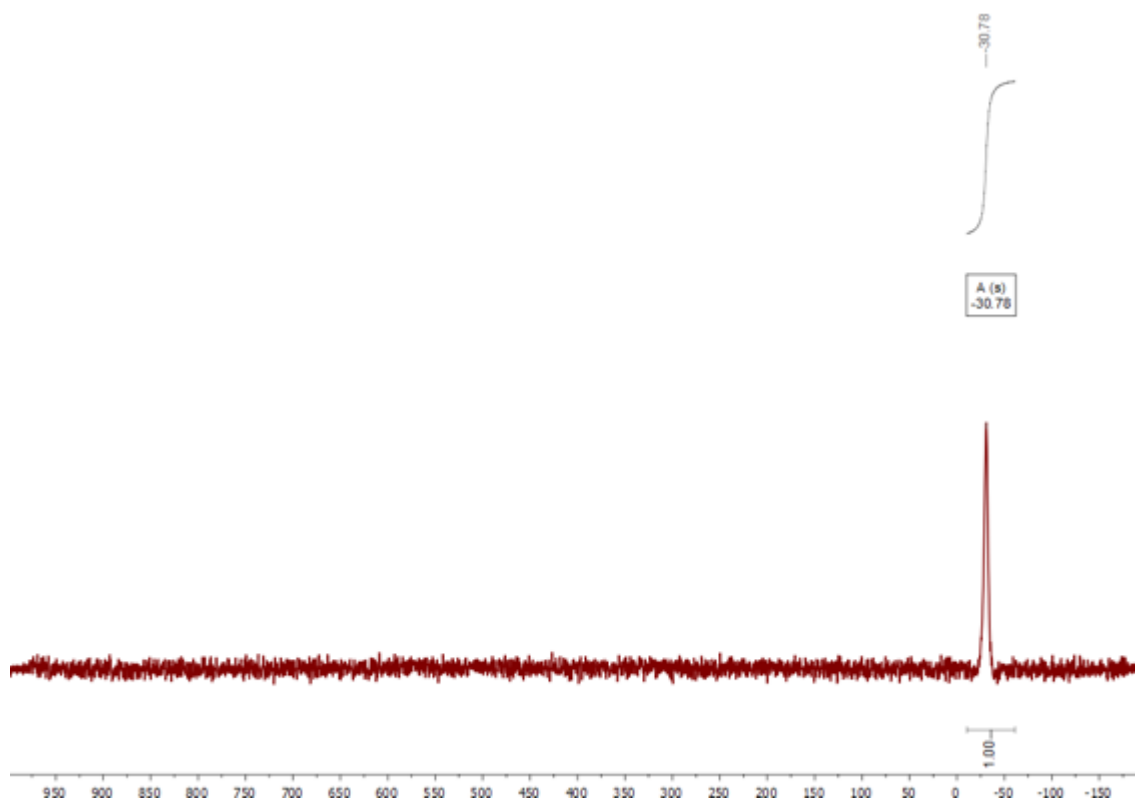


Figure S45 - ^{37}Cl NMR spectra of **11Cl** in methanol- d_4 .

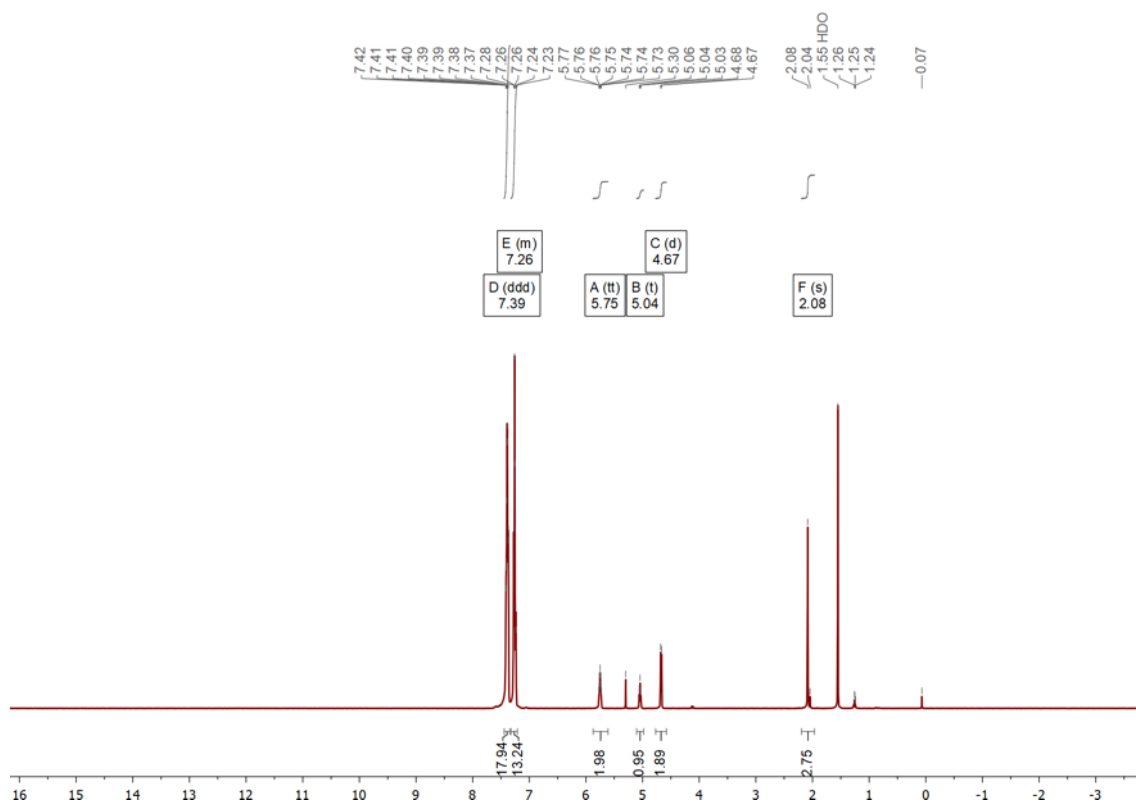


Figure S46 - ¹H NMR spectra of **11PF₆**.

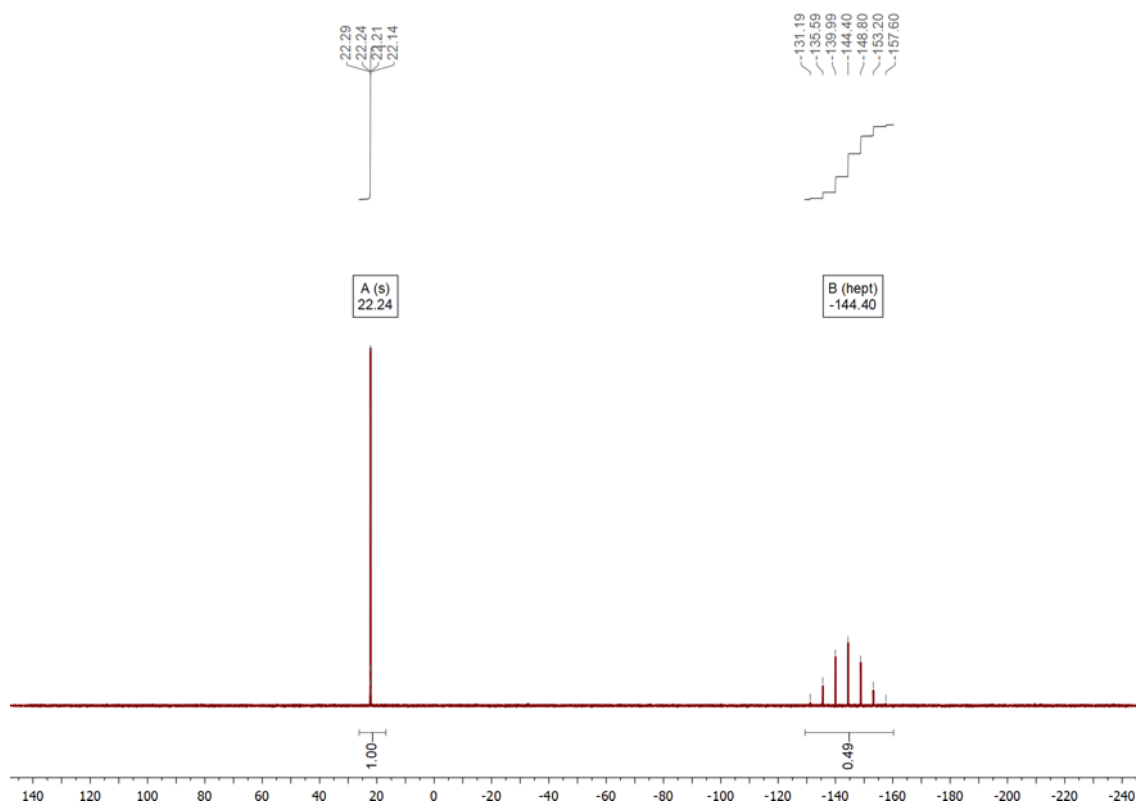


Figure S47 - ³¹P NMR spectra of **11PF₆**.

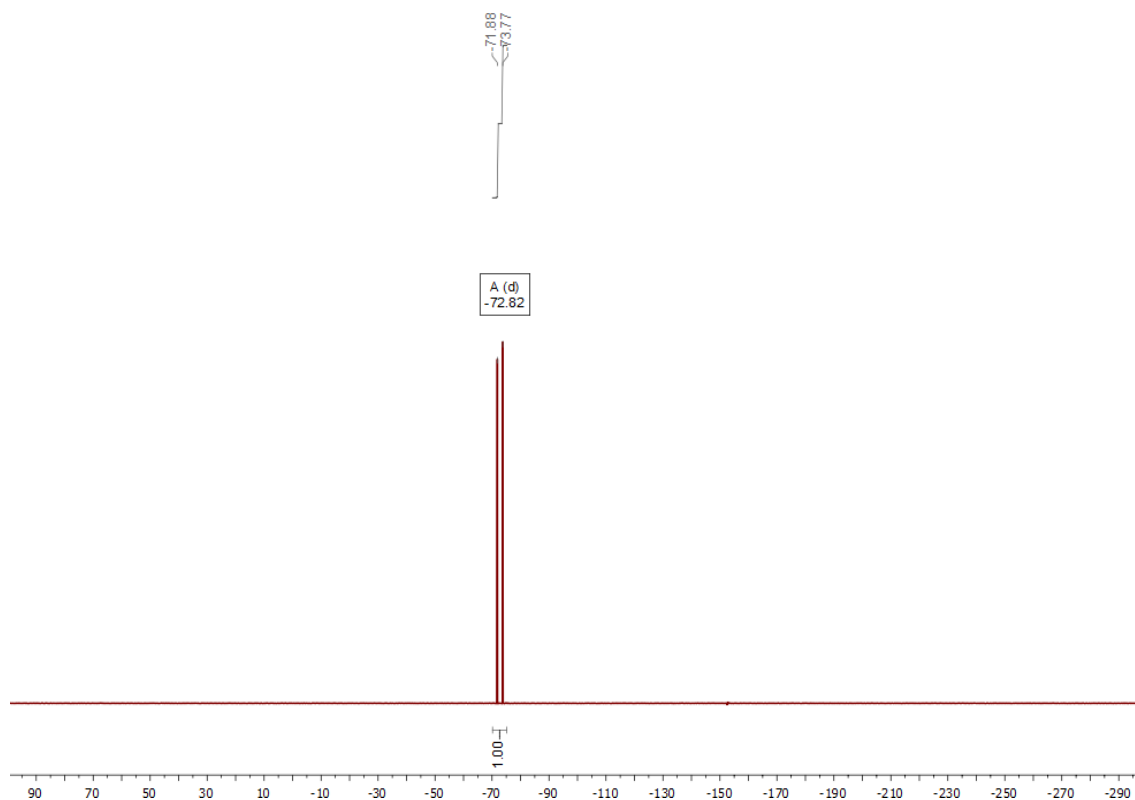


Figure S48 - ^{19}F NMR spectra of 11PF_6 .

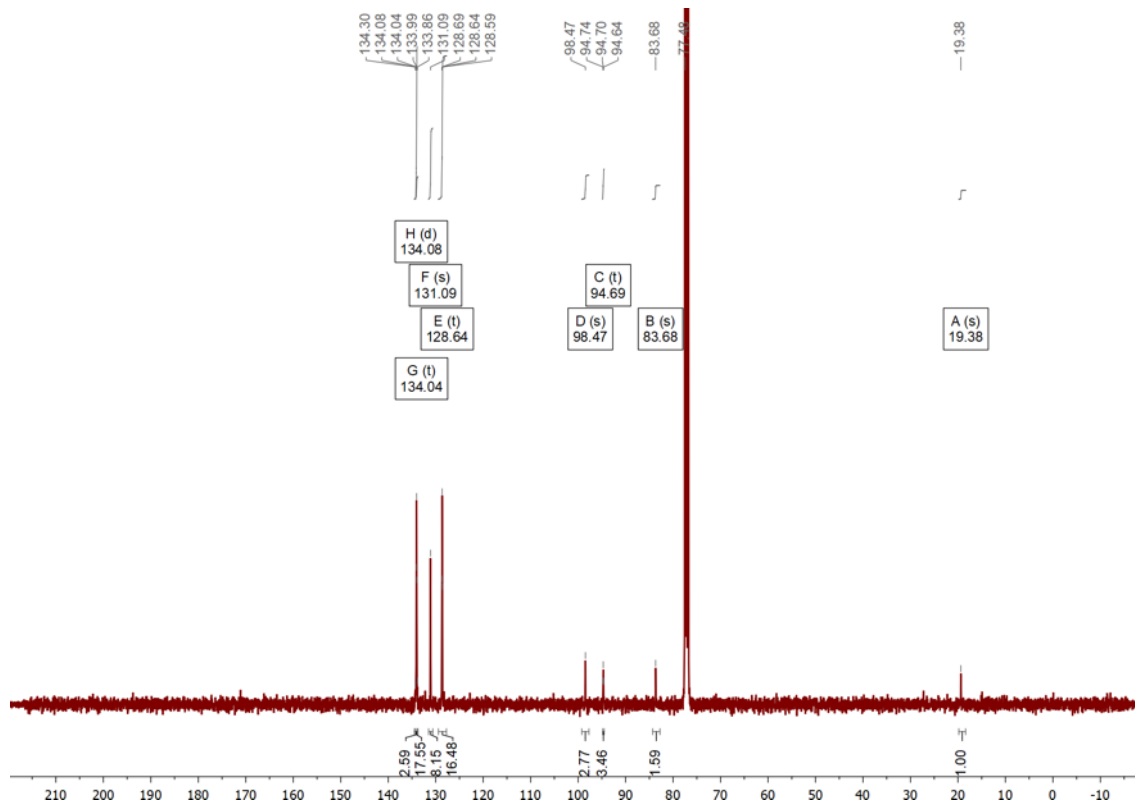


Figure S49 - ^{13}C NMR spectra of 11PF_6 .

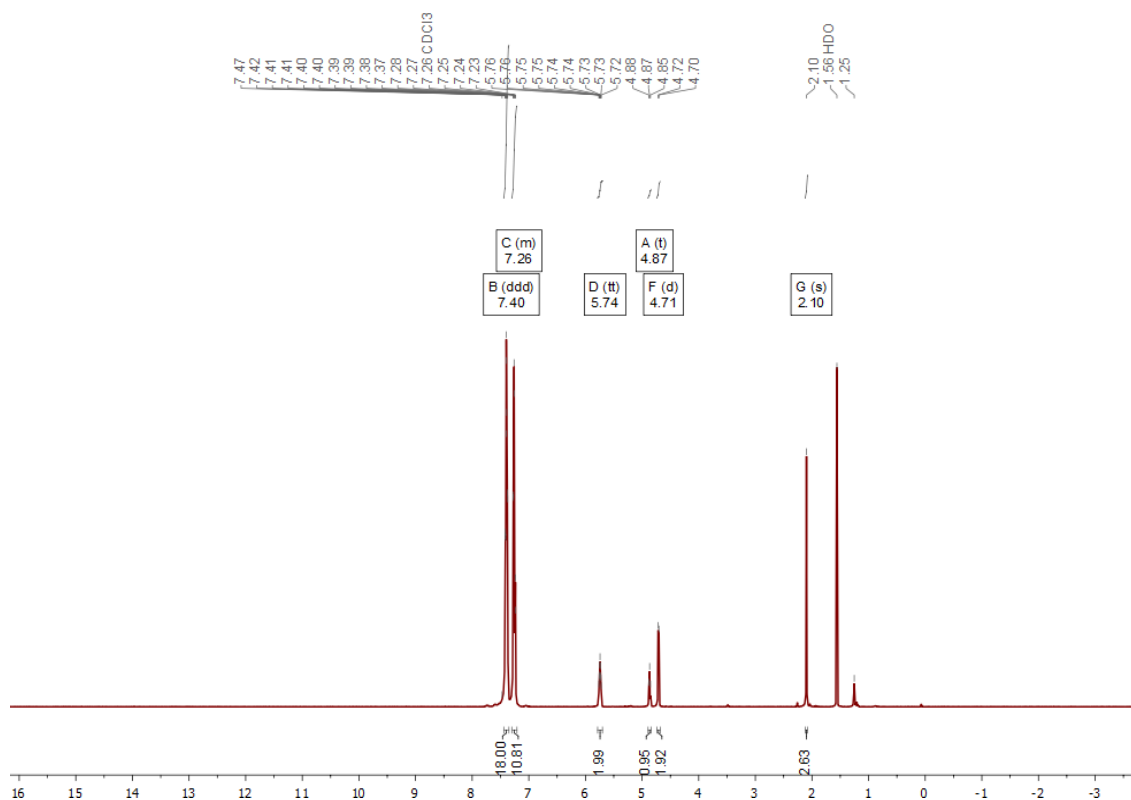


Figure S50 - ¹H NMR spectra of **11SbF₆**.

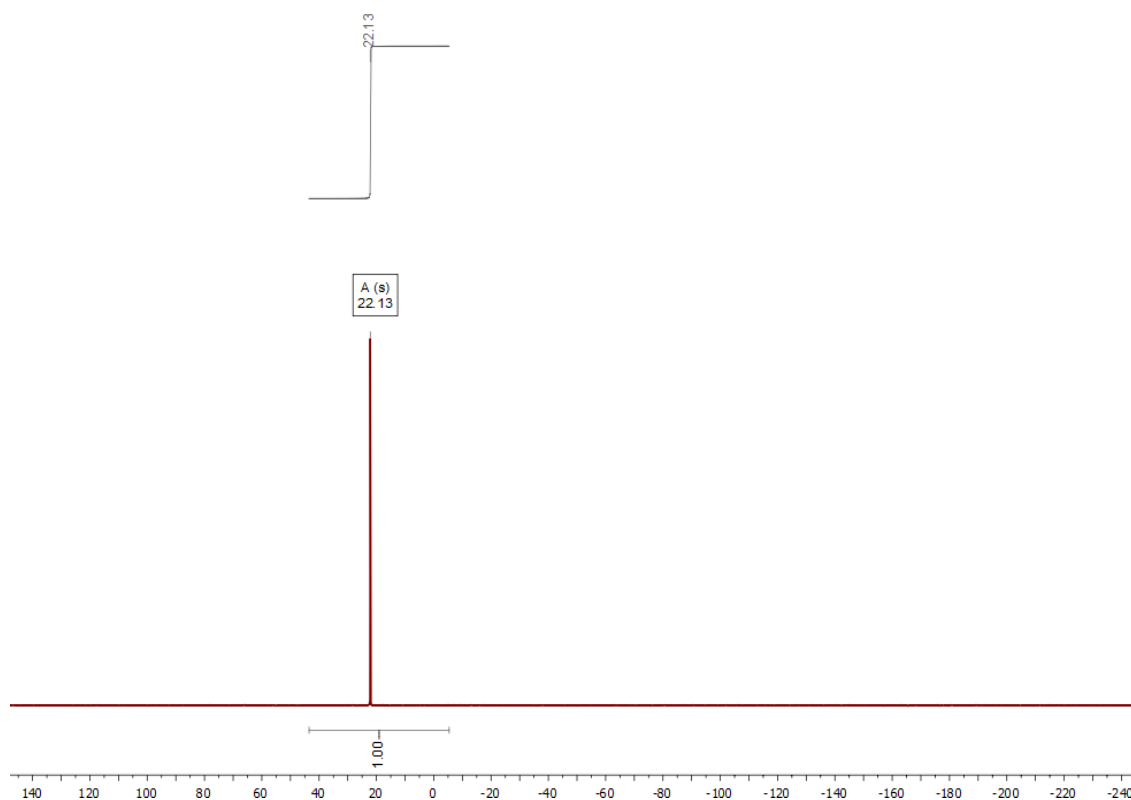


Figure S51 - ³¹P NMR spectra of **11SbF₆**.

