

DISSERTAÇÃO DE MESTRADO

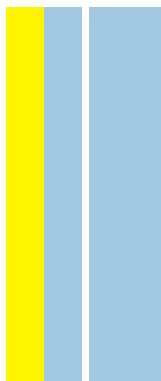
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Bioactive compounds from seaweed with in vitro anti-leukemia activity

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**BIOACTIVE COMPOUNDS FROM SEAWEED
WITH IN VITRO ANTI-LEUKEMIA ACTIVITY**

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ABSTRACT

Nowadays, cancer is a major, worldwide problem, affecting both developed and developing countries. Despite the incredible amount of research spanning this field, the statistics on cancer are not optimistic. Leukemia is a type of cancer with a worldwide incidence of 2.5% and a mortality rate of 3.4% (out of all cancers). Chronic myeloid leukemia (CML) represents 15 - 20% of all new cases of leukemia and is characterized by an uncontrolled proliferation of myeloid cells. Currently, the first-line of treatment involves tyrosine kinase inhibitors (TKI), which act in a specific way to inhibit the activity of BCR-ABL. However, resistance, mainly due to mutations, can occur. When the disease reaches a more advanced stage – the blast crisis – more aggressive chemotherapeutics are applied, such as anthracyclines. Doxorubicin is an example that is used in several types of cancers, including leukemia, yet the action of this drug can also cause resistance and other important issues such as non-targeted cytotoxicity. In the attempt to find more effective and less toxic therapies, two in vitro approaches were explored in this work: (i) the study of the anticancer activity of natural compounds; and (ii) the (less explored) improvement of current therapy combined with natural compounds.

Marine organisms are a rich source of bioactive compounds with diverse biological activities. Several compounds isolated from seaweed, namely carotenoids and phlorotannins present anticancer activity against numerous cancer cell lines. In the current work, the in vitro anticancer activity of fucoxanthin and phloroglucinol alone and co-incubated with anticancer drugs (imatinib and doxorubicin) was assessed on two human cancer cell lines (K562 and TK6) derived from blast crisis of CML. For this (i) cytotoxicity at short time; (ii) proliferation capability; (iii) induction of DNA damage; and (iv) induction of cell death by apoptosis, were evaluated. Our results showed that doxorubicin was more cytotoxic than imatinib in both cell lines. Both anticancer drugs decreased cell proliferation in a dose-dependent manner in K562 cell line without induction of DNA damage or apoptosis. Proliferation inhibition of TK6 cell line induced by doxorubicin was accompanied by an increase of DNA damage and apoptosis. Furthermore, fucoxanthin decreased cell proliferation without effects on DNA damage or apoptosis in both cell lines. When co-incubated with the anticancer drugs the inhibition was not improved when compared with fucoxanthin alone for both cell lines. Phloroglucinol did not show cytotoxic effect at short time in both cell lines, however, in TK6 cells, phloroglucinol alone and co-incubated with imatinib inhibited cell proliferation, but without induction of DNA damage and apoptosis.

This work demonstrated that both the natural compounds exhibit antiproliferative effects against K562 (fucoxanthin) and TK6 (fucoxanthin and phloroglucinol) cell lines. When co-incubated with the drugs (doxorubicin and imatinib) their efficacy was enhanced in certain conditions. The findings warrant more studies to understand the mechanisms of action involved in the antiproliferative effects of fucoxanthin and phloroglucinol in the cell lines tested. This knowledge could be interesting to explore the natural compounds as new adjuvant strategy for the treatment of cancer cells that share the same molecular characteristics that the cell lines evaluated in this work.

RESUMO

Atualmente o cancro é um grande problema ao nível global, afetando tanto países em desenvolvimento bem como desenvolvidos. Apesar da incrível quantidade de pesquisa neste campo os dados estatísticos existentes são pouco otimistas. Em particular, a leucemia é um cancro com uma incidência de 2.5% e um índice de mortalidade de 3.4% em todo o mundo. Leucemia mielóide crónica representa entre 15 – 20% de todos os tipos de leucemia e é caracterizada pela proliferação descontrolada de células mielóides. Atualmente a primeira linha de tratamento para esta doença são os inibidores de tirosina quinase, estes atuam de uma forma bastante específica inibindo a atividade do BCR-ABL, contudo pode ocorrer o aparecimento de resistências devido ao desenvolvimento de mutações. Quando a doença atinge um estado mais avançado – a fase ou crise blástica - quimioterapêuticos mais agressivos são aplicados, como é o exemplo dos antraciclinas. Doxorrubicina é uma antraciclina muito usada para tratar vários tipos de cancro, incluindo a leucemia, contudo a ação desta droga tem vindo a ser relacionada com o aparecimento de resistência e também a sua ação não específica torna-a tóxica para outras células (células normais). De maneira a tornar os tratamentos mais efetivos e menos tóxicos, duas abordagens *in vitro* foram exploradas neste trabalho: (i) o estudo da atividade anticancerígena dos compostos naturais; e (ii) o (menos estudado) melhoramento dos atuais tratamentos utilizados através da combinação com compostos naturais.