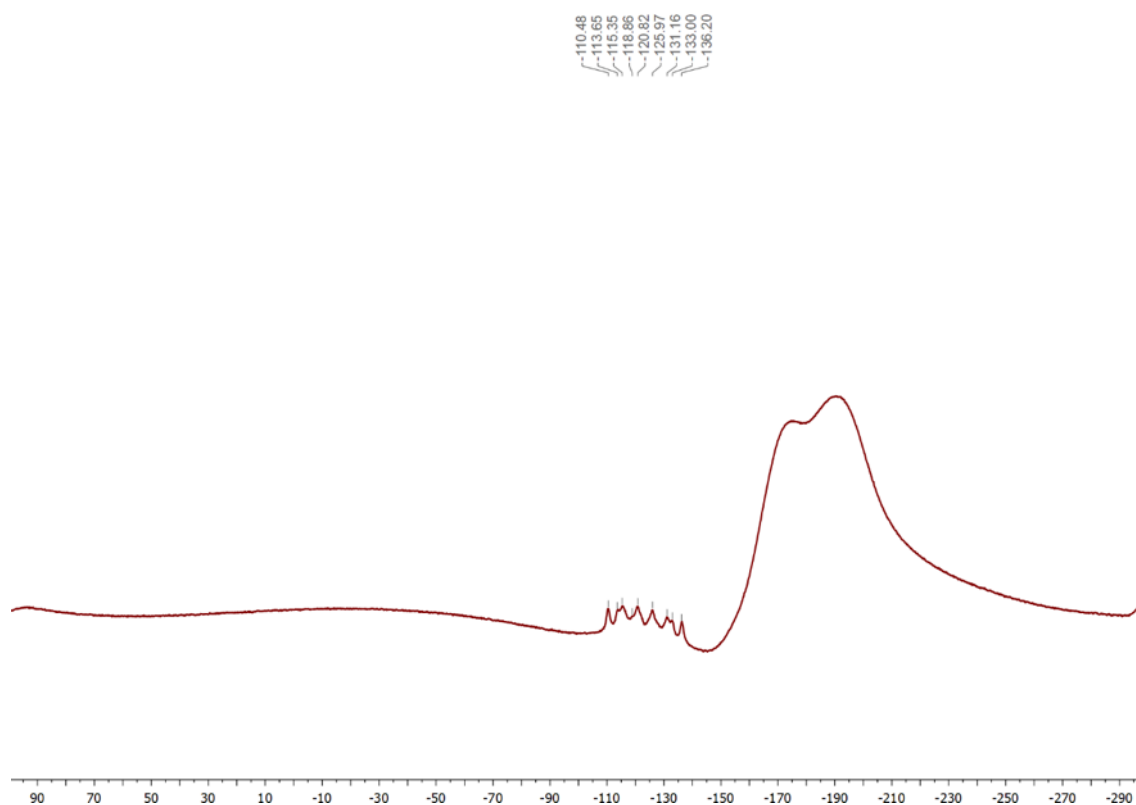


Figure S52 - ^{19}F NMR spectra of 11SbF_6 .

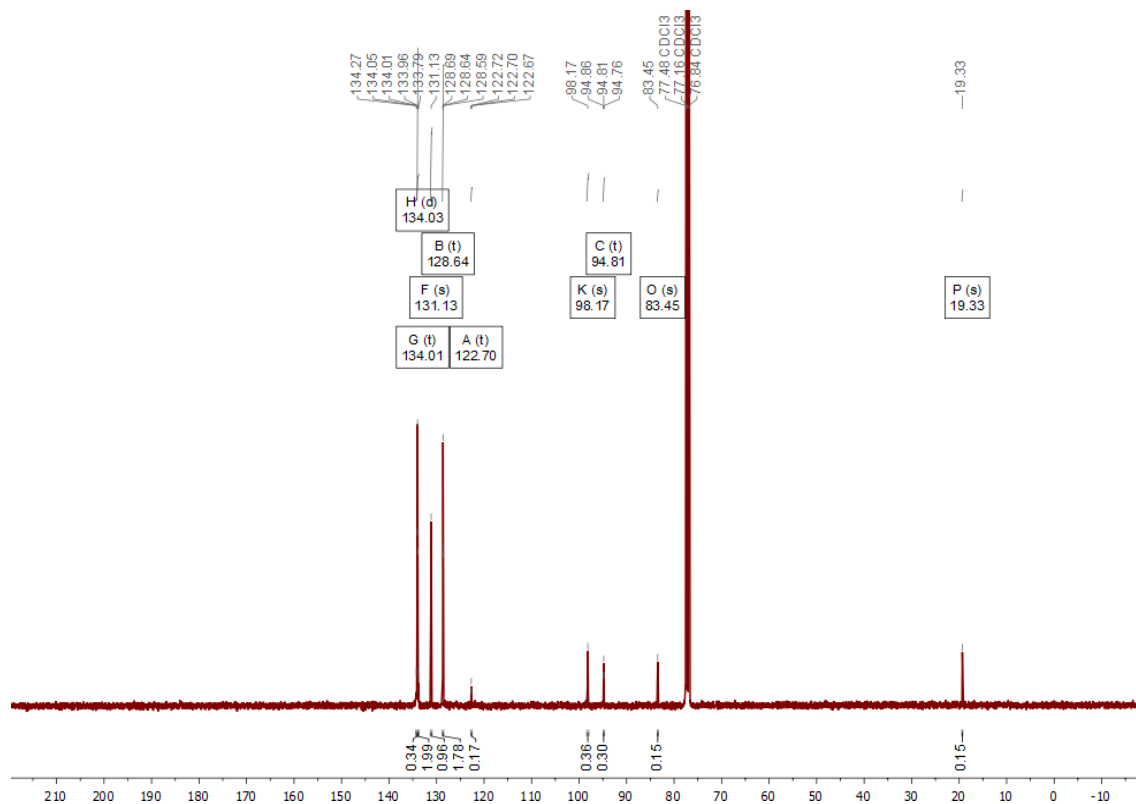


Figure S53 - ^{13}C NMR spectra of 11SbF_6 .

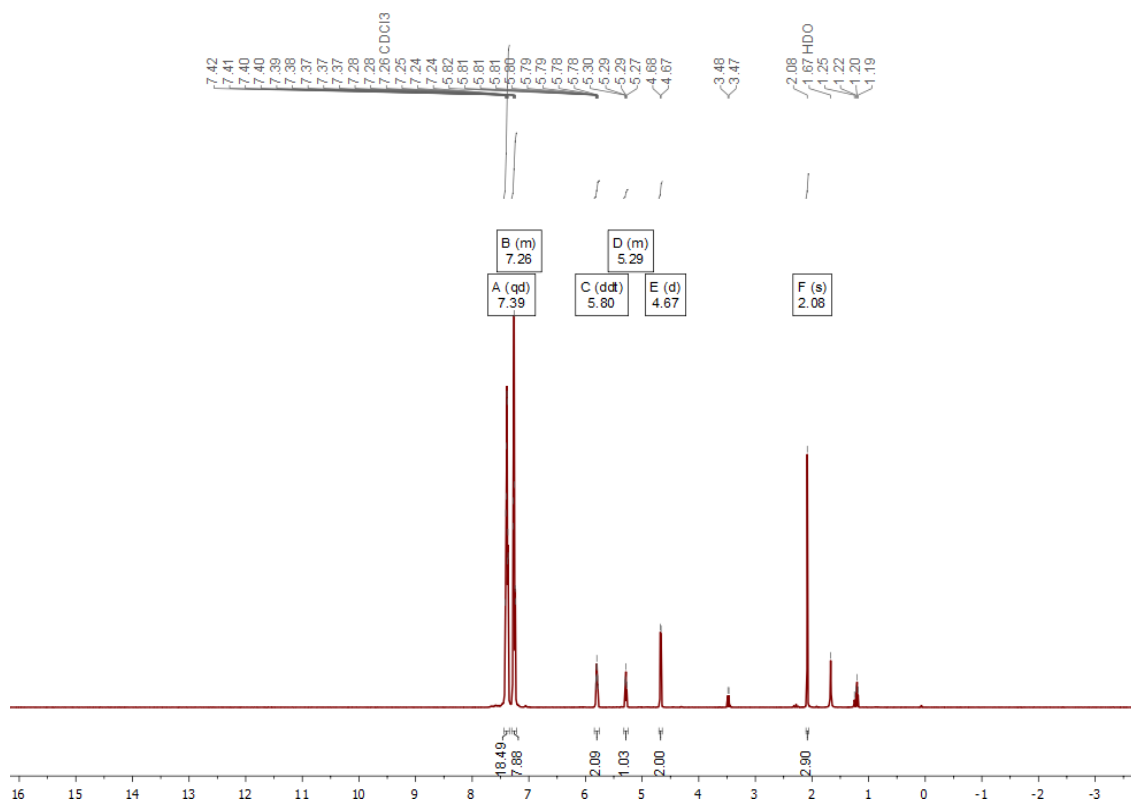


Figure S54 - ¹H NMR spectra of **11OTf**.

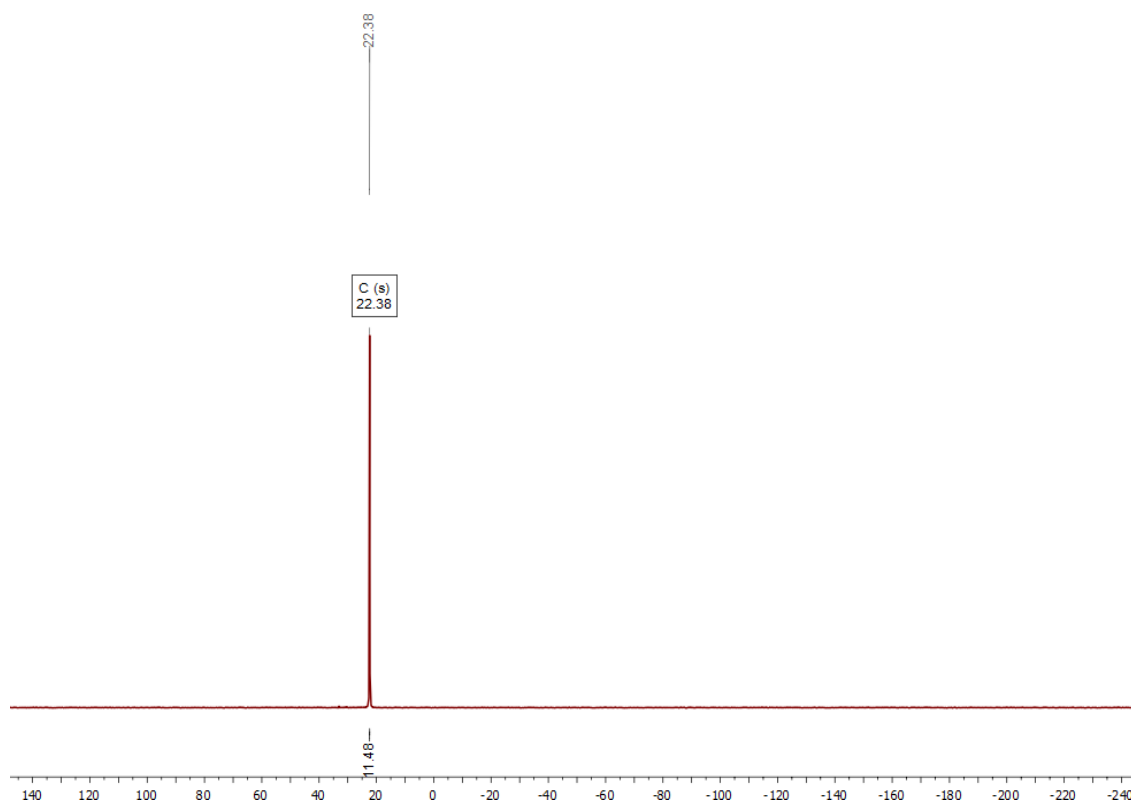


Figure S55 - ³¹P NMR spectra of **11OTf**.

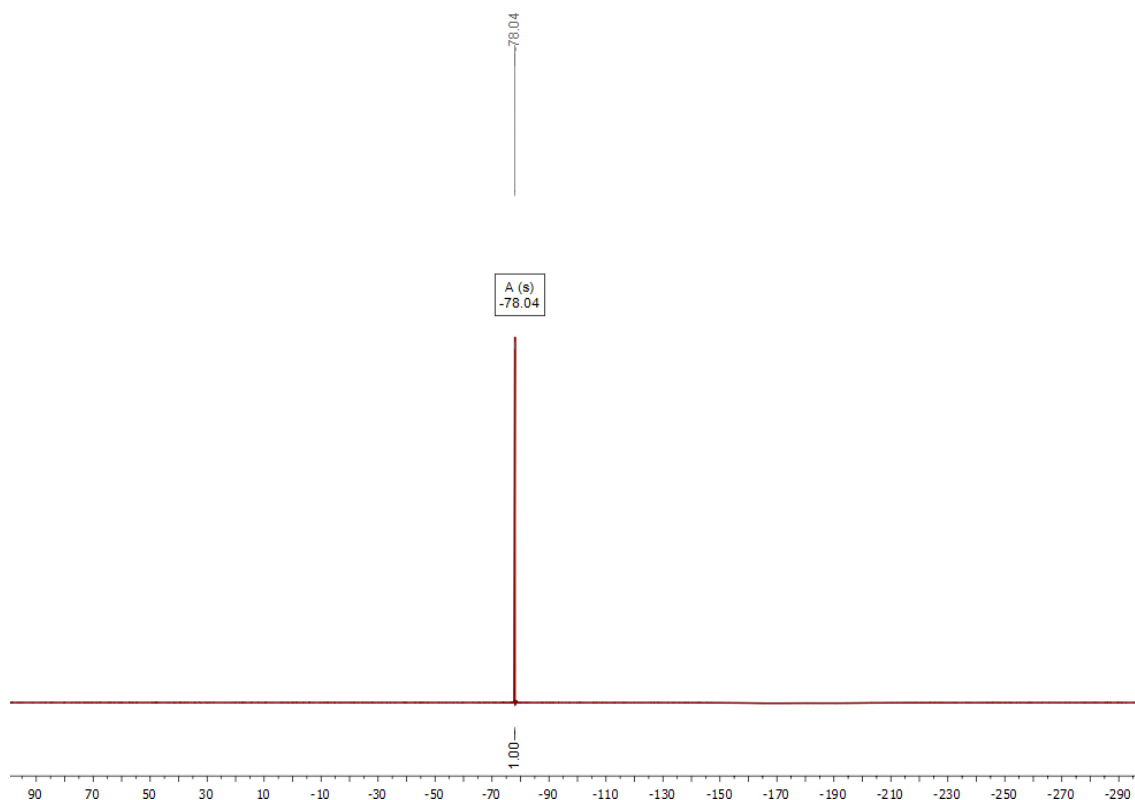


Figure S56 - ^{19}F NMR spectra of 11OTf.

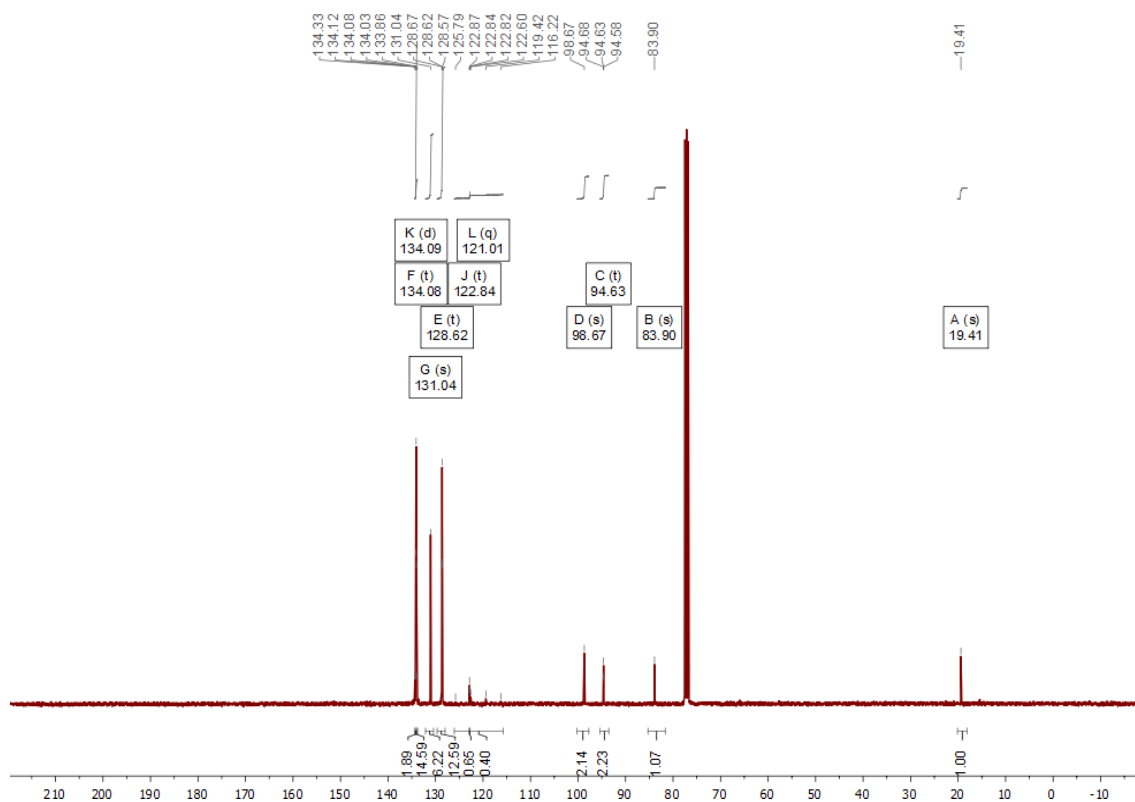
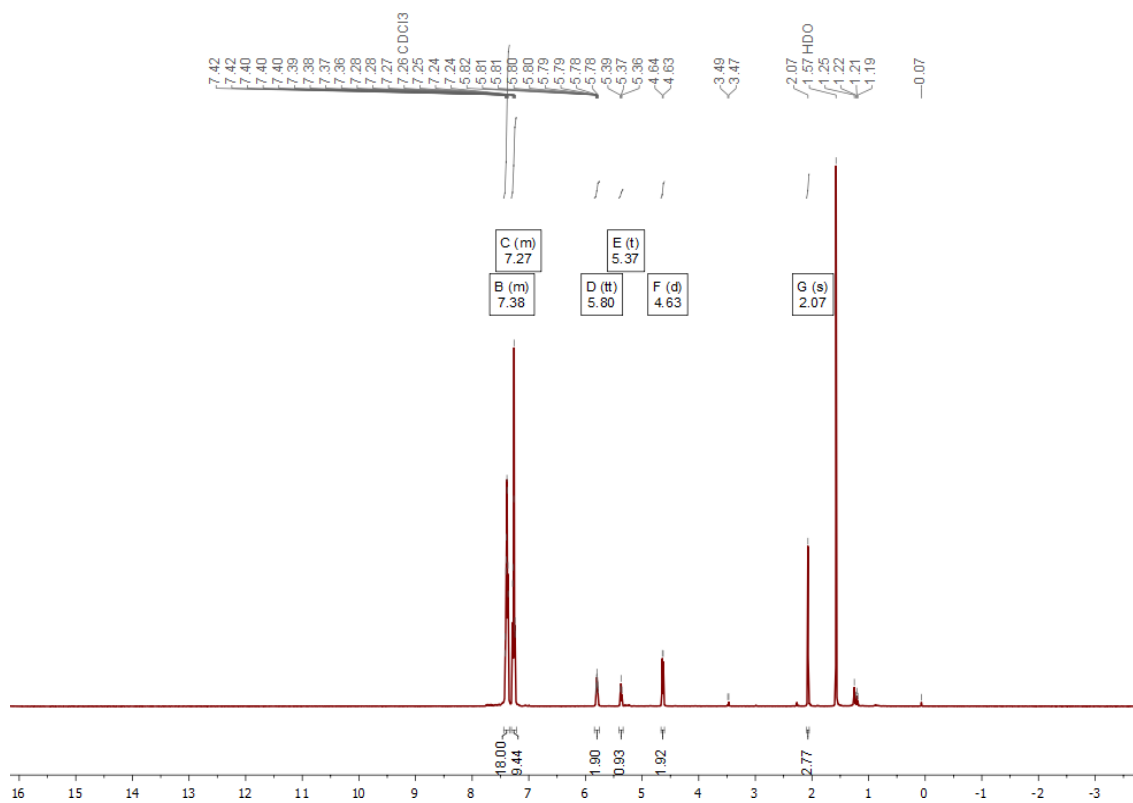


Figure S57 - ^{13}C NMR spectra of 11OTf.