Os organismos marinhos são uma fonte de compostos bioativos com diversas atividades biológicas. Alguns compostos isolados das algas, nomeadamente carotenóides e florotaninas, apresentam atividade anticancerígena contra diversas linhas celulares de cancro. No presente trabalho, a atividade anticancerígena *in vitro* da fucoxantina e floroglucinol sozinhos ou co-incubados com os fármacos anticancerígenos (imatinib e doxorrubicina) foi avaliada em duas linhas celulares cancerígenas (linha celular K562 e TK6) provenientes da fase blástica da leucemia crónica mielóide. Para isso, (i) a citotoxicidade de curta exposição; (ii) a capacidade de proliferação; (iii) a indução de danos no ADN; e (iv) a indução de morte celular por apoptose foram avaliados. Os nossos resultados mostraram que a doxorrubicina foi mais citotóxica do que o imatinib para as duas linhagens celulares. Ambos os fármacos, imatinib e doxorrubicina, diminuem a proliferação celular de uma forma dose-dependente nas K562 sem indução de danos no ADN e apoptose. A inibição da proliferação induzida pela doxorrubicina nas TK6 foi acompanhada por um aumento dos danos no ADN e da apoptose. Além disso, fucoxantina demonstrou uma redução da proliferação celular sem efeitos ao nível

dos danos no ADN ou na apoptose para ambas as linhas celulares. Quando co-incubada com os fármacos a inibição não aumenta quando comparado com o composto sozinho. Floroglucinol não mostrou citotoxicidade para curtos períodos de exposição em ambas as linhas celulares, contudo nas TK6 floroglucinol sozinho ou em co-incubação com imatinib inibiu a proliferação celular, mas sem induzir danos ao nível do ADN e apoptose.

Com este trabalho demonstramos que ambos os compostos naturais exibem um efeito antiproliferativo contra as K562 (fucoxantina) e TK6 (fucoxantina e floroglucinol). Quando co-incubados com os fármacos (doxorrubicina e imatinib) a eficiência dos mesmos é melhorada em certas condições. Os achados justificam que se implementem mais estudos de modo a compreender quais os mecanismos de ação envolvidos nestes efeitos antiproliferativos da fucoxantina e do floroglucinol nas linhagens testadas. Poderá ser de interesse explorar este efeito de modo a poder-se utilizar estes compostos em novas estratégias como adjuvantes para o tratamento de células cancerígenas que apresentem características moleculares semelhantes às utilizadas neste estudo.

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ABBREVIATIONS

·OH – Hydroxyl radical

ABL1 – Abelson murine leukemia viral oncogene homolog 1

AGP – Alpha-1-acid glycoprotein

ALL - Acute lymphoid leukemia

AP – Accelerated phase

ATCC - American type culture collection

ATL - Adult T cell leukemia

ATP – Adenosine triphosphate

BC – Blast crisis

BCR – Breakpoint cluster region

CBC – Complete blood count

CCyR – Complete cytogenetic response

Cdk – Cyclin-dependent kinases

CHF – Congestive heart failure

CML – Chronic myeloid leukemia

CP – Chronic phase

DAPI – 4',6-diamidino-2-phenylindole

DISC – Death inducing signaling complex

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

Dox – Doxorubicin

EDTA – Ethylenediaminetetraacetic acid

FBS – Fetal bovine serum

FGF-2 – Fibroblast growth factor 2

FGFR-1 – Fibroblast growth factor receptor 1

FPG - Formamidopyrimidine-DNA glycosylase

Fx – Fucoxanthin

GIST – Gastrointestinal stromal tumors

H₂O₂ – Hydrogen peroxide

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HL-60 – Human promyelocytic leukemia cell line