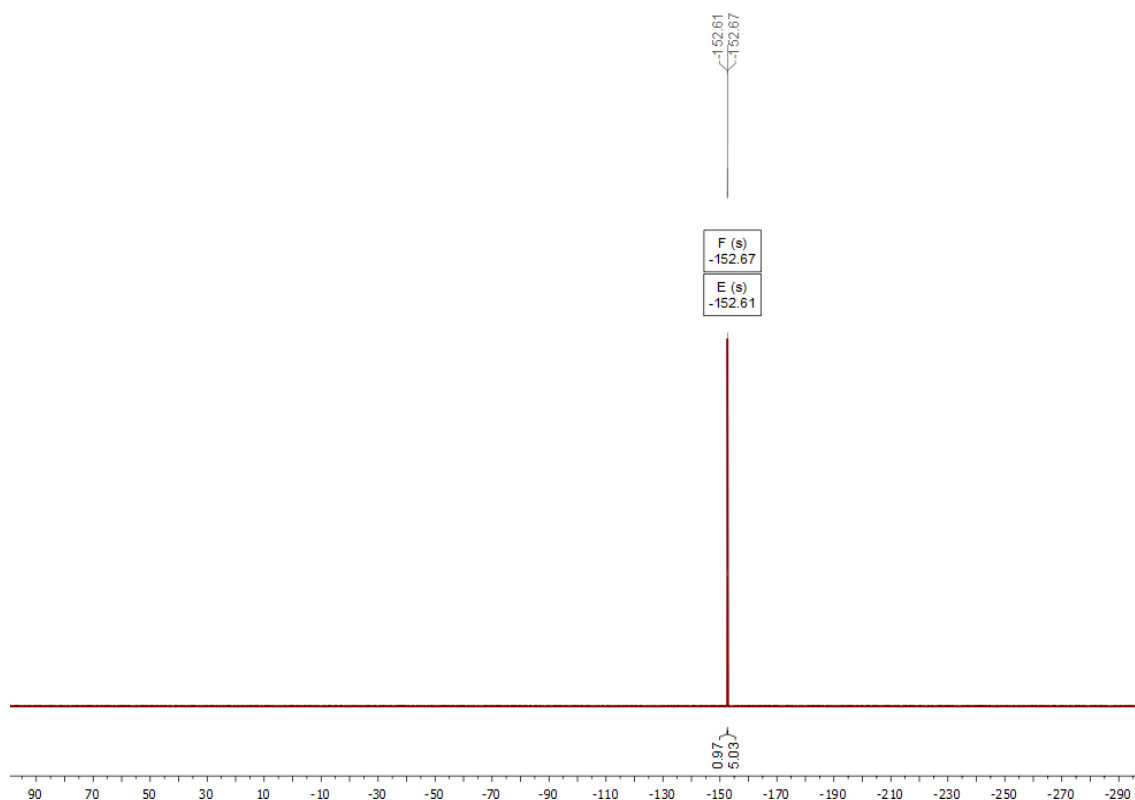


Figure S60 - ^{19}F NMR spectra of 11BF_4 .

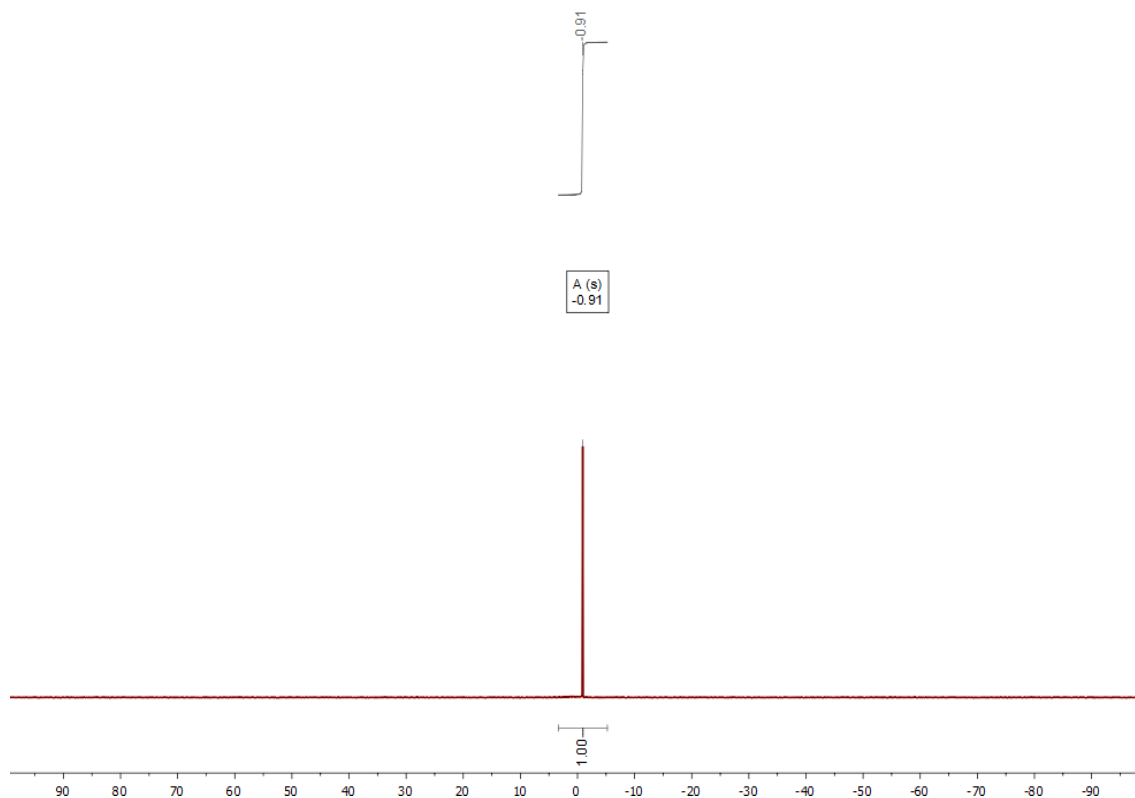


Figure S61 - ^{11}B NMR spectra of 11BF_4 .

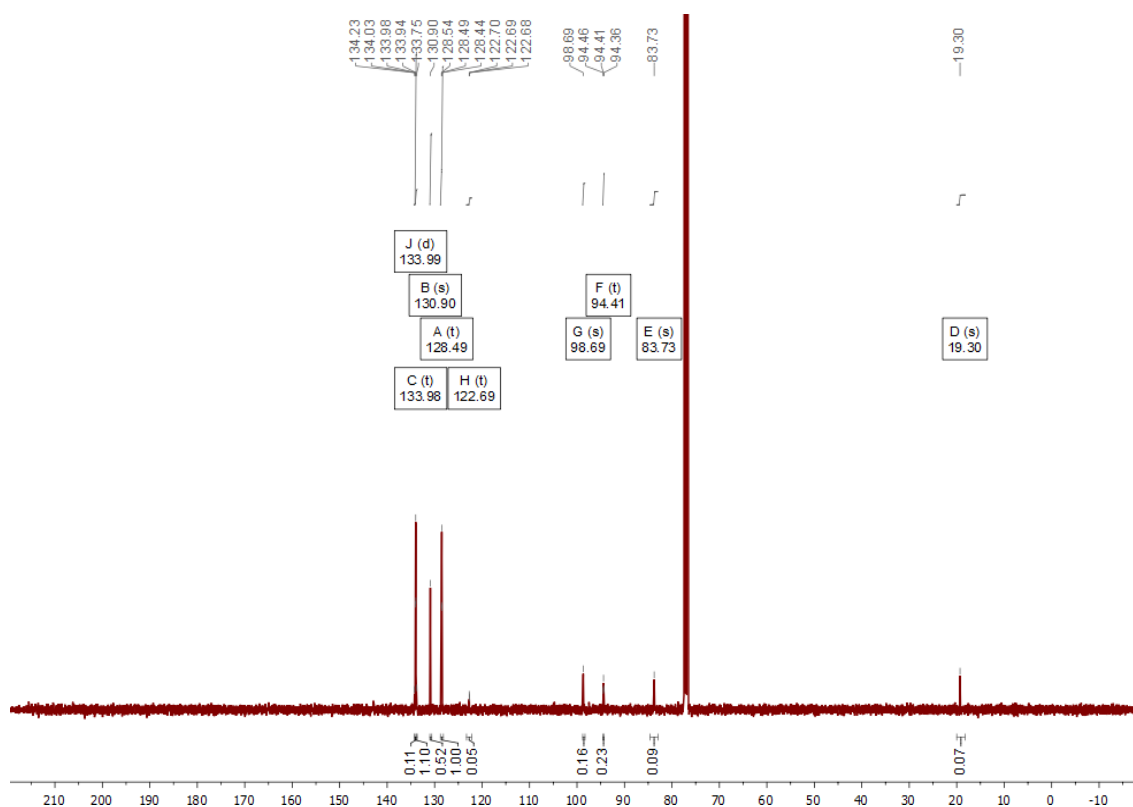


Figure S62 - ¹³C NMR spectra of **11BF₄**.

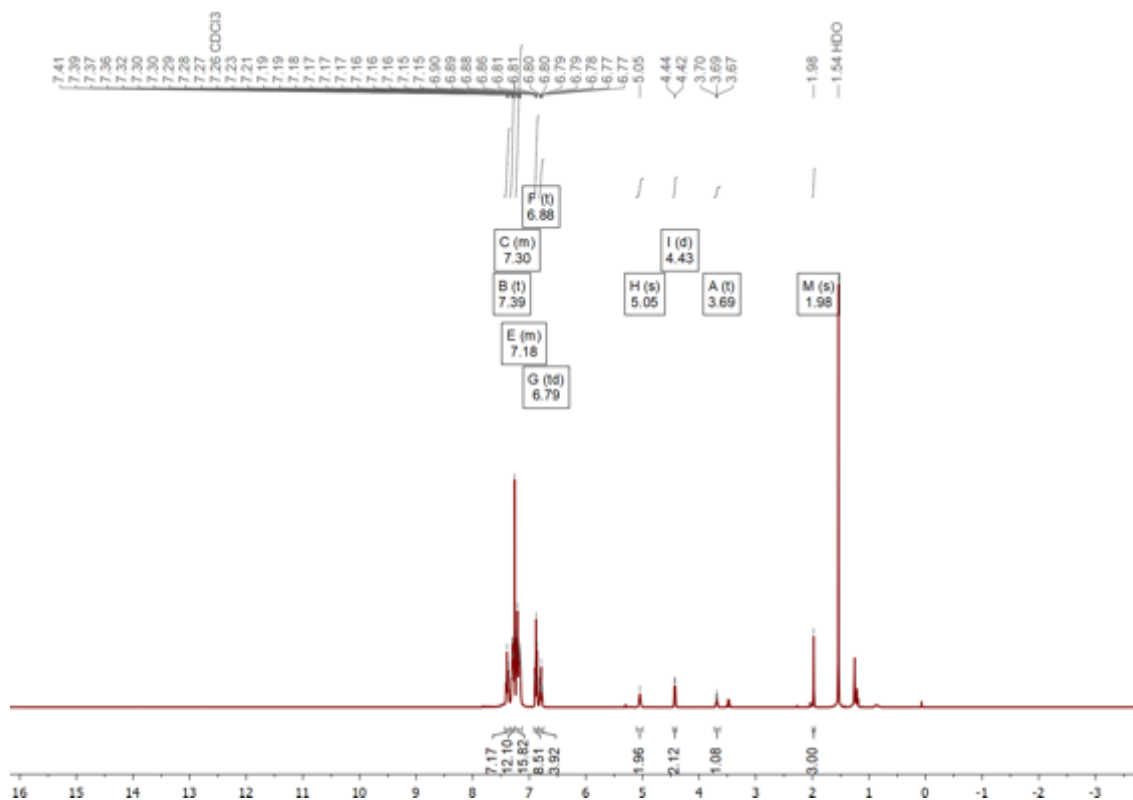


Figure S63 - ¹H NMR spectra of **11BPh₄**.

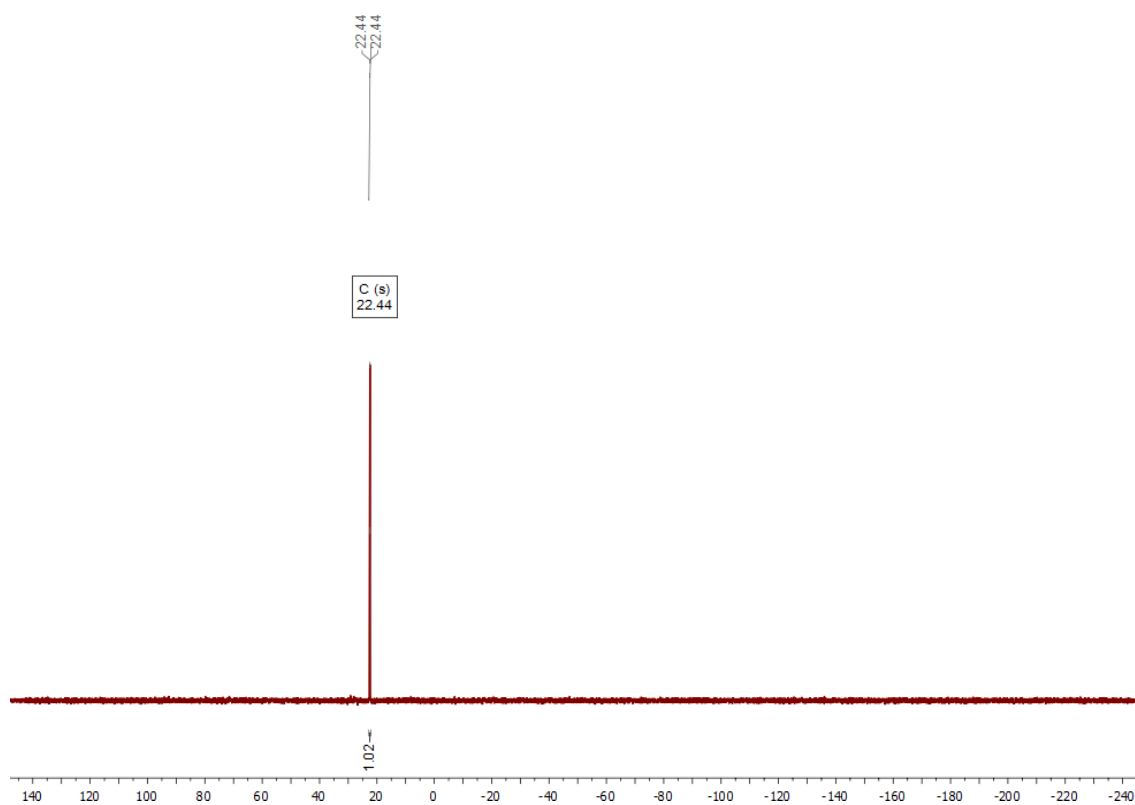


Figure S64 - ^{31}P NMR spectra of 11BPh_4 .

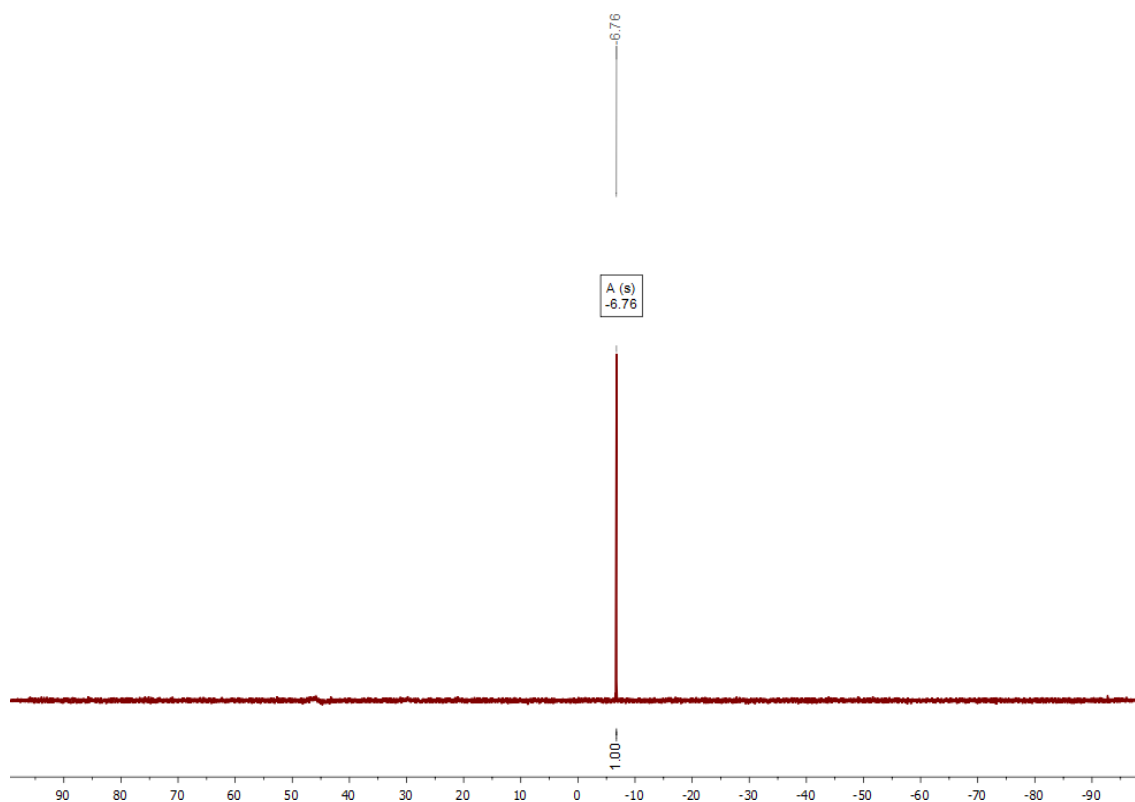


Figure S65 - ^{11}B NMR spectra of 11BPh_4 .

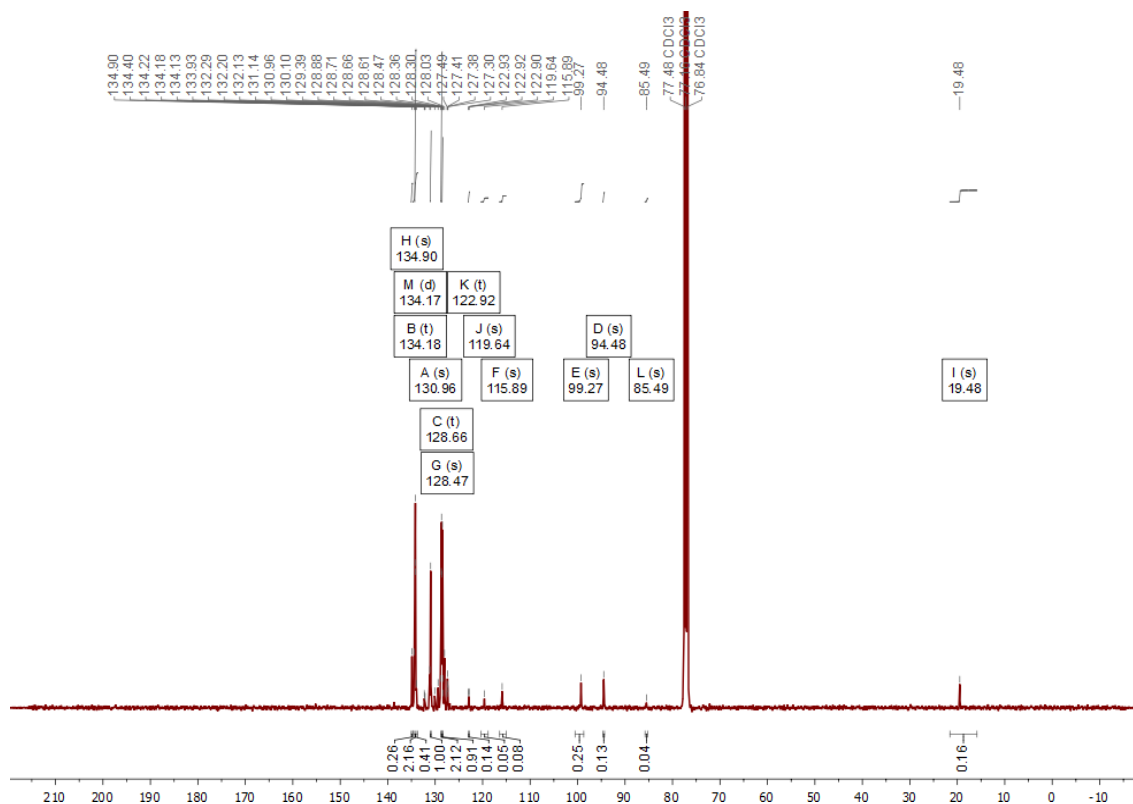


Figure S66 - ^{13}C NMR spectra of **11BPh₄**.

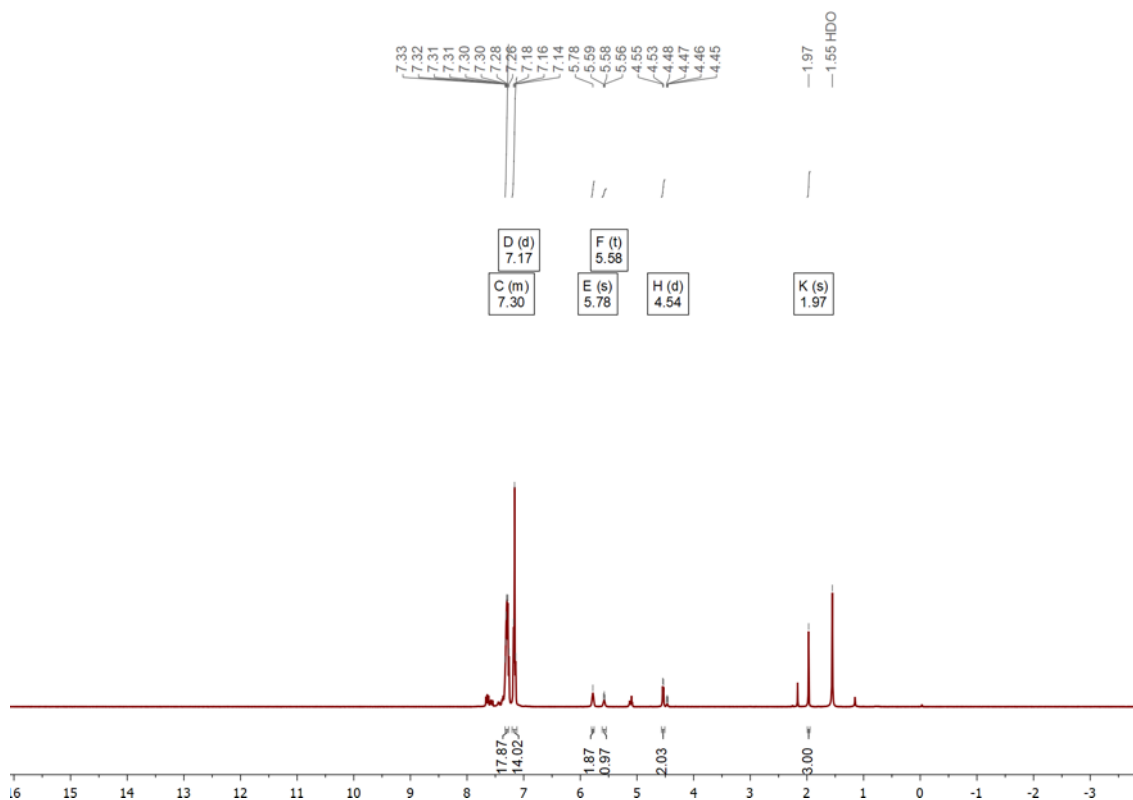


Figure S67 - ^1H NMR spectra of **11NO₃**.