hOCT1 – Human organic cation transporter 1

HSCT – Hematopoietic stem cell transplantation

HTLV-1 – Human T-cell leukemia virus type 1

IARC – International agency for research on cancer

IC₅₀ – Half maximal inhibitory concentration

IFN- α – Interferon- α

Imat - Imatinib

JNK – c-Jun N-terminal kinase

K562 – Human erythromyeloblastoid leukemia cell line

LMP – Low melting point agarose

MDR – Multidrug resistance

MKK4 – Mitogen-activated protein kinase kinase-4

MMP – Mitochondrial membrane potential

NF- κ B – Nuclear factor kappa B

NMP – Normal melting point agarose

O₂^{·-} – Superoxide anion

P-loop – Phosphate-binding loop

P-gp – P-glycoprotein

PARP – Poly (ADP-ribose) polymerase

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

Ph – Phloroglucinol

PP2A – Protein phosphatase 2A

RanGAP1 – RanGTPase activating protein 1

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute Medium

RSG - Relative suspension growth

SAHA – Suberoylanilide hydroxamic acid

SCGE - Single-cell gel electrophoresis

STI-571 – Imatinib mesylate

TK6 – Human lymphoblastoid cell line, derived from blast crisis of CML

TKD – Tyrosine kinase domain

TKI – Tyrosine kinase inhibitor

TNF – Tumor necrosis factor

TSG – Total suspension growth

UV – Ultraviolet

LIST OF PUBLICATIONS AND COMMUNICATIONS

The elaboration of this Master thesis, and collaboration with other ongoing related works rendered data sets that were included both in presentations in international meetings, conferences, and in papers, as is follows mentioned:

1. Articles under submission or to be submitted to international peer-reviewed journals
 - **Almeida, T.**, Ferreira, J., Azqueta, A., Rocha E., Ramos A.A. (2016) Bioactive compounds from seaweed with anti-leukemia activity: Carotenoids and phlorotannins – a mini-review. *To be submitted*
 - **Almeida, T.**, Ferreira, J., Azqueta, A., Rocha E., Ramos A.A. (2016) Bioactivity of fucoxanthin and phloroglucinol from seaweed with anti-leukemia activity. *To be submitted*
 - Ferreira, J., **Almeida, T.**, Azqueta, A., Rocha E., Ramos A.A. (2016) Anticancer activity against glioblastoma cell lines by compounds present in algae, alone in combination with anticancer drugs – a mini-review. *To be submitted*
 - Ferreira, J., **Almeida, T.**, Azqueta, A., Rocha E., Ramos A.A. (2016) Anticancer effects of fucoxanthin and phloroglucinol, alone and in combination with temozolomide, on the U251 and T98G glioblastoma cell lines. *To be submitted*

2. Communications in conferences
 - **Tânia Almeida**, J. Ferreira, V. Gomez-Rodriguez, E. Rocha, A. Ramos, A. Azqueta. Bioactive compounds from seaweed with anti-leukemia activity: Carotenoids and phlorotannins. ICOETOX | IBAMTOX 2016 (Poster).

CHAPTER I – GENERAL INTRODUCTION

1. Cancer

The term “cancer” originates from Greek physician Hippocrates, who noted the similarity between crabs and the cut surface of a solid tumor (Hajdu 2011). In medicine, the earliest report of cancerous diseases, refers to breast cancer, and dates back to, approximately, 3000 B.C. However, paleopathological findings show that tumors existed in animals long before men appeared on Earth (Hajdu 2011).

Our body is made up of trillions of living cells. Every cell has the ability to grow, divide and die in a well-ordered way. During the first years of a person’s life, the normal cells divide faster allowing the person to grow. Upon reaching adulthood, most cells divide only to replace worn-out or dying cells or to repair injuries. Normal cells become cancer cells as a result of alterations in cell DNA. If these alterations are not repaired and if they confer a survival advantage, the cell will not die as it should. Instead, the altered cells proliferate faster than normal cells and make new cells that the body does not need. Most types of cancer cells form a solid tumor (a mass), but in the case of leukemia, tumors are rare. In leukemia, neoplasia cells are mixed in blood and blood-forming organs (American Cancer Society, 2015).