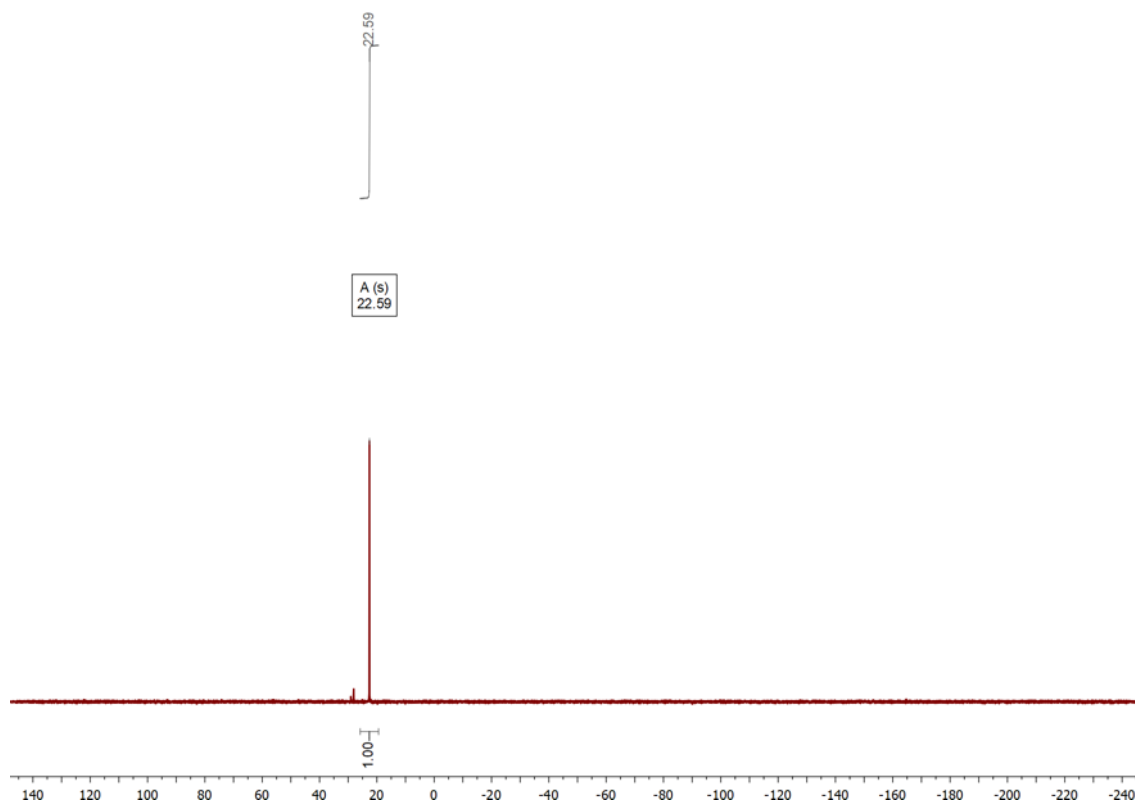


Figure S68 - ^{31}P NMR spectra of 11NO_3 .

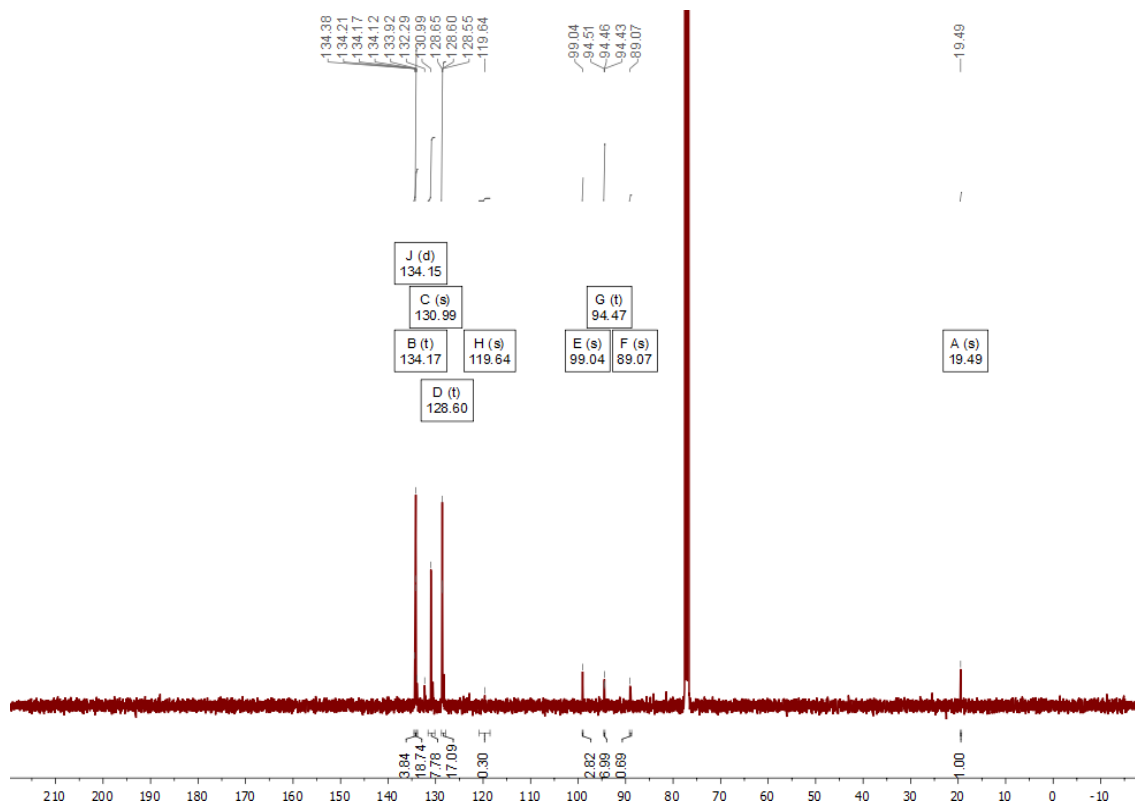


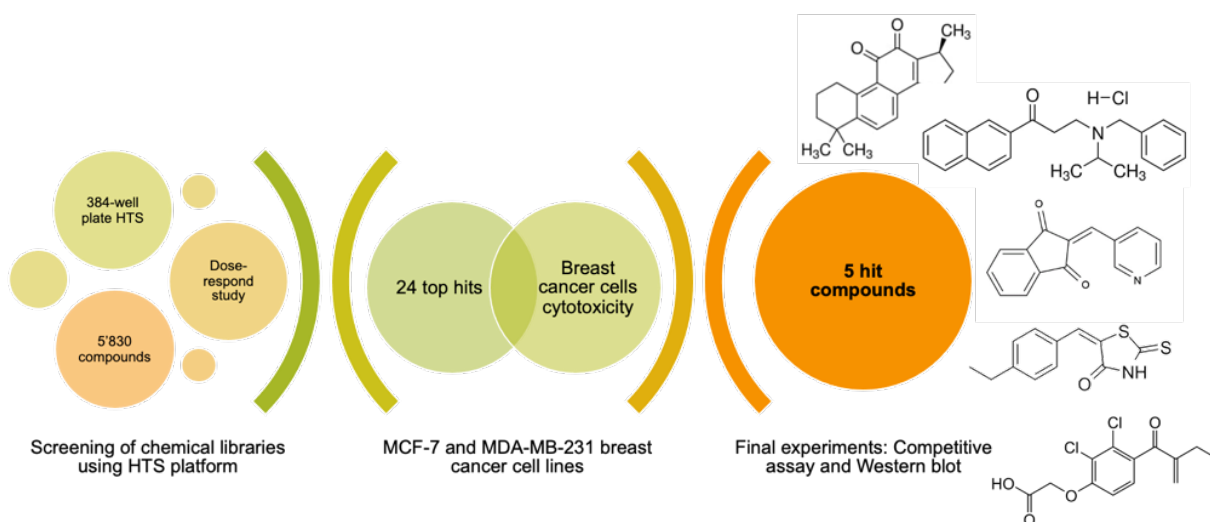
Figure S69 - ^{13}C NMR spectra of 11NO_3 .

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5 – Identification of glutathione transferase (GST P1) inhibitors via a high-throughput screening assay and implications as new treatment options for breast cancers

In this last work, I developed and optimized the 96- and 384-well plate HTS enzymatic experiments, using the last one to test the $\approx 5'830$ compounds. I also performed the cytotoxicity assay for the most inhibitory compounds against GST P1. Moreover, I also achieved the competitive inhibitory assay to identify the type of inhibition of each compound and the western blot assay. In the end, I wrote the original draft of the manuscript for later publication.



Identification of glutathione transferase (GST P1) inhibitors via a high-throughput screening assay and implications as new treatment options for breast cancers

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Abstract

Glutathione S-transferase P1 (GST P1) is an important drug target as it is implicated in drug resistance. According to the characterization of GST P1 inhibitors, it is likely that several clinically used drugs may unknowingly act as GST P1 inhibitors. Hence, we developed a high-throughput screening assay for GST P1 and screened libraries comprising 5'830 compounds. From the screening, 24 potent GST P1 inhibitors were identified and assessed for cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cell lines. ethacrynic acid, ZM 39923, PRT 4165, 10058-F4, and cryptotanshinone were shown to be the most active. Also, a competitive GST P1 assay was performed to identify the inhibition type of these 5 compounds and another *in vitro* cytotoxicity experiment was performed to explore the differences in the cytotoxicity profile of the combinations tested. Then, a western blot experiment was achieved to identify the presence of GST P1 in the breast cancer cell lines tested and the effect of ethacrynic acid, ZM 39923, and PRT 4165 in the expression of GST P1.

Keywords –glutathione S-transferase, high-throughput screening, enzyme inhibition, drug repurposing, breast cancer

1. Introduction

Many human cancers, such as lung, colon, ovarian, and breast cancers, overexpress glutathione S-transferase P1 (GST P1), an enzyme involved in phase II detoxification of xenobiotics by glutathione conjugation (1). Hence, GST P1 overexpression is considered a biomarker for cancer development (2), and is also associated with disease progression and drug resistance in patients undergoing chemotherapy (3). Therefore, the overexpression of GST P1 outcomes in the acceleration of drug metabolism, significantly decreasing the therapeutic efficiency.

The overexpression of GST P1 is involved in the chemoresistance of several cancer types, including adriamycin-resistant breast cancer (4), fluorouracil (5-FU)- and cisplatin-resistant gastric cancer (5), and doxorubicin-resistant prostate cancer (6).

Yang et al. established that the knockdown of GST P1 by small RNA expressively augmented the apoptosis and DNA damage in adriamycin-treated breast cancer cells. Moreover, it was discovered that GST P1 is upregulated in Claudin-6 (CLDN6)-overexpressing and multidrug-resistant estrogen-receptor positive (ER⁺) breast cancer cells. So, the CLDN6 block leads to a reduction in the expression and enzymatic activity of GST P1, increasing the cytotoxicity of adriamycin, 5-FU, and cisplatin in ER⁺ breast cancer cells. Comparable remarks were made in triple-negative breast cancer cells (7).

Also, micro-RNAs can regulate GST P1 expression (8, 9), e.g., the downregulation of GST P1 can enable the function of micro-RNA-124 in doxorubicin-resistance promoting the development of new treatments to overcome chemoresistance in colorectal cancer patients (10). 1,2,4-trioxane-containing GST P1 inhibitors were also considered promising drug candidates to overcome chemoresistance in cancer patients (11). Despite some contradictions in the literature, there is evidence that ethacrynic acid, a known inhibitor of GST P1 enzyme, improves the cytotoxicity of alkylating anticancer drugs in chemoresistance cancers (12, 13), e.g., the combination of cisplatin with EA-H proved to improve the activity against cisplatin-resistant cancer cells (14, 15).

According to the characterization of GST P1 inhibitors, it seems likely that many currently used drugs might also inhibit GST P1, at least to some extent. Consequently, we decided to screen compound libraries against the GST P1 enzyme, to identify possible inhibitors. With the best inhibitors, a cytotoxicity assay using MCF-7 (estrogen and progesterone receptor ⁺ breast cancer cell line) and MDA-MB-231 (triple-negative breast cancer cell line) was performed to analyze the effect of these compounds in these cell lines. After that, a competitive assay using an optimized and validated by us 96-well plate protocol was performed to identify which type of inhibitor the compounds were. Finally, a western

blot experiment was performed to evaluate the presence of GST P1, and the effect of some of the most cytotoxic compounds obtained in the cytotoxicity assay.

A schematic representation of the screen and successive work according to the results obtained in the HTS assay are presented in Figure 1.

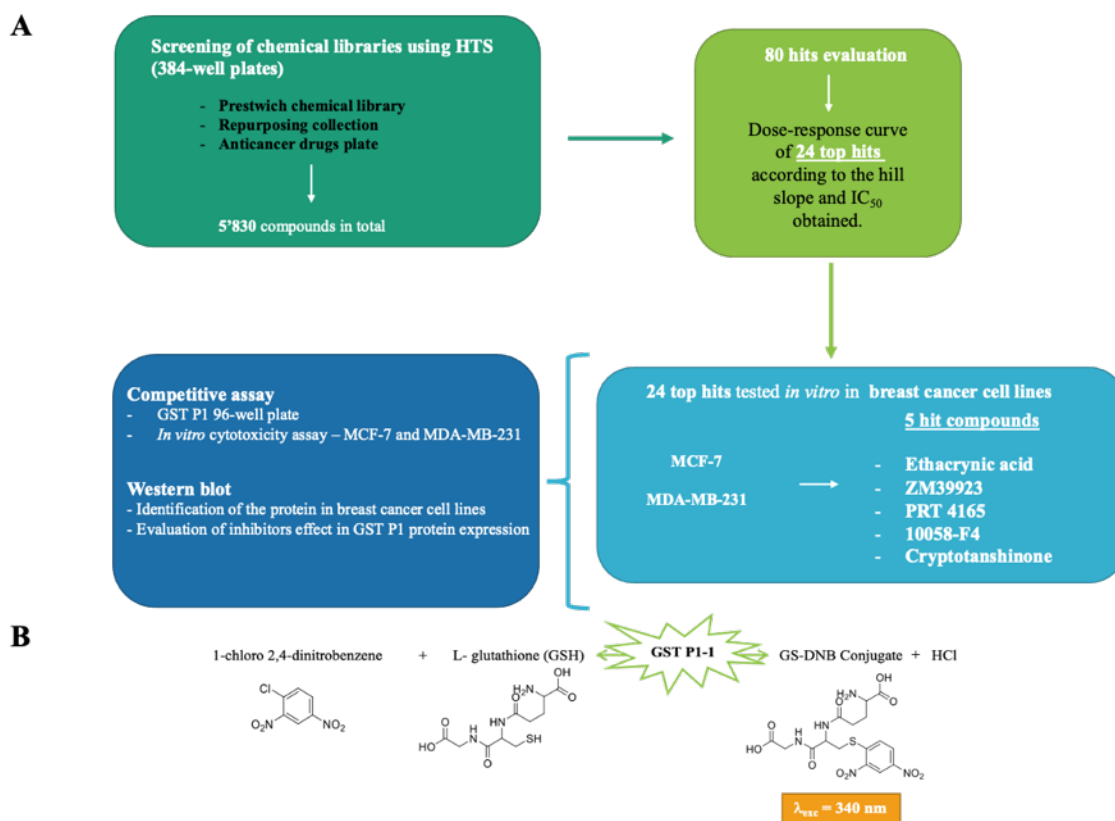


Figure 1 – A) Flowchart of the study; B) GST P1 enzymatic reaction.

2. Materials and Methods

2.1. Reagents and solutions

Recombinant Human GST P1-1, Glutathione reduced form, sodium fluoride, EDTA sodium salt, and glycerol were purchased from Sigma-Aldrich®. 1-Chloro-2,4-dinitrobenzene (CDNB) 98 %, tris(hydroxymethyl)aminomethane hydrochloride, 99.5 %, sodium dihydrogen phosphate dihydrate, and dithiothreitol 98 % were purchased from abcr GmbH & Co. Di-sodium hydrogen phosphate heptahydrate and dimethyl sulfoxide were purchased from Merck® and absolute ethanol 99.8 % was purchased from Thermo Fisher Scientific. Sodium chloride was purchased from Fluka®.

Adherent mouse fibroblast connective tissue cells (L929) and human breast cancer cells (MCF-7 and MDA-MB-231) were obtained from ATCC, Middlesex, UK, and the European Collection of Authenticated Cell Cultures (ECACC), Public Health England, Salisbury, UK, respectively. L929 fibroblasts, used as a reference for safety evaluation according to ISO 10993-1:2009 on “Biological evaluation of medical devices”. DMEM GlutaMAX media (where DMEM = Dulbecco’s modified Eagle medium) and Penicillin-streptomycin were obtained from Life Technologies, and heat-inactivated fetal bovine serum (FBS) was obtained from Sigma. White with clear bottom 96-well and 384-well microplates (cat. number 655180 and 781801, respectively) obtained from Greiner Bio-One.

2.2. Optimization and validation of 96-well plate

To evaluate the GST P1 enzyme activity, a 96-well plate assay was developed, optimized, and validated before transposition to a 384-well plate. 0.0192 U mL⁻¹ of GST P1 and 1 mM of GSH were added to the wells. Then the mixture stayed for 20 min of pre-incubation time without CDNB. After that, 4mM of CDNB was added to the wells and the reaction monitorization was recorded spectrophotometrically at 340 nm. The optimization of DMSO and ethanol concentrations in the reaction, temperature, kinetic and end-point assay, and screening of positive control (ethacrynic acid) to calculate the z'-factor to validate the protocol. All the other conditions were maintained according to (16).

2.3. Compound libraries

A total of 5'830 compounds were used for screening from the Prestwick library, Repurposing collection, and anticancer drugs plate. All compounds were supplied as 10 mM stock solutions in DMSO. Compounds were flushed with argon before being stored in the dark at -20 °C using an automated storage system (A3-RTS store from Brooks) and their chemical integrity was controlled regularly by RP-HPLC, coupled to ESI-MS with UV and CAD detectors (Thermo).

2.4. High-throughput screening (HTS)

The 5830 compounds were dispensed into 38 barcoded 384-well plates, using an Echo 655 acoustic liquid handler (Beckman Coulter®). Each drug was added to the respective well at a volume of 80 nL of 10mM stock solution in pure DMSO, yielding a final compound concentration of 10 µM and a final DMSO concentration of 0.1 %. The first and last two columns of each plate were used as negative (no compound added – 80 nL per well of DMSO) and positive (80 nL of ethacrynic acid added with a final concentration of 50 µM) controls, respectively. The Gyger Certus Flex dispenser has one bottle where the

mixture solution was filled and a syringe that was filled with the 4 mM substrate solution in pure ethanol. The freshly prepared mixture solution consisting of 1 mM of GSH and 20 nM of GST P1, pH = 7.4 was dispensed (80 μ L) to each well of the plate. After a pre-incubation time of 20 min, 4 μ L of 8 mM of CDNB solution was dispensed to each well of the plate. Afterward, the plates were kept in the dark for 2 h and then the absorbance was recorded on a BioTek Synergy NEO HTS multimode plate reader (Agilent®) at a wavelength of 340 nm.

The z'-factor was calculated for each plate according to Zhang et al. (17). This value was between 0.5 and 0.8 for all plates.

A score of 0 (0%) for the negative control (average of maximal activity measure in the presence of enzyme without any inhibitor) and a score of 1 (100%) (minimal activity measure in the presence of the reference inhibitor – ethacrynic acid) were assigned controls for each plate (i.e., normalization between 0 and 1). The score for each compound was calculated (between 0 and 1 in the majority of the cases but can be > 1 or < 0 if the absorbance measurements are higher than the negative control or lower than the positive control). A hit is defined as a compound having a score larger than the average of the negative control plus three times its standard deviation for both replicates. An internal laboratory information management system was used for basic data processing, management, visualization, and statistical hit validation. The screening of the compounds was performed in duplicate and compound scores were calculated as average $\pm$ standard deviation.

2.5. Determination of IC₅₀ of hits for GST P1 enzyme

From the primary assay, 80 hit-selected compounds were validated for their GST inhibition activity using a dose-response approach in 384-well plates. The compounds were tested at 8 different concentrations (μ M) starting at 30 μ M with a half-log dilution with 2 replicates per concentration. The compounds were plated using the Echo 655 dispenser. The assay conditions were the same as for the primary screening. The absorbance measurements were performed on a BioTek Synergy NEO HTS multimode plate reader for the absorbance measurements of the dose-response curves using an excitation wavelength of 340 nm.