1.1. Cancer statistics

According to worldwide GLOBOCAN estimates, produced by the International Agency for Research on Cancer (IARC) in 2012, there were 14.1 million new cases and 8.2 million deaths due to cancer (Ferlay et al. 2015). The overall data showed that lung and breast cancer are the most frequently diagnosed and caused the highest number of death in men and women, respectively in both developed and developing countries. In developed countries, colorectal and prostate cancer were also at the top of the list for the highest number of cases. The problem becomes more tragic in less developed countries where the cancer incidence has been increasing in the last decade and incurs lower survival rates, representing 57% of all cancer cases and 65% of cancer deaths worldwide (Sloan and Gelband 2007, Torre et al. 2015). Currently, cancer continues to be a worldwide killer disease, despite the large amount of research undertaken. By 2020, it is estimated that there will be a world population of 7.5 billion, with approximately 15 million of new cases of cancer and 12 million cancer deaths (Anand et al. 2008).

1.2. Risk factors

Several risk factors have been associated with the appearance of cancer. These include internal factors, which represent 5 - 10% of all cases (e.g. inherited mutations, hormones and immune conditions) and environmental/acquired factors, which represent 90 - 95% (e.g. smoking tobacco, alcohol consumption, lack of physical activity, bad eating habits, infectious agents, environmental pollution and radiation) (Figure 1) (Anand et al. 2008, Jemal et al. 2011).

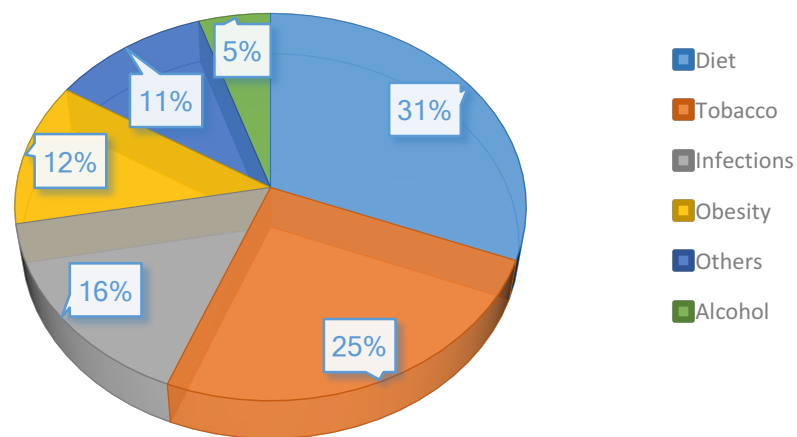


Figure 1 – The role of environmental factors in the development of cancer, with the percentage contribution of each factor. Adapted from Anand et al. (2008).

Several agents from environmental or cellular (endogenous) sources are able to induce DNA damage directly, or in an indirect way that may contribute to mutagenesis (Loeb and Loeb 2000, Helleday et al. 2014). As mentioned earlier, just 5-10% of all cancers are related with inherited gene defects, meaning that lifestyle factors have a great importance in cancer development. Changes in environmental and lifestyle factors seem a promising strategy for the prevention of cancer. There are some environmental factors that can promote the appearance of leukemia, such as viral infections, exposure to polycyclic aromatic hydrocarbons, pesticides, chlorinated drinking water, the presence also of nitrates in the water, radiation, usually from radioactive substances and ultraviolet (UV) light, and low-frequency electromagnetic fields (Belpomme et al. 2007).

1.3. Cancer cell biology

Cancer is a heterogeneous group of diseases, the main feature is the production of abnormal cells that grow beyond the natural boundaries. Nowadays, several hallmarks of cancer have been proposed, forming the fundamental principles of this malignant transformation. Tumor formation is a multistep process, where the normal cells evolve progressively into the neoplastic stage by acquiring particular capacities that enable them to become tumorigenic. These hallmarks are self-sufficiency in growth signals, insensitivity to antigrowth signals, genomic instability, avoiding apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion, metastasis, tumor-promoting inflammation, metabolic reprogramming, and evasion of the immune system (Hanahan and Weinberg 2011, Floor et al. 2012). Each cancer has its own way of action. The following sections will highlight those characteristics particularly important for the development of leukemia, including genetic instability, proliferation and cell cycle arrest, and apoptosis.