2.6. Cytotoxicity assays

L929, MCF-7, and MDA-MB-231 cell lines were cultured in DMEM GlutaMAX media containing 10% heat-inactivated FBS and 1% (v/v) penicillin-streptomycin at 37 °C and

CO₂ (5%). The cytotoxicity was determined using the 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were seeded in flat-bottomed 96-well plates as a suspension in a prepared medium (100 µL aliquots and approximately 50'000 cells/well) and preincubated for 24 h. Stock solutions of compounds were prepared in DMSO and were diluted in the medium. The solutions were sequentially diluted to give a final DMSO concentration of 0.5% and a final compound concentration range (0–100 µM). The compounds were added to the preincubated 96-well plates in 100 µL aliquots, and the plates were incubated for a further 72 h. MTT (10 µL, 5 mg/mL in Dulbecco's phosphate-buffered saline) was added to the cells, and the plates were incubated for a further 3 h. The culture medium was aspirated and the purple formazan crystals, formed by the mitochondrial dehydrogenase activity of vital cells, were dissolved in DMSO (100 µL/well). The absorbance of the resulting solutions is directly proportional to the number of surviving cells and was quantified at 570 and 630 nm (for background correction) using a SpectraMax M5e multimode microplate reader (using SoftMax Pro software, version 6.2.2). The percentage of surviving cells was calculated from the absorbance of wells corresponding to the untreated control cells. The reported IC₅₀ values are based on the means from four independent experiments, each comprising three replicates per concentration level.

2.7. Determination of inhibitor binding mechanism

The competitive enzymatic assay was performed using the optimized 96-well plate protocol. Different concentrations of CDNB (µM) were used: 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 at the same concentration of inhibitor (5 µM). Experiments were performed in duplicate. The Michaelis-Menten constant, K_m , and half of the maximum velocity V_{max} were calculated using GraphPad Prism 9 software.

To extrapolate the equations to identify the type of inhibition, the Michaelis-Menten equation was applied (Eq. 1):

$$v_0 = \frac{V_{max} [S]}{[S] + K_m} \quad (1)$$

2.8. Western blot assay

MCF-7 and MDA-MB-231 (320'000 cells per 60 mm petri dish) were incubated for 24 h with the IC₅₀ concentration of each compound to evaluate their effect on GST P1

expression. Then, the cancer cells were rinsed with ice-cold PBS twice and lysed in 100 μ L of buffer containing 50 mM Tris, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 , and 1% NP-40 for 30 min in ice. After scraping, brief sonication, and subsequent high-speed centrifugation (14'000 g for 20 min), the cell supernatants were collected and assayed for protein content using the BCA method. Aliquots containing 5 μ g of protein were boiled for 5 min and electrophoresed on 10 % SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes, which were blocked with 5% milk for 1 h at room temperature and then incubated with an anti-GST P1 antibody 1:1000 (Fisher Scientific AG) overnight at 4 °C. The membranes were further incubated with secondary goat anti-mouse horseradish peroxidase-conjugated anti-immunoglobulin-G 1:5000 (Invitrogen). Protein bands were detected using an enhanced chemiluminescence western blot detection system (WesternBright™ ECL-spray from Advansta®). For the housekeeping protein (GAPDH), the membrane was stripped and reprobed with an anti-GAPDH antibody 1:1000 (Sigma) for 2 h and then incubated with secondary goat anti-rabbit horseradish peroxidase-conjugated anti-immunoglobulin-G 1:5000 (Invitrogen).

3. Results and discussion

3.1. Identification and validation of potent GST P1 inhibitors

This assay was based on one reported in the literature with modifications in reagents concentrations, DMSO and ethanol concentrations, temperature, and pre-incubation time (16). Table 1 lists the optimized parameters, the range studied, and the selected value for each parameter.

Table 4 – Parameter optimization for the 96-well plate GST P1 inhibition assay.

Parameter	Actual values tested	Selected value
CDNB concentration (mM)	0.25, 0.5, 1, 1.5, 2, 4, 6, 8	4
GSH concentration (mM)	0.1, 0.25, 0.5, 1, 2, 4, 6, 8	1
GST P1 concentration (U/mL)	0.002, 0.007, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06	0.02
Pre-incubation time (min)	0, 15, 20, 30	20
DMSO percentage (%)	0.08, 0.15, 0.3, 0.6, 1.25, 2, 2.5, 5	2
Ethanol percentage (%)	1, 1.25, 2.5, 5, 10, 20	5
Temperature (°C)	25 and 37	25

The parameters were optimized using the univariant method, i.e., each parameter was optimized as a single parameter where only one parameter is changed, and the others are kept constant. The CDBN, GSH, and GST P1 concentrations were optimized to maximize

the absorbance signal at a good cost-benefit ratio. The effect of the amount of DMSO and ethanol was studied (Figure S1), with DMSO having little impact, whereas ethanol decreases the reaction rate.

The K_m was also calculated to be 8.4 mM and to ensure high GST P1 activity in the inhibition assays a value of 4 mM was chosen (Figure S2).

The CDNB, GSH, and GST P1 concentrations, pre-incubation time, DMSO, and ethanol concentrations were optimized to obtain the best conditions to implement the screening assay in the 384-well plate. It was calculated the z' -factor of the end-point reaction with 20 min of pre-incubation time without the CDNB and after adding the CDNB, all the reagents were incubated for 2 h before the detection. The z' factor obtained was > 0.5 so the experiment was validated.

In the next step, the GST P1 inhibition assay was expanded from a 96-well plate assay to a 384-well plate assay, leading to an optimized volume of 80 μ L. The z' -factor obtained was > 0.5 ($n = 38$). Two different compound libraries were tested: the Prestwick chemical library, the Repurposing collection, and a selection of 71 anticancer drugs. Then, it was performed the screening and hit validation using the assay design strategy presented in Figure S3. From the 5'830 compounds tested, 336 compounds were identified as hits from the HTS screen (Table S1). From these 336 hits, the dose-response curves were performed for the first 80 hit compounds (score obtained in LIMS ≥ 0.7). Considering the previous results, it was selected 24 compounds with known anticancer activity and with hillslopes < 4 (except for Nastorazepide, CD437, MK-5108, and Hypericin). These 4 compounds with higher hillslopes were also selected for the reconfirmation assay since their mechanism of action could be interesting to study for breast cancer treatment and, specifically CD437 and Hypericin, the IC_{50} values obtained for GST P1 are one of the lowest ones (Table S2). Remarkably, ethacrynic acid, the positive control of this experiment, was also considered a hit due to its presence in the libraries studied, and with 5 times less concentration compared with the positive control (10 μ M instead of 50 μ M), it also presented a good hillslope and IC_{50} value. The hill slopes, IC_{50} values for GST P1 enzyme, and the dose-response curves of these compounds are represented in Figure S4 and Table S2.

3.2. Cytotoxicity assay

The cytotoxicity of the 24 compounds selected from HTS assays was assessed against human breast cancer cell lines, MCF-7 and MDA-MB-231, and nontumoral adherent mouse fibroblast connective tissue cells (L929) using the 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for 72 h (Table 2).

MCF-7 and MDA-MB-231 cell lines were selected to perform this experiment since, according to Figure 2, the GST P1 enzyme is expressed in the MDA-MB-231 breast cancer cell line and is not expressed in the MCF-7 breast cancer cell line (18). As the MDA-MB-231 breast cancer cell line is considered more aggressive than MCF-7 (19), it is important to identify compounds that cause cytotoxicity in this cell line by acting in GST P1 activity.

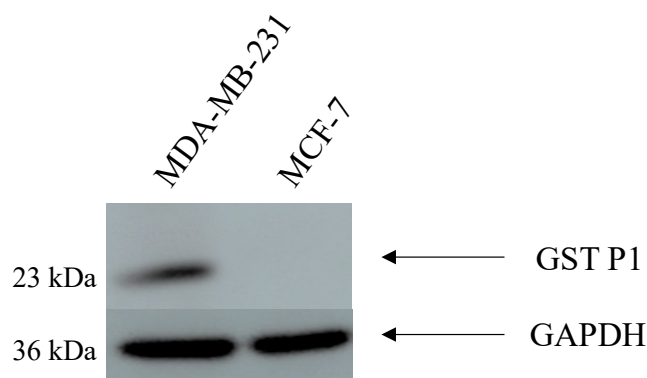


Figure 2 – Expression of GST P1 in MDA-MB-231 and MCF-7 cell lines by western blot assay.

According to the results (Table 2 and Table S3), some compounds do not present statistical differences between the non-tumoral L929 cell line and both breast cancer cell lines (MCF-7 and MDA-MB-231) such as **TCID**; **5-Methyl-2-phenyl-1,2-dihdropyran-3-one**; **Nastorazepide** and **CD437**. **CD437** presents higher cytotoxic activity against L929, MCF-7, and MDA-MB-231, nevertheless, as there are no statistical differences between them, it was excluded for not being a selective compound for breast cancer cells. Moreover, other compounds e.g., **PD-118057**, **MK-5108**, **AS 252424**, **AZD9496**, **GW3965 HCl**, **nTZDpa**, **RQ00203078**, **TCS PIM-1 1**, **PSB 06126** and **Hypericin** present lower cytotoxic effects in MDA-MB-231 compared with L929 and MCF-7 cell lines, so these compounds are not interesting from the point of view of this study.

Considering the selection of compounds with lower IC_{50} values for MCF-7 and MDA-MB-231 cell lines and, in some cases, with statistical differences between them, all the compounds with higher IC_{50} values for these cell lines were excluded from the next experiment. According to this, from the 24 compounds previously selected and tested, just 5 compounds were selected for the next experiments: **cryptotanshinone**; **ZM 39923**; **10058-F4**; and **ethacrynic acid**, since they present better selectivity between L929 and MCF-7/MDA-MB-231 and the IC_{50} values are $< 15\mu M$. Although compound **PRT 4165** presents higher IC_{50} values compared with the compounds mentioned previously (IC_{50} : L929 $>100\mu M$; MCF-7 = $59 \pm 7\mu M$ and MDA-MB-231 = $27 \pm 6\mu M$), this compound was

also selected since is one of the few compounds tested that presented an IC_{50} for the MDA-MB-231 cell line ($IC_{50} = 27 \pm 6 \mu M$) lower than MCF-7 ($IC_{50} = 59 \pm 7 \mu M$) with considered statistical differences ($^{****}P < 0.0001$). Looking at the mechanism of action of compounds **ZM 39923** and **ZM 449829** (a degradation product of ZM 39923 HCl), are considered selective and potent inhibitors of Janus tyrosine kinase 3 (20), however, they do not present the same cytotoxicity behavior since **ZM 39923** is 1.6-fold and 4-fold more cytotoxic for MCF-7 and MDA-MB-231 than **ZM 449829**, presenting the same cytotoxicity for L929 cell line. So, **ZM 449829** was not selected for the next experiment.

Looking for the chemical structures of these 5 compounds (Table S4), which includes the reference inhibitor of GST P1 (**ethacrynic acid**), there are some chemical groups present in their chemical structures, mostly methyl groups, aromatic rings, ketones, and carboxylic acids and they can justify their inhibitory effect in GST P1 enzyme. According to the literature, there are some compounds considered GST P1 inhibitors with similar structures to these 5 compounds, such as Piperlongumine and Trimethoxycinnamic acid (21) and 6-(7-nitrobenzo[c][1,2,5]-oxadiazol-4-yl)thiohexan-1-ol (NBDHEX) (22).

Furthermore, it was also assessed the effect of **ethacrynic acid**, **ZM 39923**, and **PRT 4165**, 3 of the 5 most cytotoxic compounds obtained in the *in vitro* assay (Figure 3 – numbers **4**, **5**, and **6**, respectively).

Table 5 – IC_{50} values obtained for the 24 compounds tested in vitro and the statistical differences between all the cell lines. See Table S2 in the Supporting Information for the full statistical analysis.

Compounds	IC_{50} (μM) (average $\pm$ standard deviation)		
	MCF-7	MDA-MB-231	L929
ZM 449829	6.8 ± 0.2	10.3 ± 0.2	8.1 ± 0.4
Cryptotanshinone 97.0%	0.29 ± 0.04	2.9 ± 0.3	4.4 ± 0.3
TCID	> 100	> 100	> 100
ZM 39923 (hydrochloride)	4.3 ± 0.1	2.6 ± 0.1	8.2 ± 0.6
PD-118057	41 ± 4	> 100	> 100
PRT 4165	59 ± 7	27 ± 6	> 100
5-Methyl-2-phenyl-1,2-dihydropyrazol-3-one, 98%	> 100	> 100	> 100
10058-F4	14 ± 2	15 ± 4	> 100
MK-5108	3.4 ± 0.8	> 100	2.2 ± 0.5
Embelin	11 ± 1	12 ± 1	14 ± 1
Nastorazepide	> 100	> 100	> 100
CD437	0.51 ± 0.05	0.74 ± 0.07	0.24 ± 0.03
AS252424	38 ± 2	91 ± 3	24 ± 2
AZD9496	14 ± 1	47 ± 5	36 ± 4
en460	10 ± 1	19 ± 1	18 ± 2
Hexachlorophene	14 ± 1	9 ± 1	11 ± 1
GW3965 (hydrochloride)	25 ± 1	54 ± 1	24 ± 2
nTZDpa	39 ± 2	95 ± 4	36 ± 3
RQ-00203078	17 ± 2	51 ± 6	42 ± 6
Avasimibe	12 ± 1	16 ± 1	13 ± 1
TCS PIM-1 1	74 ± 4	> 100	36 ± 3
PSB 06126	45 ± 3	> 100	39 ± 4
Hypericin	8 ± 1	26 ± 3	19 ± 1
Ethacrynic acid	13 ± 1	14 ± 1	> 100

According to Figure 4, the control of the MDA-MB-231 cell line (3) presents a higher expression compared with the other three bands (4, 5, and 6) that correspond to the effect of the compounds described before. **ethacrynic acid (4)**, **ZM 39923 (5)**, and **PRT 4165 (6)** compounds were compared with the MDA-MB-231 control (3), and there are 1.93-fold; 1.86-fold; and 2.28-fold less expression of GST P1 by the effect of (4), (5) and (6), respectively.

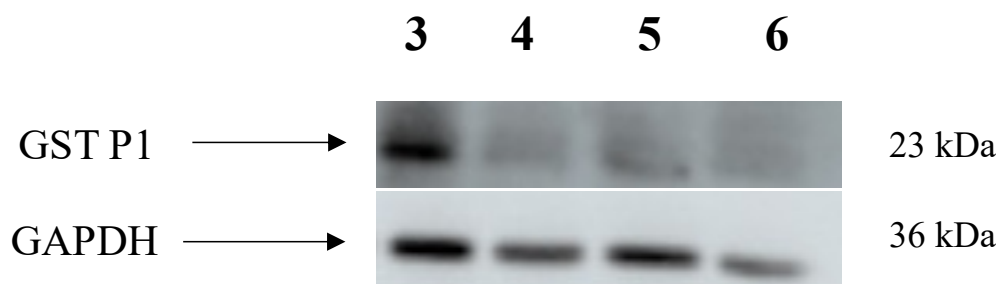


Figure 3 – Effect of GST P1 expression in MDA-MB-231 breast cancer cell line (3) of ethacrynic acid (4), ZM 39923 (5), and PRT 4165 (6) compounds.

3.3. Competitive inhibitory assay

The idea to perform this experiment was to identify the type of inhibition in the presence of the GST P1 enzyme of the 5 most cytotoxic compounds obtained in the *in vitro* cytotoxic assay (Figure 4). This experiment was performed in the 96-well plate previously optimized.

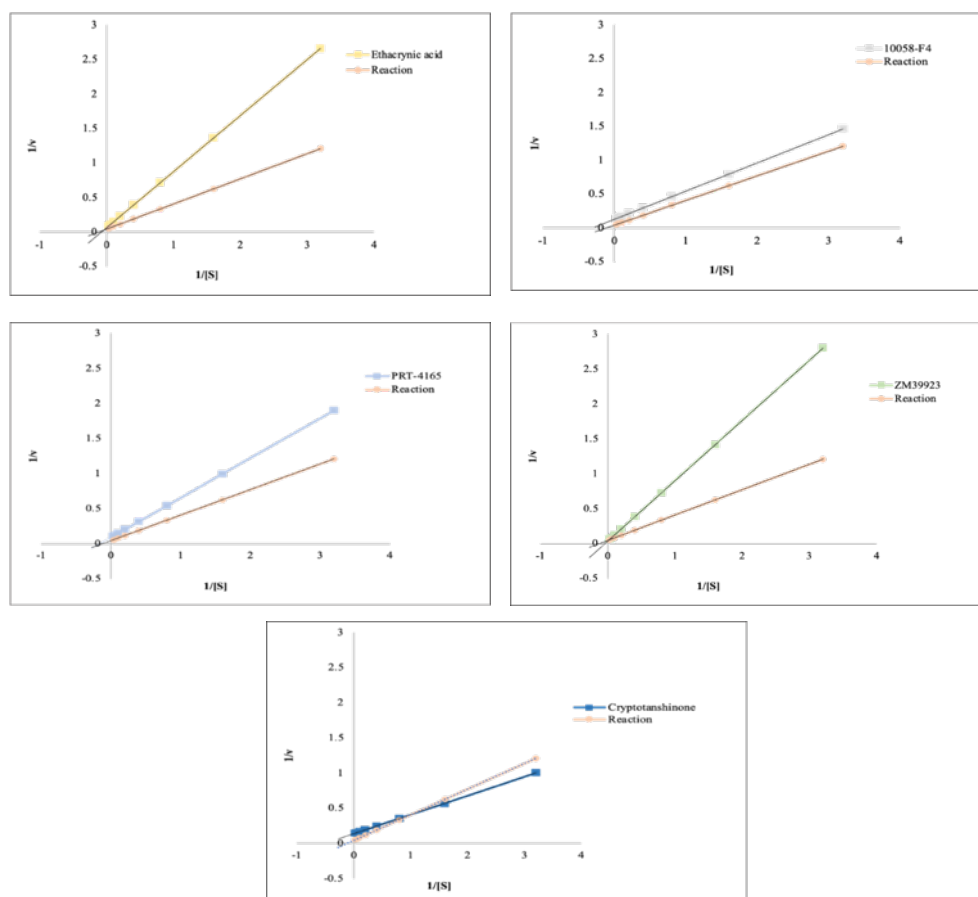


Figure 4 – Schematic representation of the type of inhibition of the 5 most cytotoxic compounds obtained *in vitro*.

Table 6 – Inhibition type according to the K_m and V_{max} obtained in the competitive GST P1 experiment.

	K_m	V_{max}	Inhibition type
Reaction	8.4 ± 2.4	23 ± 3	-
Ethacrynic acid	12.3 ± 9.3	15.1 ± 7.3	Competitive
PRT 4165	6.4 ± 0.7	11.3 ± 0.5	Uncompetitive
ZM 39923	24.6 ± 6.7	28 ± 4	Competitive
10058-F4	3.3 ± 1.8	7.9 ± 1.4	Uncompetitive
Cryptotanshinone	2 ± 1	7.4 ± 1.3	Uncompetitive

The Michaelis-Menten equation predicts a hyperbolic relationship between initial velocity and substrate concentration, describing the kinetic behavior of several enzymes (23). Evaluating the differences in K_m and V_{max} compared with the reaction without an inhibitor is mandatory to identify the competition type of inhibitors. Competitive inhibitors

present an increase in the K_m and an unaffected V_{max} . On the other hand, uncompetitive inhibitors present a decrease in K_m and V_{max} (24).

According to Figure 5 and Table 3, two compounds (**ethacrynic acid** and **ZM 39923**) are competitive inhibitors while **PRT 4165**, **10058-F4**, and **cryptotanshinone** compounds are uncompetitive inhibitors.

After this information, it could be interesting to conjugate competitive to uncompetitive inhibitors and evaluate the *in vitro* cytotoxicity of these conjugates. The same protocol and detection method was used before (MTT) to perform them.

This study was initiated with the IC_{50} concentrations of both compounds and then successive dilutions of them were made. So, for the MCF-7 cell line, the assay concentrations were started with 13 μM for **ethacrynic acid**, 4.3 μM for **ZM 39923**, 59 μM for **PRT 4165**, 14 μM for **10058-F4** and 0.29 μM for **cryptotanshinone**. The cytotoxic results for all the combinations with **ethacrynic acid** are presented in Figure 5. The combination of **ethacrynic acid** with **ZM 39923** (both competitive GST P1 inhibitors) and **PRT 4165**, **10058-F4**, and **cryptotanshinone** (uncompetitive GST P1 inhibitors), leads to a percentage of inhibition of 63%, 52%, 64%, and 59%, respectively, when it was tested 4-fold less the IC_{50} concentration of these compounds separately. When was tested **ZM 39923** with the **PRT 4165** uncompetitive compound, the percentage of inhibition obtained (60%) was similar to the conjugation of **ethacrynic acid** with **PRT 4165** (52%). On the other hand, the results obtained are quite different when it conjugated **ZM 39923** with **10058-F4** and **cryptotanshinone**. The conjugation of **ZM 39923** with **10058-F4** in their IC_{50} concentration leads to 55 % of inhibition, so the conjugation of these compounds does not improve the cytotoxicity in the MCF-7 cell line, and the conjugation with **cryptotanshinone** leads to 62 % of inhibition with 2-fold less the IC_{50} concentration of both compounds separately, being less cytotoxic than the conjugation of **ethacrynic acid** with **cryptotanshinone**.