1.3.1. Genetic instability

The greatest differences between cancerous and normal cells are their abilities to divide, survive, invade, metastasize and destroy the host. Genetic instability is one of the hallmarks found in cancer cells, including genetic changes such as mutations in specific genes and structural and numerical changes at the chromosomal level (Shen 2011). Mutations can appear due to DNA damage inflicted by environmental (e.g. radiations, industrial chemicals, natural carcinogens) or cellular sources (e.g. depurination, free radicals, DNA polymerase errors). In normal cells, DNA is replicated with high fidelity where the repair mechanisms are normally able to resolve the damage. However, due to the frequency of occurrence of DNA damage, the inaccessible structure of human chromatin and defects with DNA repair mechanisms, it is possible that some lesions escape the DNA repair mechanisms and produce mutations, contributing to genomic instability (Loeb and Loeb 2000, Lord and Ashworth 2012, Ferguson et al. 2015). Some of these changes will give the cell the capacity to ignore the regulatory processes needed to control cell division, expression, adaptation and even cell death, contributing to cancer initiation and progression. Besides that, this lack of stability generates cancer cells which are widely heterogenic, resulting in the development of cells resistant to certain chemotherapeutics (Loeb and Loeb 2000).

1.3.2. Proliferation and cell cycle

The cell cycle is a process that allows the cell to grow, replicate its DNA and divide. It is divided into four sequential phases (as shown in Figure 2): S phase is when the DNA replicates, M phase is responsible for the cell division producing two daughter cells, and two gaps (G1 and G2). G1 appears after M phase, a time where the cell is responsive to positive and negative growth signals, and G2 follows S phase when the cell prepares to start mitosis. There is also a fifth state, G0 or quiescence, in the case of deprivation of the growth-promoting signals in the G1 gap (Garrett 2001, Williams and Stoeber 2012).

The control of each phase and the transitions between them are controlled by sensor mechanisms. These monitor the cellular environment, mainly the genomic integrity, and determine if the cell must go on in the cell cycle progress. The main cell cycle checkpoints occur in the: G1/S phase transition, the biggest sensor of DNA damage; G2/M to monitor the fidelity of DNA replications and finally a mitotic checkpoint where the fidelity of chromosome segregation in mitosis is controlled (Garrett 2001). If some abnormality is detected, such as DNA damage, signaling pathways are activated resulting in cell cycle arrest in the attempt to “solve the problem” (Williams and Stoeber 2012). Mammalian cell cycle progression is controlled by cyclin-dependent kinases (Cdk), a family of serine/threonine kinases, through the phosphorylation of certain proteins (Garrett 2001). In the case of failure at cell cycle checkpoints, uncontrolled proliferation may occur - a typical characteristic for malignant phenotypes (Williams and Stoeber 2012).

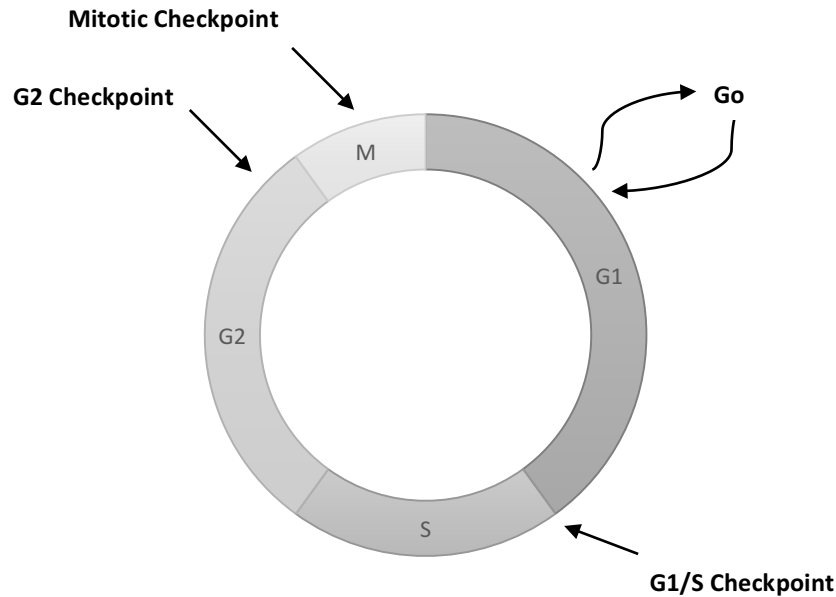


Figure 2 – Cell cycle and respective checkpoints. Adapted from Garrett (2001).

1.3.3. Apoptosis

Apoptosis is a mechanism of programmed cell death that presents several morphological and biochemical modifications such as rounding-up of the cell, membrane blebbing, externalization of phosphatidylserine, mitochondrial fragmentation, protein cleavage, retraction of pseudopods, pyknosis (reduction of cellular volume), condensation of chromatin, karyorrhexis (nuclear fragmentation) and formation of apoptotic bodies, amongst others. It is important to consider that other types of programmed cell death exist and others may yet be discovered (Elmore 2007, Kroemer et al. 2009, Fuchs and Steller 2015).