MCF-7 cell line

[Ethacrynic acid] : [ZM 39923] (μM)		% Inhibition
3.25 : 1.075		63 $\pm$ 4
6.5 : 2.15		35 $\pm$ 4
13 : 4.3		15 $\pm$ 7

[Ethacrynic acid] : [PRT 4165] (μM)		% Inhibition
3.25 : 14.75		52 $\pm$ 6
6.5 : 29.5		36 $\pm$ 4
13 : 59		20 $\pm$ 3

[Ethacrynic acid] : [10058-F4] (μM)		% Inhibition
3.25 : 3.5		64 $\pm$ 3
6.5 : 7		40 $\pm$ 2
13 : 14		20 $\pm$ 2

[Ethacrynic acid] : [Cryptotanshinone] (μM)		% Inhibition
3.25 : 0.072		59 $\pm$ 6
6.5 : 0.145		39 $\pm$ 4
13 : 0.29		23 $\pm$ 2

[ZM 39923] : [PRT 4165] (μM)		% Inhibition
1.075 : 14.75		60 $\pm$ 12
2.15 : 29.5		47 $\pm$ 6
4.3 : 59		26 $\pm$ 7

[ZM 39923] : [10058-F4] (μM)		% Inhibition
4.3 : 14		55 $\pm$ 9

[ZM 39923] : [Cryptotanshinone] (μM)		% Inhibition
2.15 : 0.145		62 $\pm$ 6
4.3 : 0.29		38 $\pm$ 4

Figure 5 – Percentage of inhibition for MCF-7 breast cancer cell line obtained by the conjugation of

ethacrynic acid and ZM 39923 and each with PRT4165, 10058-F4, and cryptotanshinone compounds.

It was also performed the same study using the MDA-MB-231 breast cancer cell line using as a starting point the IC₅₀ concentrations obtained in the cytotoxicity assay for this cell line: 14 μ M for **ethacrynic acid**, 2.6 μ M of **ZM 39923**, 27 μ M of **PRT 4165**, 15 μ M of **10058-F4** and 2.9 μ M of **cryptotanshinone**. Looking at Figure 6, the conjugation of **ethacrynic acid** with **ZM 39923** (competitive inhibitors) and **PRT 4165** and **10058-F4** leads to percentages of inhibition of 62 %, 65 %, and 52 %, respectively when it was tested 2-fold less IC₅₀ concentration of these compounds separately. However, with the conjugation of **ethacrynic acid** with **cryptotanshinone**, it was obtained a percentage of inhibition of 65 % when it was tested 8-fold less the IC₅₀ concentration of these compounds separately, so this combination is more cytotoxic than the previous ones.

The combination of **ZM 39923** with **PRT 4165** leads to a similar percentage of inhibition (66 %) when compared with **ethacrynic acid** conjugation with this compound (65 %) in the same ratio. The combination of **ZM 39923** with **10058-F4** leads to a percentage of inhibition of 69 % when tested with 2-fold less IC₅₀ concentration of these compounds separately, being this conjugation 1.3 times less cytotoxic compared with the combination of **ethacrynic acid** with **10058-F4**.

MDA-MB-231 cell line

[Ethacrynic acid] : [ZM 39923] (μ M)		% Inhibition
7 : 1.3		62 $\pm$ 5
14 : 2.6		30 $\pm$ 2

[Ethacrynic acid] : [PRT 4165] (μ M)		% Inhibition
7 : 13.5		65 $\pm$ 6
14 : 27		17 $\pm$ 11

[Ethacrynic acid] : [10058-F4] (μ M)		% Inhibition
7 : 7.5		52 $\pm$ 7
14 : 15		21 $\pm$ 2

[Ethacrynic acid] : [Cryptotanshinone] (μ M)		% Inhibition
1.75 : 0.362		65 $\pm$ 7
3.5 : 0.725		56 $\pm$ 6
7 : 1.45		43 $\pm$ 5
14 : 2.9		15 $\pm$ 1

[ZM 39923] : [PRT 4165] (μ M)		% Inhibition
1.3 : 13.5		66 $\pm$ 9
2.6 : 27		40 $\pm$ 10

[ZM 39923] : [10058-F4] (μ M)		% Inhibition
1.3 : 7.5		69 $\pm$ 11
2.6 : 15		49 $\pm$ 7

[ZM 39923] : [Cryptotanshinone] (μ M)		% Inhibition
0.65 : 0.725		65 $\pm$ 14
1.3 : 1.45		56 $\pm$ 9
2.6 : 2.9		33 $\pm$ 4

Figure 6 - Percentage of inhibition for MDA-MB-231 breast cancer cell line obtained by the conjugation of **ethacrynic acid** and **ZM 39923** and each with **PRT 4165**, **10058-F4**, and **cryptotanshinone** compounds.

Comparing the combinations of **ethacrynic acid** and **ZM 39923** with **cryptotanshinone**, it was obtained the same percentage of inhibition (65 %) but for different concentrations. While with the combination of **ethacrynic acid** with **cryptotanshinone**, the percentage of inhibition of 65 % was obtained for 8-fold less IC₅₀ concentration of both compounds separately, for **ZM 39923** combined with the same uncompetitive compound, the same percentage of inhibition was obtained with 4-fold less IC₅₀ concentration of both

compounds separately. So, the combination of **ethacrynic acid** and **cryptotanshinone** is the most cytotoxic for the MDA-MB-231 cell line.

4. Conclusions

This study analyzed 5'830 compounds using a developed, optimized, and validated GST P1 high-throughput screening 384-well plate experiment, being 24 compounds considered the most inhibitors for this enzyme. Two breast cancer cell lines (MCF-7 and MDA-MB-231) were selected to perform an *in vitro* assay to test the cytotoxicity of the 24 compounds previously selected. From these 24 compounds, **ethacrynic acid**, **ZM 39923**, **PRT 4165**, and **10058-F4** were the most cytotoxic compounds and **cryptotanshinone** was the compound with more statistical differences between the MCF-7 and MDA-MB-231. Moreover, with the western blot results obtained it was possible to prove the expression and non-expression of GST P1 in MDA-MB-231 and MCF-7 breast cancer cell lines, respectively, and the effect of approx. 2-fold reduction of this expression after 24 h MDA-MB-231 cell line treatment with **ethacrynic acid**, **ZM 39923**, and **PRT 4165** compounds.

Then, it was important to understand the type of competition of these compounds in GST P1, so a 96-well plate previously optimized, was established. According to these results, **ethacrynic acid** and **ZM 39923** were identified as competitive GST P1 inhibitors, and **PRT 4165**, **10058-F4**, and **cryptotanshinone** were recognized as uncompetitive GST P1 inhibitors. Furthermore, combinations of these compounds were made to explore whether there were improvements in cytotoxicity in breast cancer cell lines compared to the results obtained for each compound alone. In agreement with the results obtained, only the combination of **ZM 39923** with **10058-F4** in the MCF-7 cell line does not improve the cytotoxicity compared with the IC₅₀ concentration obtained for each compound isolate. With the other combinations, it was possible an increase in the cytotoxicity mostly of 2-fold and 4-fold, and in one case (**ethacrynic acid** with **cryptotanshinone** in the MDA-MB-231 cell lines) was obtained an increase in cytotoxicity of 8-fold. These results are very promising and support the evidence that these compounds should be tested *in vivo* models to better understand their effects and mechanisms of action against triple-negative breast cancer.

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Supplementary material

of

Identification of glutathione transferase (GST P1) inhibitors via a high-throughput screening assay and implications as new treatment options for breast cancers

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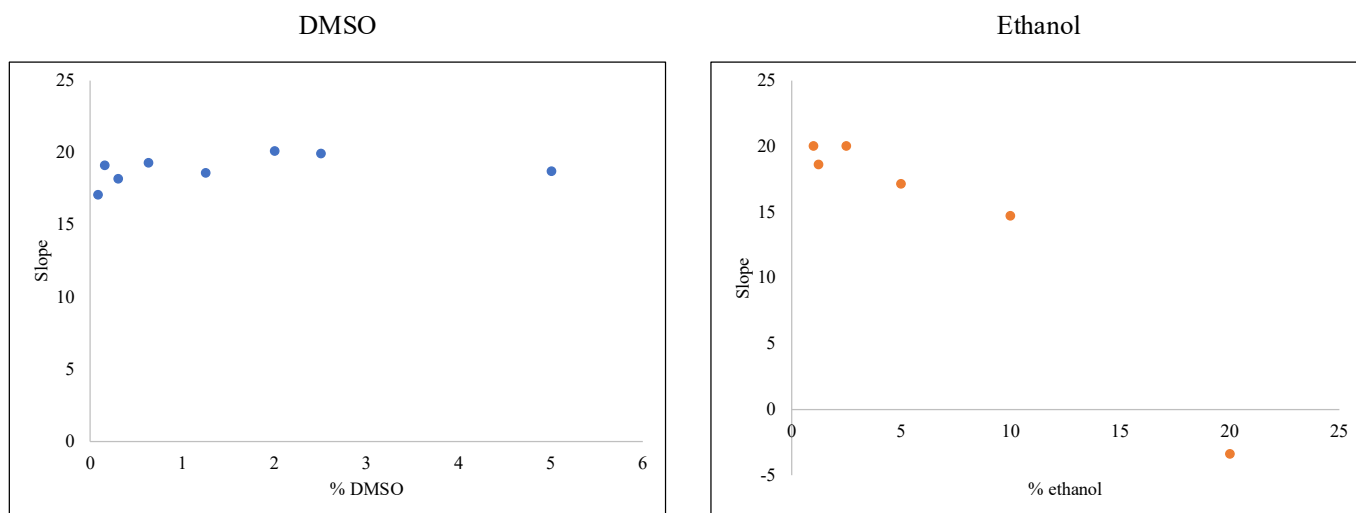


Figure S1 – Optimization of the percentage of DMSO and ethanol used in the reaction.

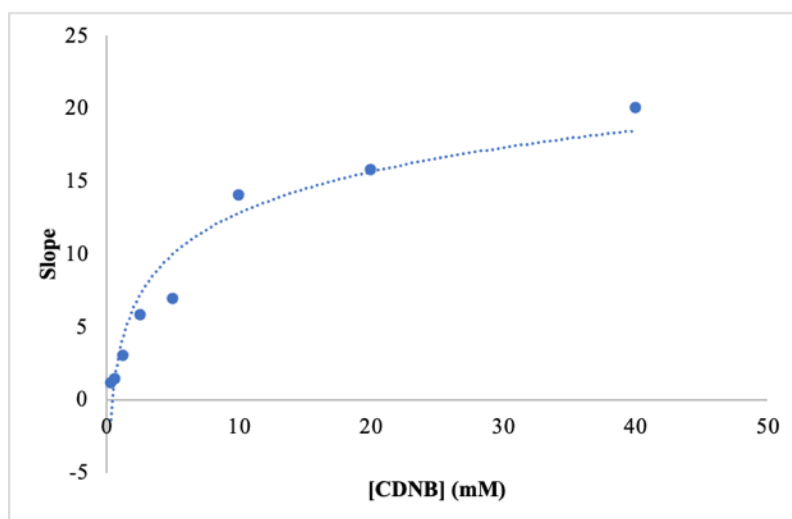


Figure S2 - Calibration curve of GST P1 enzymatic reaction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO		1	17	33	49	65	81	97	113	129	145	161	177	193	209	225	241	257	273	289	305	Ethacrynic acid	
B	DMSO		2	18	34	50	66	82	98	114	130	146	162	178	194	210	226	242	258	274	290	306	Ethacrynic acid	
C	DMSO		3	19	35	51	67	83	99	115	131	147	163	179	195	211	227	243	259	275	291	307	Ethacrynic acid	
D	DMSO		4	20	36	52	68	84	100	116	132	148	164	180	196	212	228	244	260	276	292	308	Ethacrynic acid	
E	DMSO		5	21	37	53	69	85	101	117	133	149	165	181	197	213	229	245	261	277	293	309	Ethacrynic acid	
F	DMSO		6	22	38	54	70	86	102	118	134	150	166	182	198	214	230	246	262	278	294	310	Ethacrynic acid	
G	DMSO		7	23	39	55	71	87	103	119	135	151	167	183	199	215	231	247	263	279	295	311	Ethacrynic acid	
H	DMSO		8	24	40	56	72	88	104	120	136	152	168	184	200	216	232	248	264	280	296	312	Ethacrynic acid	
I	DMSO		9	25	41	57	73	89	105	121	137	153	169	185	201	217	233	249	265	281	297	313	Ethacrynic acid	
J	DMSO		10	26	42	58	74	90	106	122	138	154	170	186	202	218	234	250	266	282	298	314	Ethacrynic acid	
K	DMSO		11	27	43	59	75	91	107	123	139	155	171	187	203	219	235	251	267	283	299	315	Ethacrynic acid	
L	DMSO		12	28	44	60	76	92	108	124	140	156	172	188	204	220	236	252	268	284	300	316	Ethacrynic acid	
M	DMSO		13	29	45	61	77	93	109	125	141	157	173	189	205	221	237	253	269	285	301	317	Ethacrynic acid	
N	DMSO		14	30	46	62	78	94	110	126	142	158	174	190	206	222	238	254	270	286	302	318	Ethacrynic acid	
O	DMSO		15	31	47	63	79	95	111	127	143	159	175	191	207	223	239	255	271	287	303	319	Ethacrynic acid	
P	DMSO		16	32	48	64	80	96	112	128	144	160	176	192	208	224	240	256	272	288	304	320	Ethacrynic acid	

Figure S3 – 384-well plate assay design strategy.

Table S1 – First 80 hit compounds obtained in the 384-well plate assay according to the score.

Name	Score	ScoreSD
TCID	1.094	0.016
Hexachlorophene	1.077	0.035
Cryptotanshinone	1.037	0.032
GW3965 (hydrochloride)	1.027	0.054
N-demethylantipyrine	1.019	0.032
Bithionol	1.007	0.041
rose-bengal	0.996	0.073
Merbromin	0.993	0.027
AS-2034178	0.982	0.011
Tanshinone IIA sulfonate (sodium)	0.981	0.014
CD-1530	0.979	0.053
PSB-06126	0.962	0.021
Edaravone	0.958	0.071
Nastorazepide	0.958	0.065
ZM323881 (hydrochloride)	0.946	0.002
CD437	0.939	0.012
rose-bengal-lactone	0.937	0.062
Embelin	0.935	0.022
MK-5108	0.933	0.058
ER-50891	0.927	0.017
Protoporphyrin IX	0.914	0.015
CHF5074	0.911	0.086
nTZDpa	0.909	0.031
AS-252424	0.907	0.050

IOWH-032	0.901	0.087
PRT4165	0.890	0.019
IPA-3	0.881	0.009
SDZ-220-040	0.871	0.049
Sal003	0.865	0.028
Chicago sky blue 6B	0.863	0.004
Lasalocid	0.863	0.039
PI3K inhibitor X	0.863	0.011
PD-118057	0.858	0.073
GW627368	0.854	0.134
Ethacrynic acid	0.850	0.002
MNS	0.850	0.009
EN460	0.837	0.038
Tiplaxtinin	0.836	0.040
Avasimibe	0.835	0.062
erythrosine	0.828	0.032
Sulfinpyrazone	0.823	0.011
Docosahexaenoic Acid	0.821	0.082
JAK3-inhibitor-V	0.817	0.053
TCS PIM-1 1	0.813	0.030
TPPS4	0.809	0.004
RTA-408	0.807	0.046
AZD9496	0.806	0.169
Macelignan	0.803	0.077
Bromosporine	0.801	0.016
Zafirlukast	0.801	0.204
cis-9,trans-11-Conjugated-linoleic-acid	0.796	0.074
ZM39923 (hydrochloride)	0.788	0.059
brilliant-green	0.783	0.043
TUG-891	0.783	0.016
Evans Blue	0.783	0.031
AMG 837 (calcium hydrate)	0.783	0.028
Hypericin	0.774	0.008
bonaphthone	0.770	0.020
Y16	0.765	0.033
Palovarotene	0.763	0.084
Hematoporphyrin	0.758	0.006
NS-3623	0.752	0.005
diphencyprone	0.744	0.024
AT13148	0.738	0.090
SR-11302	0.733	0.069
10058-F4	0.731	0.007
4EGI-1	0.730	0.012
TC-I-2000	0.729	0.040
Salirasib	0.717	0.037
BADGE	0.715	0.017
Ki16425	0.710	0.103
GW 4064	0.702	0.196
Triclocarban	0.702	0.068
Temoporfin	0.699	0.036
RQ-00203078	0.697	0.100

PHT-427	0.696	0.028
Anthralin	0.694	0.030
SKI II	0.694	0.046
buclicine	0.694	0.010
BMS 299897	0.690	0.114
TC-LPA5-4	0.686	0.079
Prasugrel	0.681	0.009
IMD-0354	0.681	0.077
Hemin	0.680	0.007
sodium-dodecyl-sulfate	0.678	0.256
DCPIB	0.672	0.031
chlorophyllin	0.671	0.025
Pranlukast	0.670	0.150
LY223982	0.661	0.087
Clofoctol	0.659	0.103
MK591 (free acid)	0.659	0.049
GDC-0623	0.654	0.015
ciglitazone	0.651	0.032
Tipranavir	0.649	0.041
GDC-0810	0.643	0.123
A-317491	0.642	0.055
4E1RCat	0.641	0.000
dihomo-gamma-linolenic-acid	0.637	0.045
tetradecylthioacetic-acid	0.635	0.161
SC-9	0.627	0.133
6,8-Bis(benzylthio)octanoic acid	0.627	0.180
Bardoxolone (methyl)	0.622	0.044
AGN 194310	0.621	0.020
A-769662	0.621	0.002
Estramustine (phosphate sodium)	0.620	0.034
rac-BHFF	0.613	0.081
LY225910	0.610	0.033
Ceforanide	0.609	0.008
Mitotane	0.606	0.154
Fenticonazole (Nitrate)	0.604	0.005
TY-52156	0.602	0.038
PYR-41	0.597	0.065
DMPS	0.591	0.002
L-655240	0.589	0.116
Trilostane	0.588	0.025
NGB-2904	0.586	0.002
CGK733	0.585	0.012
Clinofibrate	0.585	0.191
Meclocycline sulfosalicylate	0.582	0.014
SU-16f	0.577	0.018
Tiratricol, 3,3',5-triiodothyroacetic acid	0.574	0.070
AM-24	0.574	0.114
m-3M3FBS	0.571	0.015
Triclosan	0.570	0.034
AM095 (free acid)	0.566	0.005
CL-225385	0.565	0.003