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic signals or mitochondrial pathway. The intrinsic pathway is initiated by several stress signals, such as radiation, drugs, free radicals, absence of growth factors, viral infections, amongst others that induce changes in mitochondrial permeability. Consequently, cause the release of cytochrome c, activation of caspase-9, -3 and other caspases that culminate with cell death (Kalimuthu and Se-Kwon 2013). The involvement of the Bcl-2 family proteins, responsible for mitochondrial outer membrane permeability, and the p53 tumor suppressor protein, which regulates the Bcl-2 proteins is crucial for the occurrence of this pathway. The Bcl-2 family includes anti- and pro- apoptotic proteins. The anti-apoptotic members are: Bcl-2,

Bcl-xL and Mcl-1 and the pro-apoptotic members are: Bax and Bak amongst others (Burke 2010). In the extrinsic pathway, apoptosis is initiated by transmembrane receptor-mediated pathways, where cytokine ligands, such as TNF, bind to death receptors presents on cell surface, forming a death inducing signaling complex (DISC) that activates the caspase-8 and -10, followed by the rest of the caspase cascade - once caspase-8 is activated, apoptosis is triggered. Disabling the apoptosis is one of the pathogenic occurrences which contributes to cancer initiation, promotion, and progression (Elmore 2007, Kalimuthu and Se-Kwon 2013).

2. Leukemia

Leukemia is one cancer that affects the blood-forming cells present in bone marrow and the hematopoietic process (Gibson et al. 2013). According to the GLOBOCAN project 2012, leukemia has a worldwide incidence of 351 965 cases which correspond to 2.5% of all cancers, and a mortality of 255 471 cases corresponding to 3.4% of all cancer deaths (Torre et al., 2015). This disease can affect the lymphoid or the myeloid stem cells, both resulting in the production of many white blood cells that are abnormal and do not mature normally (Vardiman et al. 2009). Referring to morphology, genetics, and clinical features, leukemia could be classified into four main groups: Acute lymphoid leukemia, chronic lymphoid leukemia, acute myeloid leukemia and chronic myeloid leukemia. In lymphoid leukemia, also known as lymphoblastic or lymphocytic leukemia, the abnormalities start in the cells that become lymphocytes. In myeloid leukemia, the cancer cells come from granulocytic, monocytic/ macrophagic, erythroid, megakaryocytic and mast cell lineages. The terms acute and chronic differ in the maturity of the leukemic cells (Vardiman et al. 2009).

2.1. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) represents 15% of all cases of leukemia and the median age for this disease is 64 years (Gibson et al. 2013). The myeloid abnormally starts in the blood-forming cells of the bone marrow and is characterized by uncontrolled proliferation of neoplastic hematopoietic precursor cells and weakened production of normal hematopoiesis, causing several abnormalities in the blood such as neutropenia, anemia and thrombocytopenia (Ghosh et al. 2014).

CML can occur in two or three stages: the first one, known as chronic phase (CP), is normally asymptomatic and presents as a myeloid hyperplasia in the bone marrow and peripheral blood (< 20 %). The intermediate stage, called accelerated phase (AP), occurs with few symptoms. The second phase – the blast crisis (BC) – appears 3-5 years after diagnosis of untreated CML-CP patients, as a rapid progression, and the number of undifferentiated myeloblasts is higher than 20 %. In some cases the transition from CP into BC phase occurs without AP signals (O'Brien et al. 2009, Burke 2010, Jabbour and Kantarjian 2014).

Some patients with CML have no symptoms at all, however, some of the symptoms commonly associated with this disease are the non-specifics (lethargy, fatigue, fevers, night sweats) or splenomegaly (such abdominal pain) (Gibson et al. 2013). Facing these symptoms, the main tests to diagnose this disease are a complete blood count (CBC) and bone marrow biopsy. Bone marrow cytogenetics is recommended to help choose the best treatment, taking into consideration the karyotypic abnormalities (O'Brien et al. 2009).