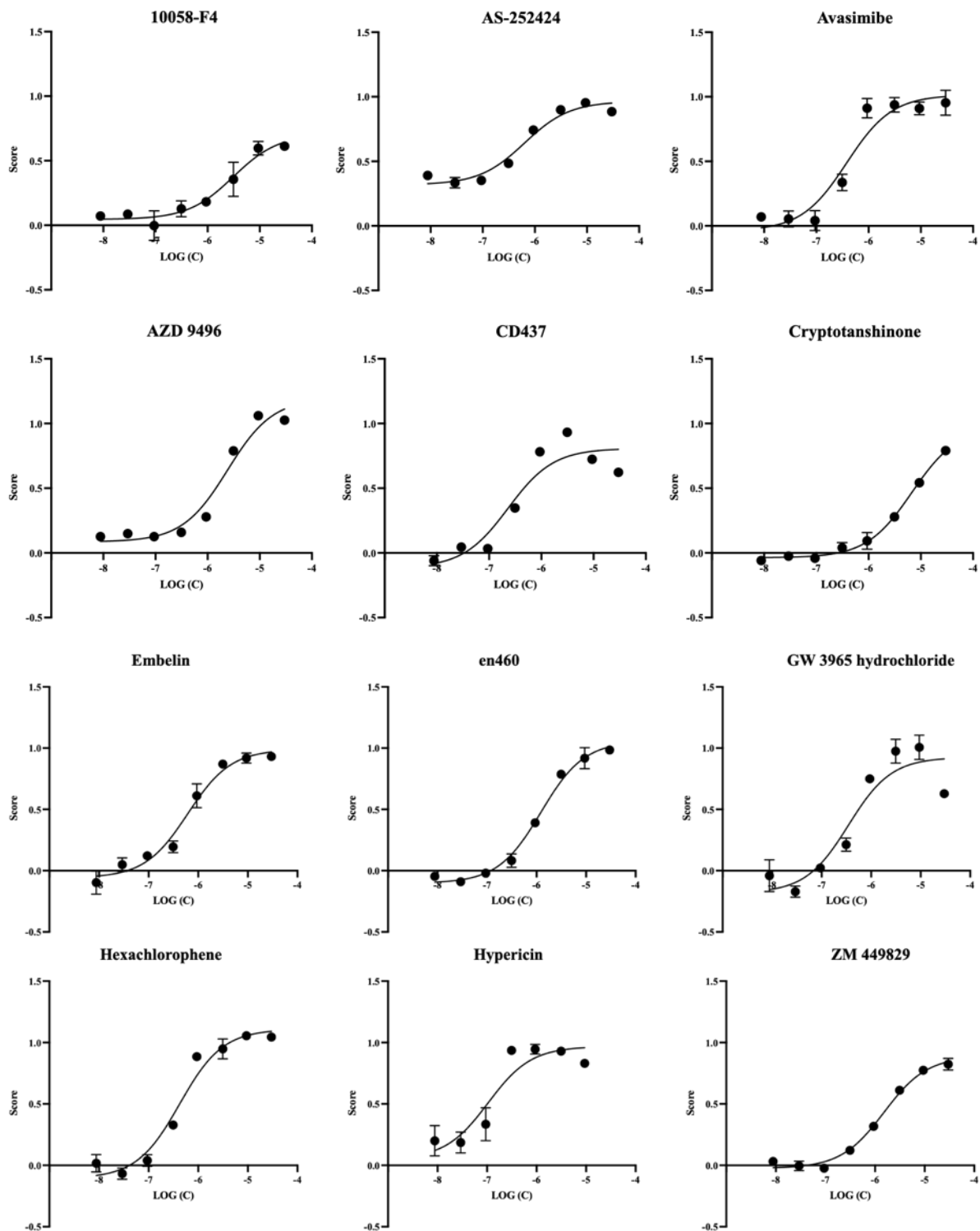
T-5224	0.564	0.087
almitrine	0.561	0.021
AVE 0991	0.560	0.190
AKBA	0.560	0.060
ESI-09	0.557	0.079
m-THP	0.553	0.077
Triclabendazole	0.551	0.091
GW0742	0.548	0.040
Kenpaullone	0.546	0.037
Nordihydroguaiaretic acid	0.544	0.030
AS-1949490	0.541	0.011
YM022	0.539	0.009
Benzbromarone	0.539	0.012
Lithocholic acid	0.536	0.046
CCG-63802	0.534	0.022
Bakuchiol	0.534	0.115
Troglitazone	0.532	0.043
Ascorbyl palmitate	0.531	0.011
T-62	0.531	0.042
A 922500	0.527	0.035
revaprazan (S enantiomer)	0.527	0.064
CGP-53353	0.525	0.014
ONO-8130	0.524	0.120
Salubrial	0.521	0.001
Ligustilide	0.517	0.003
SR-2640	0.516	0.083
Saroglitazar	0.514	0.008
trans-10,cis-12-Conjugated-linoleic-acid	0.513	0.003
G-1	0.508	0.037
SCR-1481B1	0.508	0.044
HA14-1	0.505	0.096
GW9508	0.505	0.115
VU0238429	0.504	0.064
Magnolol	0.504	0.090
PPT	0.493	0.129
TBB	0.492	0.072
DPI-201106	0.490	0.062
AGN 195183	0.488	0.005
ertapenem	0.484	0.121
Tyrphostin AG 879	0.484	0.051
Bevirimat	0.484	0.015
NVP 231	0.481	0.062
Ivacaftor	0.478	0.022
Haloproglin	0.478	0.179
pyrintegrin	0.478	0.093
arcyriaflavin-a	0.475	0.045
SB 216763	0.473	0.065
JZL 184	0.472	0.001
Sobetirome	0.470	0.110
SR1078	0.467	0.080
GKT137831	0.466	0.065

Regorafenib	0.466	0.145
Meclozine dihydrochloride	0.460	0.003
ZK-164015	0.459	0.150
Honokiol	0.459	0.138
DBeQ	0.459	0.100
CaCCinh-A01	0.457	0.178
ZAPA	0.456	0.143
PD-407824	0.455	0.084
Oxyclozanide	0.454	0.021
CGP-71683	0.453	0.011
Oxyphenbutazone	0.453	0.189
Linifanib	0.449	0.044
Suramin (sodium salt)	0.448	0.183
MGCD-265 analog	0.445	0.099
EO-1428	0.442	0.053
edaglitazone	0.441	0.028
PSNCBAM-1	0.438	0.114
Cot inhibitor-2	0.438	0.093
RSV604	0.436	0.138
Methacycline hydrochloride	0.434	0.090
PFK-158	0.432	0.010
NS-5806	0.431	0.043
SB-366791	0.430	0.062
TRAM-39	0.429	0.099
Adarotene	0.428	0.046
PD-81723	0.428	0.036
TC-I-2014	0.427	0.144
PKC412	0.424	0.022
Oxytetracycline dihydrate	0.420	0.026
PFK-015	0.420	0.039
SJ-172550	0.419	0.100
Sanguinarine (chloride)	0.416	0.058
3,3'-Diindolylmethane	0.416	0.064
GW7647	0.416	0.031
Dapivirine	0.414	0.029
GP2a	0.411	0.025
NS1643	0.402	0.111
AS-604850	0.402	0.025
2-TEDC	0.401	0.029
Sulindac	0.400	0.022
iCRT 14	0.399	0.006
Inolitazone (dihydrochloride)	0.395	0.064
ST-1859	0.395	0.062
A-784168	0.395	0.046
BTT-3033	0.393	0.018
SCH-221510	0.392	0.053
StemRegenin 1	0.392	0.089
Evatanepag	0.391	0.003
Estradiol Valerate	0.389	0.038
(+/-)-SLV319	0.389	0.037
Bexarotene	0.387	0.023

AZ-10417808	0.386	0.021
3'-fluorobenzylpiperone	0.385	0.060
GSK1059615	0.384	0.023
difenpiramide	0.379	0.079
Costunolide	0.377	0.044
Corosolic acid	0.373	0.099
CAY10505	0.371	0.076
CIM-0216	0.370	0.147
Dihydromyricetin	0.368	0.123
compound-w	0.368	0.014
Lixivaptan	0.366	0.055
GSK2190915	0.366	0.055
3PO	0.365	0.007
PD-198306	0.365	0.042
SB225002	0.364	0.133
BMS-309403	0.364	0.058
(-)-Epicatechin gallate	0.363	0.051
TWS119	0.359	0.107
PF-04418948	0.356	0.123
GW 5074	0.355	0.059
Diacerein	0.355	0.084
KB-SRC-4	0.354	0.134
Tivantinib	0.353	0.077
Dichlorophen	0.353	0.041
enoxolone	0.353	0.101
CMPD-1	0.352	0.020
JSH-23	0.349	0.036
T0901317	0.348	0.033
GSK9027	0.347	0.023
MRS3777	0.346	0.038
LE-135	0.345	0.056
TSU-68	0.345	0.033
L-168049	0.344	0.052
Licochalcone A	0.344	0.035
SANT-2	0.344	0.060
PLX8394	0.344	0.028
Tanshinone IIA	0.344	0.051
AGI-6780	0.343	0.058
VX-809	0.343	0.009
4BP-TQS	0.341	0.016
THZ1	0.339	0.010
N-benzylinaltrindole	0.338	0.086
Lu AE58054 (Hydrochloride)	0.335	0.011
Quercetin	0.335	0.070
Vilazodone	0.335	0.017
THZ2	0.334	0.002
L755507	0.333	0.058
P7C3	0.326	0.010
Ro 61-8048	0.323	0.052
cefodizime	0.322	0.145
xaliproden	0.321	0.031

cefminox	0.320	0.017
ICA-121431	0.320	0.043
lestaurtinib	0.317	0.009
Lonidamine	0.316	0.025
EPPTB	0.315	0.080
FPL-55712	0.315	0.019
cefalonium	0.314	0.018
BAY 43-9006	0.313	0.001
RI-1	0.311	0.075
rolitetraacycline	0.311	0.006
YM-750	0.309	0.070
NT157	0.309	0.057
fanetizole	0.308	0.037
ML277	0.307	0.055
D-(+)-maltose	0.307	0.011
Curcumin	0.304	0.073
SP-141	0.303	0.072
carboxyamidotriazole	0.302	0.054
SU-4312	0.299	0.027
cefcapene-pivoxil	0.298	0.015
Phenylbutazone	0.298	0.029
INH1	0.298	0.023
captan	0.298	0.048
CID-5458317	0.297	0.011
CID 16020046	0.297	0.018
VRT752271	0.295	0.055
fananserine	0.294	0.060
PF-562271	0.291	0.015
UK-5099	0.284	0.030
ML-193	0.282	0.037
PR-619	0.277	0.025
KG-5	0.276	0.027
URMC-099	0.274	0.038
PI4KIIbeta-IN-10	0.268	0.050
Etravirine	0.262	0.067
G-15	0.262	0.011
SEN-1269	0.262	0.042
JLK-6	0.260	0.059
L-741742	0.251	0.043
GW-1929	0.248	0.026
bemesetron	0.247	0.004
ML-298	0.247	0.058
S-Trityl-L-cysteine	0.246	0.002
Chlorindione	0.244	0.018
AG-556	0.242	0.006
Cefazolin sodium salt	0.241	0.040
CCT018159	0.233	0.068
1-azakenpaullone	0.229	0.029
SMI-4a	0.226	0.017
PNU-89843	0.219	0.033
ammonium-glycyrrhizinate	0.215	0.050

SB-452533	0.214	0.018
CRT0044876	0.212	0.010
flindokalner	0.211	0.031
IPAG	0.206	0.055
4-CMTB	0.199	0.036
MDL-11939	0.198	0.033



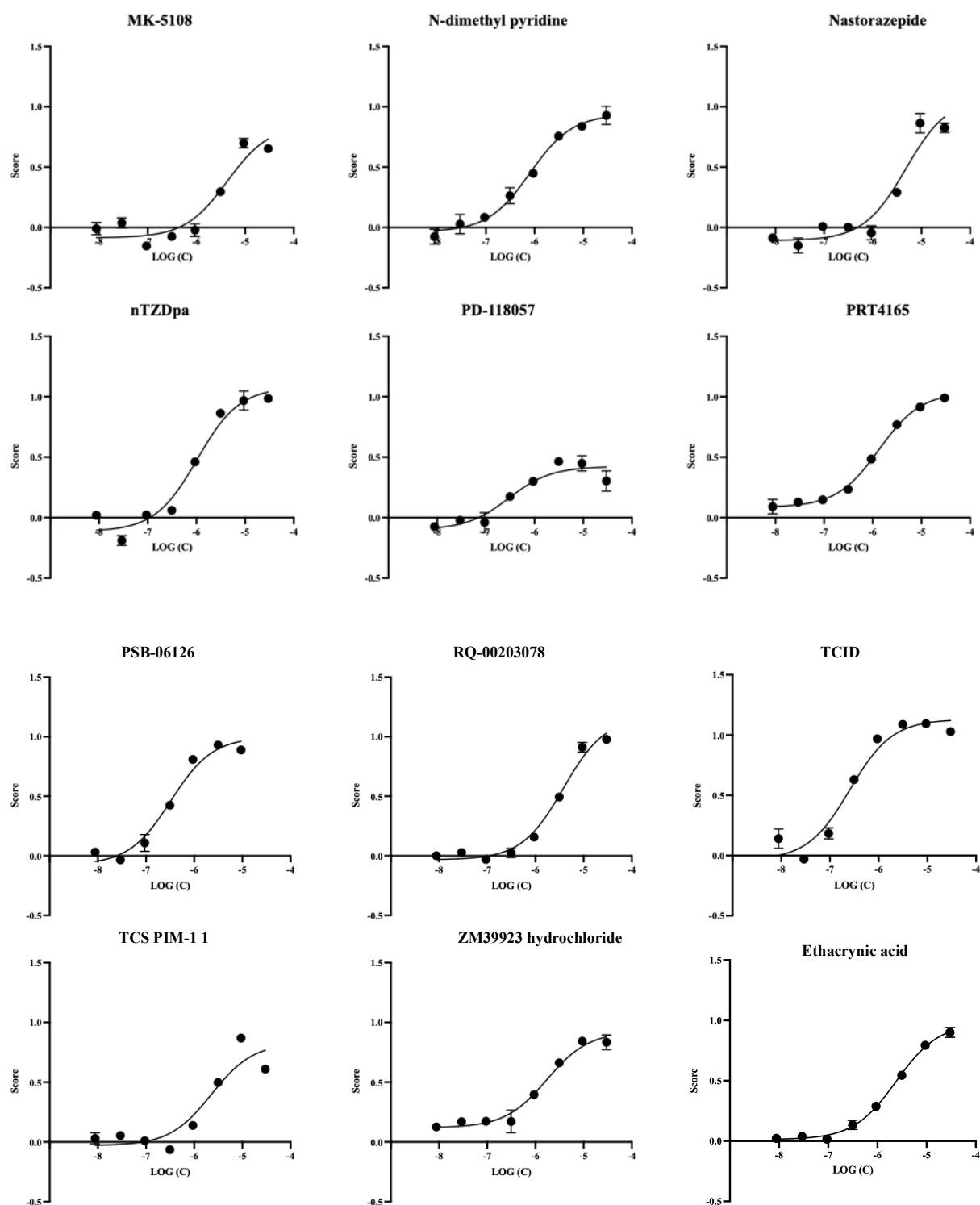
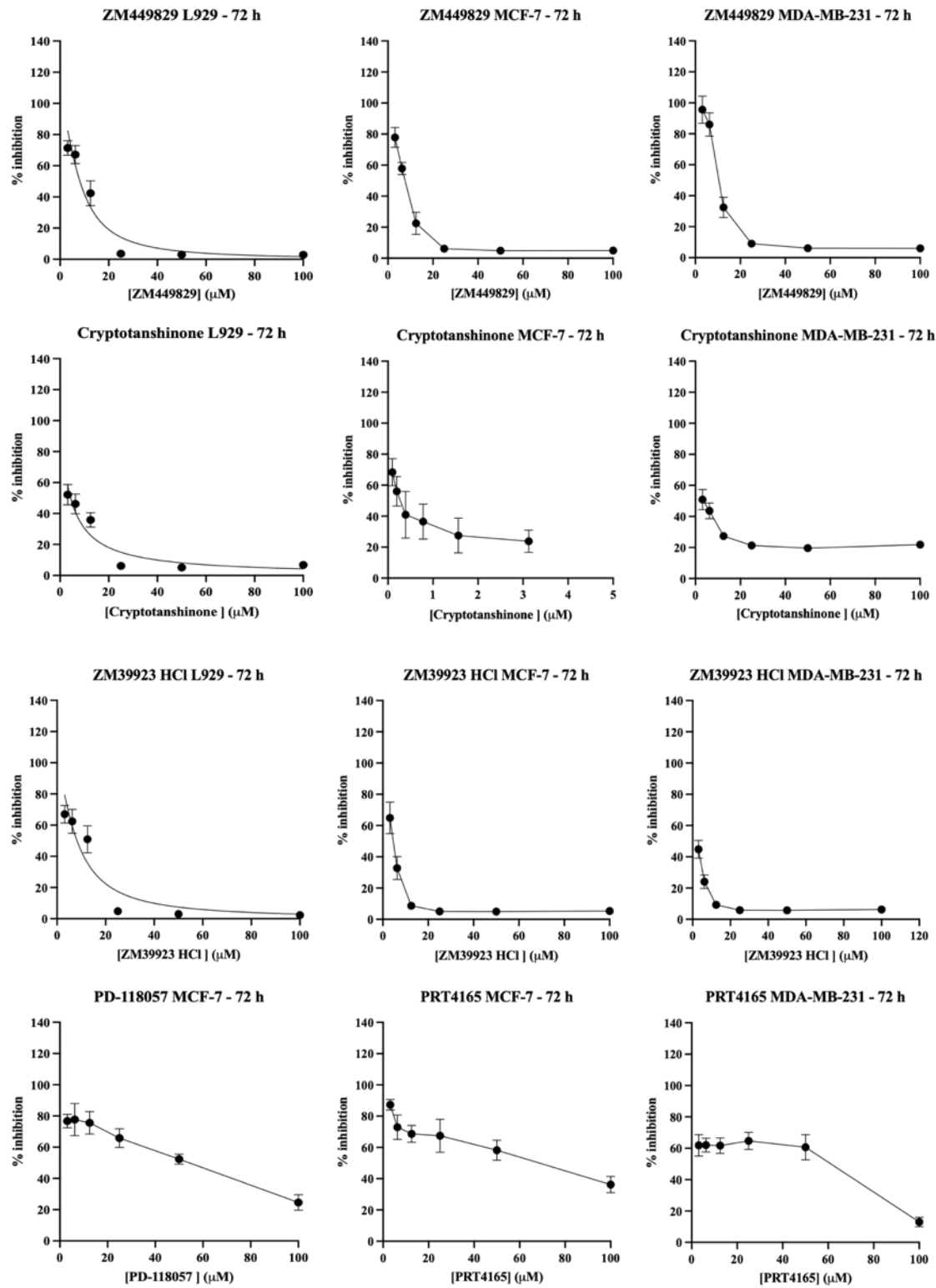
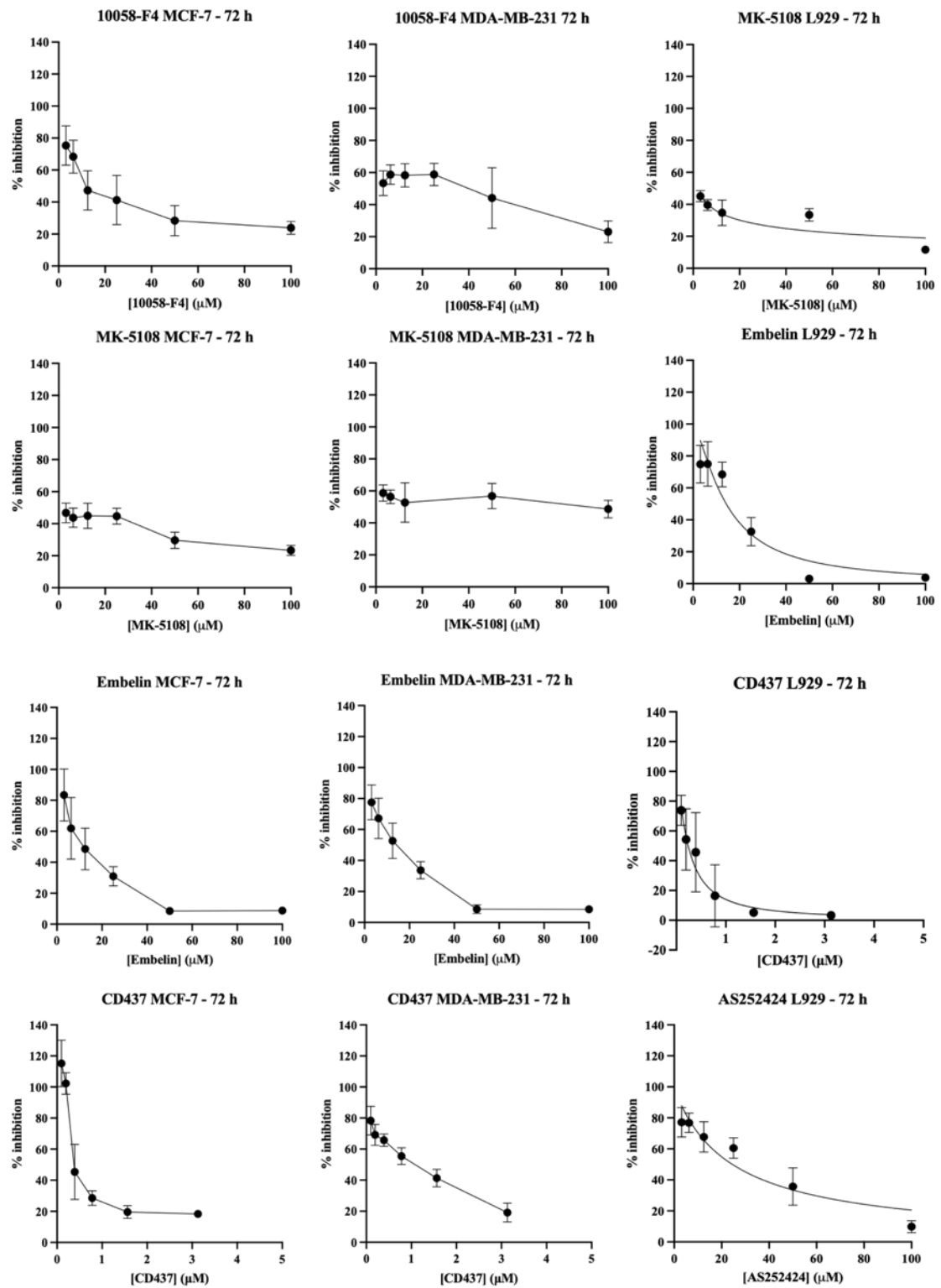


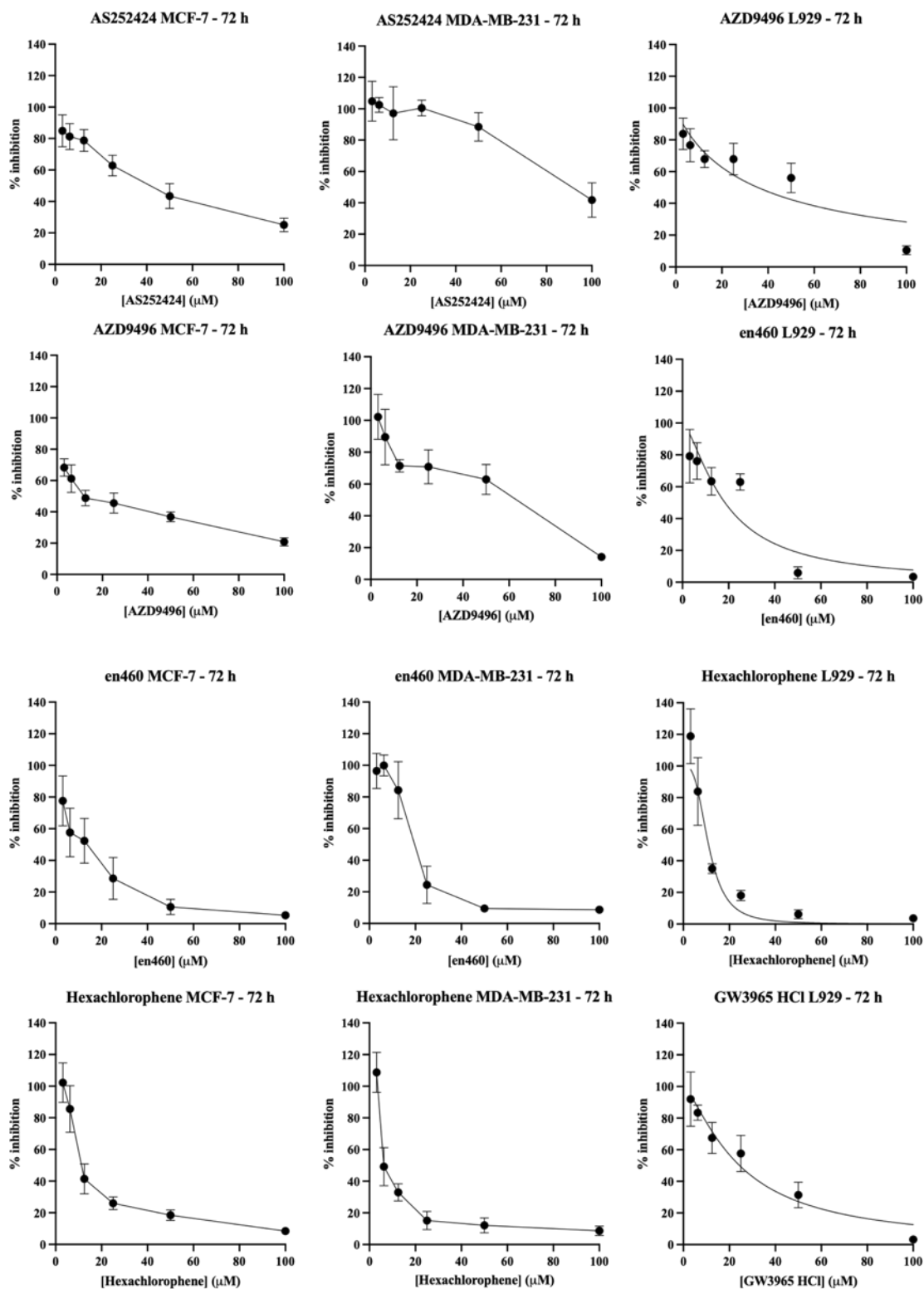
Figure S4 – Dose-response curves of 25 compounds HTS reconfirmation assay.