2.1.1. Molecular approach

Chronic myeloid leukemia is a hematopoietic stem cell disease which presents as a chromosome translocation between chromosomes 9 and 22 during cell division, resulting in a shorter chromosome 22 called *Philadelphia chromosome*. This translocation t(9;22)(q34;11) of DNA means that part of chromosome 22 breakpoint cluster region (*BCR*) gene at band q11 fusion with chromosome 9 Abelson 1 (*ABL*) gene at band q34, leading to the formation of a new oncogene *BCR-ABL* (Rowley 1973). *ABL* is a proto-oncogene that encodes for a protein tyrosine kinase found mainly in the nucleus, which regulates the cell cycle, differentiation, migration, invasion, genomic instability and the response to genotoxic stress (Deininger et al. 2000, Burke and Carroll 2010, Greuber et al. 2013). The *BCR* gene encodes for a serine/threonine kinase that can act as a GTPase-activating protein for members of the Rho family of guanine nucleotide exchange factors, and can also phosphorylate histones and casein (Burke 2010).

BCR-ABL encodes a new protein, p210^{*BCR-ABL*} which shows deregulated tyrosine kinase activity and contains the NH₂-terminal domains of *BCR* and the COOH-terminal domains of *ABL*. The critical functional changes found with the expression of this new protein are: *ABL* protein becomes constitutively active as a protein tyrosine kinase enzyme; attenuation of DNA

protein-binding activity of ABL; and the improvement of the binding of ABL to cytoskeletal actin microfilaments (O'Brien et al. 2009). Animal models have confirmed that this protein plays a crucial role in the pathogenesis of CML, increasing cell proliferation, blocking apoptosis and stromal interactions of the cell. Hence, the development of targeted therapy with a specific action on tyrosine kinase was a breakthrough in the treatment of myeloid leukemia (Gibson et al. 2013).

In very few cases, less than 10%, the leukemia cells present the *BCR-ABL* oncogene, but not the *Philadelphia chromosome*, and so it is possible that the *BCR-ABL* gene can be formed in a different way. There are also very rare cases where neither the oncogene nor the *Philadelphia chromosome* are found, which means that other oncogenes may be causing this disease (Onida et al. 2002). It is also possible to express another fusion protein (p190) however this is more common in cases of acute lymphoid leukemia (ALL) (O'Brien et al. 2009, Cutler et al. 2015).

2.1.2. Downstream signaling pathways of BCR-ABL

The *BCR-ABL* chimeric oncogene encodes for a constitutively active tyrosine kinase that modulates different signaling pathways, such as PI3K/AKT/mTOR, JAK-STAT, Wnt/ β -catenin and autophagy. This brings benefits in terms of cell survival, proliferation, differentiation, and migration, allowing cell proliferation, protection against cell death in the absence of external factors and promotion of invasion and metastasis (Sinclair et al. 2013). Modulation of survival pathways by BCR-ABL activation (Figure 3) has also been related to resistance to genotoxic therapeutics since BCR-ABL-positive cells can repair DNA damage more quickly through the up-regulation of RAD51 (a protein of homolog recombination repair system), decreasing its degradation and activating it through post-translational modification. The expression of RAD51 in the cells seems to be positively correlated with the appearance of a resistance phenotype (Collis et al. 2001). The capacity to repair DNA is not only influenced by the efficiency of the repair mechanisms but also by the time that the cell has to carry out the mechanism. BCR-ABL-positive cells are able to activate DNA damage-dependent cell cycle checkpoints faster by displaying a pronounced G2/M delay (Bedi et al. 1995). Another mechanism involved in drug

resistance is the modulation of Bcl-2 family members, namely the up-regulation of anti-apoptotic proteins (e.g. Bcl-xl and Bcl-2) (Skorski 2002).