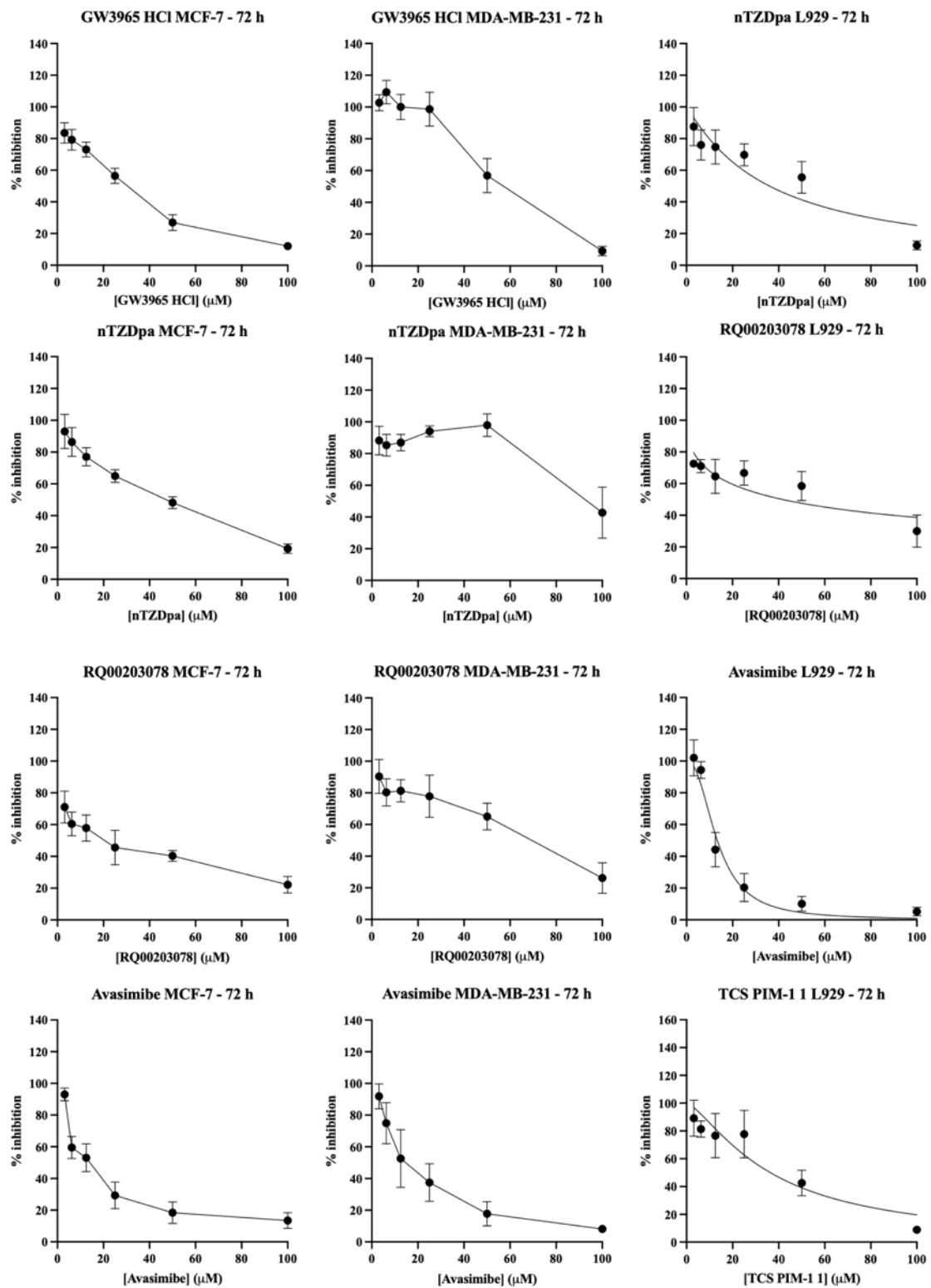
Table S2 – Hill slope and IC_{50} values obtained in the reconfirmation assay for the 24 compounds selected.

Compounds	Hill Slope (dose-response curve)	IC_{50} (μ M) GSTP1
TCID	1.16	0.9
GW3965 (hydrochloride)	2.48	0.43
5-Methyl-2-phenyl-1,2-dihydropyrazol-3-one, 98%	0.8	0.83
PSB-06126	3.4	0.29
Nastorazepide	10.6	3.3
CD437	11.6	0.32
Embelin	1.46	0.64
MK-5108	13.6	3.2
nTZDpa	1.7	0.95
AS-252424	1.95	0.61
PRT4165	1.2	1.3
PD-118057	1.8	0.33
en460	1.4	1.1
Avasimibe	4.2	0.37
ZM449829	1.3	1.4
TCS PIM-1 1	2.5	2.15
AZD9496	2.23	2.05
ZM39923 (hydrochloride)	1.56	1.5
10058-F4	1.37	2.75
Hypericin	11.6	0.1
RQ-00203078	1.6	3.1
Hexachlorophene	2.2	0.42
Cryptotanshinone	0.85	8.9
Ethacrynic acid	1.01	2.4









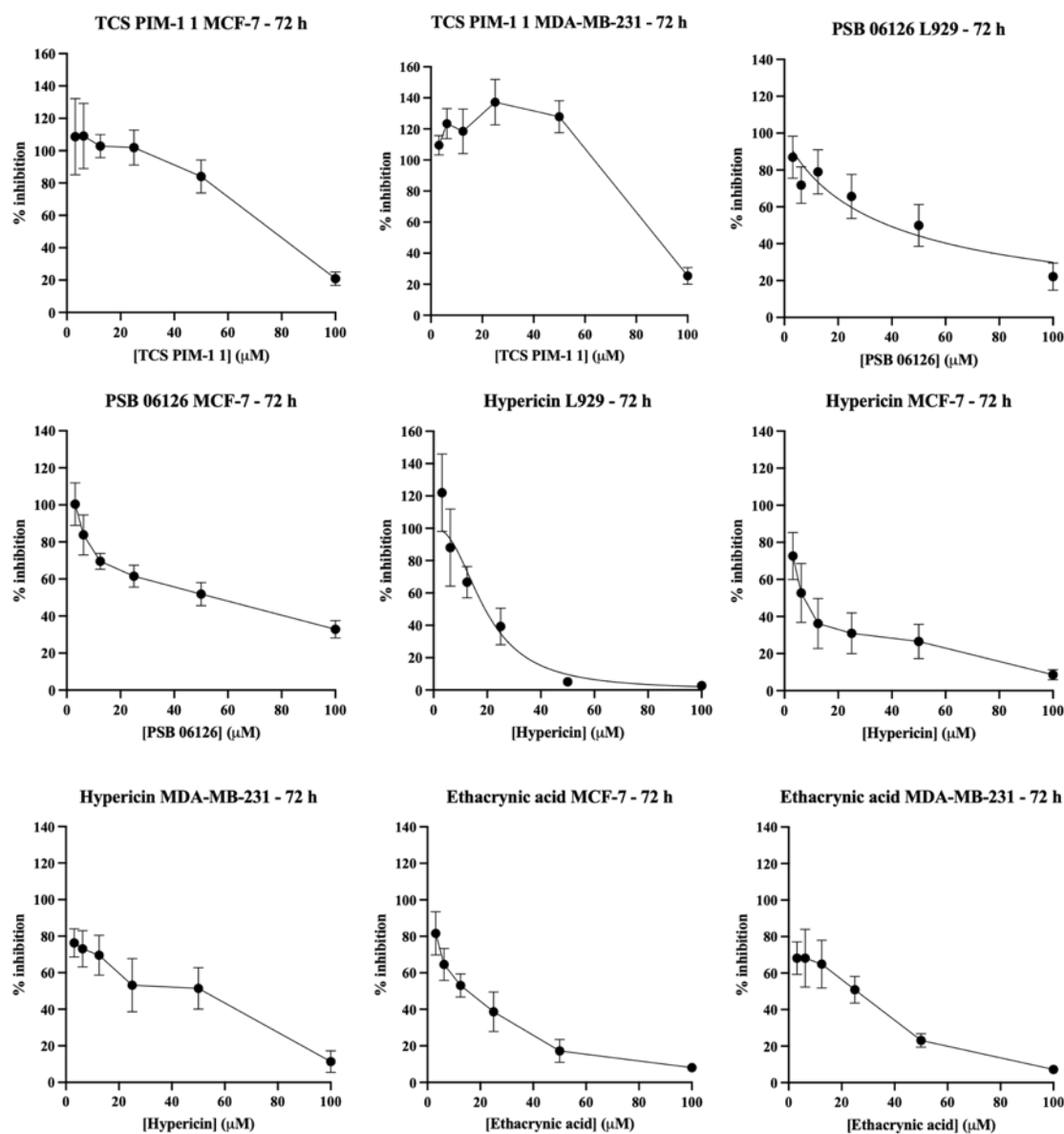


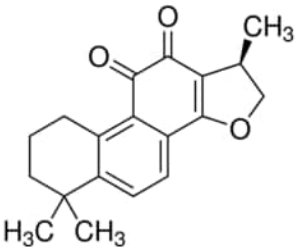
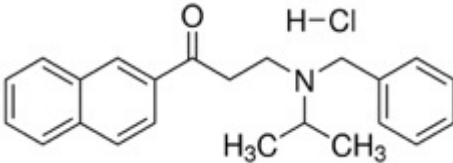
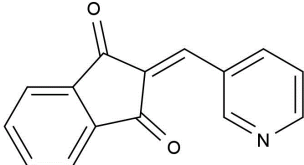
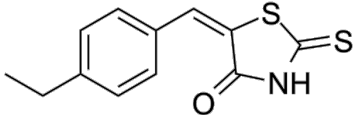
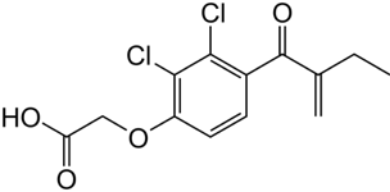
Figure S5 – Dose-response curves of 24 compounds tested in vitro for L929, MCF-7, and MDA-MB-231 cell lines for 72 h.

Table S3 – Statistical differences between the three cell lines tested.

Compounds	Statistical differences		
	L929/MCF-7	L929/MDA-MB-231	MCF-7/MDA-MB-231
ZM 449829	∅	*	***
Cryptotanshinone 97.0%	***	∅	*
TCID	∅	∅	∅
ZM 39923 (hydrochloride)	**	****	∅
PD-118057	****	∅	****
PRT 4165	****	****	****
5-Methyl-2-phenyl-1,2-dihydropyrazol-3-one, 98%	∅	∅	∅
10058-F4	****	****	∅
MK-5108	∅	****	****
Embelin	**	∅	∅
Nastorazepide	∅	∅	∅
CD437	∅	∅	∅
AS252424	****	****	****
AZD9496	****	****	****
en460	****	∅	****
Hexachlorophene	**	∅	****
GW3965 (hydrochloride)	∅	****	****
nTZDpa	**	****	****
RQ00203078	****	****	****
Avasimibe	∅	**	****
TCS PIM-1 1	****	****	****
PSB 06126	****	****	****
Hypericin	****	****	****
Ethacrynic acid	****	****	∅

∅ - no statistical difference; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

Table S4 – Chemical structures of the 5 most cytotoxic compounds obtained *in vitro*.

Compounds/Mechanism of action	Chemical structures
Cryptotanshinone 97 % Inhibits cell proliferation in a variety of cancer cell lines, including rhabdomyosarcoma, melanoma, cervical, colon, breast, and prostate cancer cells.	
ZM 39923 HCl A potent and selective inhibitor of Janus tyrosine kinase 3.	
PRT 4165 A potent inhibitor of PRC1-mediated H2A ubiquitylation <i>in vivo</i> and <i>in vitro</i> .	
10058-F4 Induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia.	
Ethacrynic acid Inhibition of the activity of the Na ⁺ -K ⁺ -2Cl ⁻ symporter in the thick ascending limb of the loop of Henle. Reference inhibitor of GST P1 enzyme.	

Chapter V

Conclusions and future perspectives

In this thesis, organometallic compounds, especially ruthenium-based compounds, and two compound libraries were tested, and their effect was evaluated in enzymatic biomarkers and *in vitro* breast cancer cells, to better understand their potential to be applied as anticancer drugs. With the two different reviews (**Chapter II**) performed, the knowledge about automatic and microfluidic techniques was deepened as well as about the type of metabolic changes and enzymatic biomarkers in the cancer microenvironment. 2D and 3D cell-based assays proved to be valuable to evaluate enzymatic biomarkers. Some of these were then considered in different works of this thesis successfully.

In order to achieve the first goal of this thesis, small libraries of organometallic compounds were tested against cyclooxygenase-2 (COX-2) (**Chapter IV – work 1**), dihydrofolate reductase (DHFR) (**Chapter IV – work 2**) and glutathione S-transferase P1 (GST P1) (**Chapter IV – work 3**) using automatic methodologies based in sequential injection analysis (SIA) (**work 3**) and sequential injection coupled to lab-on-valve (μ SI-LOV) (**work 1 and 2**). For each work, the automatic methodologies were optimized according to the enzymatic features of each reaction and enabled to obtain the better analytical signal and the most accurate and precise results (RSD between 2.5 - 7%). The inhibitory capacity of the compounds was evaluated by calculating the half-maximal inhibitory concentration (IC_{50}), ranging between $6.7 \pm 0.7 \mu\text{M}$ and $275 \pm 9 \mu\text{M}$. All the results obtained by the analysis of COX-2, DHFR, and GST P1 inhibitory activity in works **1, 2, and 3**, respectively, provide significant information about the mechanism of the studied compounds, mostly ruthenium-based compounds, and the role of these enzymes in their mode of action. Comparing the results obtained in these three automatic methods, RAPTA-B is a more potent inhibitor against COX-2 and GST P1. RAPTA-T presents 1.4 and 2 times more inhibitory activity against COX-2 than GST P1 and DHFR, respectively. RAPTA-C presents a similar inhibition profile for COX-2 and GST P1. Oxali-RAPTA-C presents approximately 2 times higher inhibitory activity against DHFR than COX-2 and GST P1. Moreover, for the iron compounds tested in DHFR and GST P1 enzymes, the inhibitory effect is between 3 to 28 times more inhibitory for DHFR than GST P1, being in one case (compound **6b** in **Chapter IV - work 2**) 127 times more inhibitory. Furthermore, all these methodologies were validated using a batch procedure proving the agreement between the LOV and batch methods since there are no statistical differences between both techniques. **Works 1 and 2** confirmed the potential of the μ SIA-LOV system as a simple, versatile, robust, and rapid analytical tool for automating the determination of IC_{50}

values of metal-based compounds. In **work 3**, it was developed an SIA system and some of the compounds tested exhibited good inhibition profiles with the low μM range of IC_{50} values and were comparable to the benchmark organic inhibitor, ethacrynic acid.

After these works, newly ruthenium-based compounds were synthesized and were tested *in vitro* to evaluate their cytotoxicity in luminal and triple-negative breast cancer cell lines (MCF-7 and MDA-MB-231) for 24 h and 72 h (**Chapter IV - work 4**). All the compounds were also evaluated against a non-tumorigenic cell line – L929, used as a reference for the safety evaluation according to ISO 10993-1:2009 on “Biological evaluation of medical devices”. According to these results, compounds **10** and **11CA** showed to be the most cytotoxic and selective compounds. Moreover, cell uptake assay was also implemented for two of the **11CA** compounds, the most lipophilic $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]\text{BPh}_4$ (**11BPh₄**) and the most lipophobic $[\text{Ru}(\eta^6\text{-toluene})(\text{PPh}_3)_2\text{Cl}]\text{Cl}$ (**11Cl**), and compound **11Cl** showed significant cellular uptake compared with **11BPh₄**. An apoptosis assay was also performed for all **11CA** compounds, and the results suggested that **11Cl** might induce cell death by a non-apoptotic mechanism, which could be interesting in the treatment of apoptosis-resistant cancers. Considering the results obtained in the interaction of **11Cl** with DNA, it was observed a minor groove interaction with ctDNA. Also, the interaction with BSA was established by fluorescence measurements and the interaction mode is similar to ctDNA.

According to the last proposed goal of this thesis, it was also developed and optimized an HTS 384-well plate assay to evaluate approximately 5'830 compounds with or without anticancer activity (**Chapter IV - work 5**). The 24 most inhibitory compounds against GST P1 enzyme were selected according to the hill slopes and the IC_{50} values obtained. These 24 compounds were also evaluated *in vitro* to assess their cytotoxicity in the previous breast cancer cell lines tested (MCF-7 and MDA-MB-231) and the same non-tumorigenic cell line (L929). From these 24 compounds, 5 proved to be more cytotoxic for breast cancer cell lines than for the non-tumorigenic cell line (**Ethacrynic acid**, **Cryptotanshinone**, **PRT 4165**, **ZM 39923**, and **10058-F4**). A western blot assay was also performed to evaluate the expression of GST P1 in both breast cancer cell lines and also the effect of a few compounds tested *in vitro* in its expression. According to the results, MDA-MB-231 expressed GST P1 protein while MCF-7 does not express this protein. In agreement with the effect of the expression by the compounds tested (**Ethacrynic acid**, **ZM 39923**, and **PRT 4165**), it was possible to notice a decrease in GST P1 expression in approx. 2-fold. Finally, an experiment was performed to identify the type of inhibition in the presence of the GST P1 enzyme of the 5 most cytotoxic compounds obtained in the *in vitro* cytotoxic assay. **Ethacrynic acid** and **ZM 39923** were identified as competitive GST

P1 inhibitors, and **PRT 4165**, **10058-F4**, and **Cryptotanshinone** were recognized as uncompetitive GST P1 inhibitors. Furthermore, combinations of these compounds were tested and improvements in cytotoxicity in breast cancer cell lines were achieved compared to each compound alone. Just the combination of **ZM 39923** with **10058-F4** in the MCF-7 cell line does not improve the cytotoxicity. On the other hand, it was possible an increase in the cytotoxicity mostly of 2-fold and 4-fold with the other combinations, and in one case (combination of **Ethacrynic acid** with **Cryptotanshinone** in the MDA-MB-231 cell lines), it was obtained an increase in cytotoxicity of 8-fold.

To conclude and from a future perspective, the highlighted compounds of this thesis, such as the ruthenium-based compound intensively studied in **work 4** and the most cytotoxic compounds obtained in the HTS assay (**work 5**), should be applied to *in vivo* and clinical studies to evaluate their safety and effectiveness of these experimental drugs in humans. With some optimism, it could be the future treatment against breast cancer.