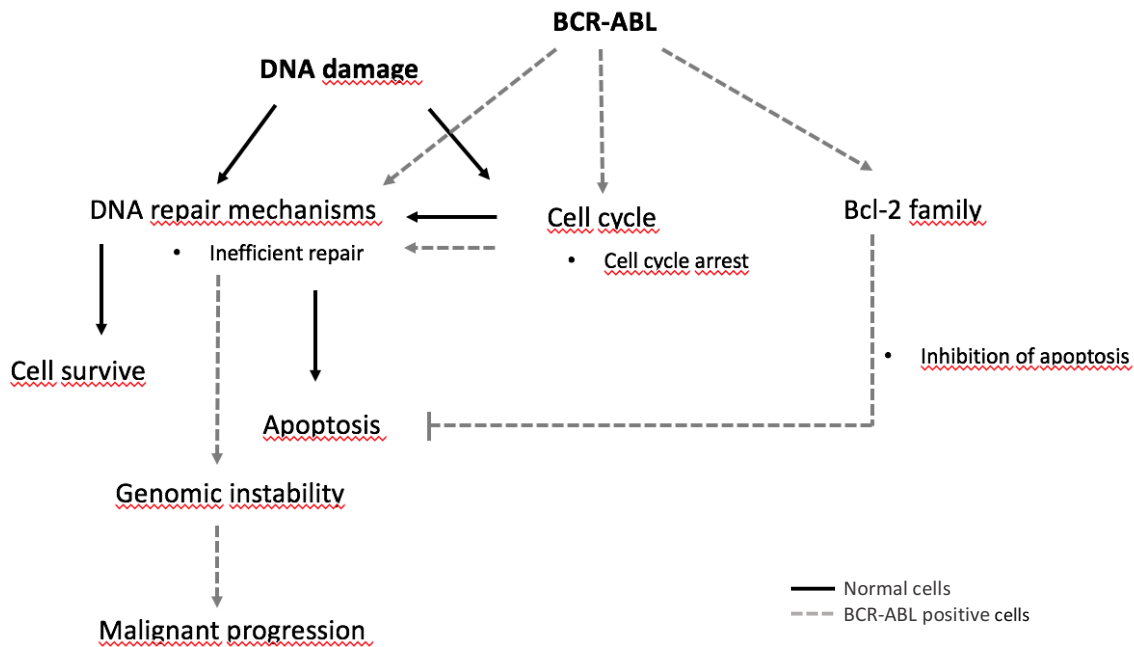


Figure 3 – Mechanisms of drug resistance induced by BCR-ABL. In a normal cell (represented as black) DNA repair mechanisms are activated and DNA damage can be completely repaired – the cell will survive. In the case of inefficient repair, and if the cell cycle checkpoints are not strong enough to allow more time for repair, death signals are activated with induction of apoptosis. In a BCR-ABL expressed cell (represented as gray) the global levels of DNA repair mechanisms are increased. This also affects the checkpoints, providing more time for repair, however, the efficiency is compromised. In addition, in case of some unrepaired or misrepaired lesion escape, the apoptotic signals are inhibited by the regulation of Bcl-2 protein family. Overall, this process leads to an accumulation of DNA damage, increasing the genomic instability. Adapted from Skorski (2002).

2.2. Treatment of CML

With advances in the knowledge of cancer biology, CML treatments have changed. Conventional chemotherapy treatment was widely used allowing the destruction of the cells. Examples of the drugs used in CML were busulfan followed by hydroxyurea. Both chemotherapeutics showed an improvement regarding the symptoms and hematology, but the cytogenetic remission was not significant (Henkes et al. 2008, Zhang et al. 2008). Allogeneic hematopoietic stem cell transplantation (HSCT) seems to be the type of treatment that proves

to cure more patients. Yet this method presents some disadvantages, such as the need for a suitable donor and the high toxicity of the procedure, which generates some side-effects such as immunodeficiency, infections, organ toxicity and graft versus host disease (Henkes et al. 2008). Another treatment is the Interferon- α (IFN- α) therapy, introduced in the 1980s, this presents some positive final results regarding remission and disease-free survival. However, the toxicity levels in the patients proved to be a problem, because of their ability to induce fatigue, myalgias, arthralgias, headaches, weight loss, depression, diarrhea, neurological symptoms, memory changes, hair thinning, autoimmune diseases, and cardiomyopathy (Henkes et al. 2008, Burke 2010). The need to find more potent and specific agents combined with the better understanding of molecular mechanisms underlying CML led, in the early 1990s, to the development of tyrosine kinase inhibitors (e.g. imatinib). Imatinib results in the inhibition of proliferation, restoration of the cell cycle, apoptosis induction and reversal of genetic instability in BCR-ABL dependent cells. However, even with the exceptional efficiency of TKI, problems may occur, such as drug resistance, loss of response, kinase domain mutations and transformation of the disease (e.g. evolution to an accelerated or blast phase). The BC is typically lethal, at this point more aggressive treatments are applied such anthracyclines (O'Brien et al. 2009). The previous two treatments (TKI and anthracyclines) will now be explored in further detail.

2.2.1. Tyrosine kinase inhibitors

Nowadays, the first-line of treatment for myeloid leukemia in the chronic phase is imatinib mesylate (STI-571), a potent BCR-ABL tyrosine kinase inhibitor (TKI) (Druker et al. 2006). TKI will bind competitively to the adenosine triphosphate (ATP) binding site of the BCR-ABL protein and consequently inhibits the phosphorylation of proteins related to BCR-ABL signal transduction (Mahon et al. 2000, Gibson et al. 2013). The final outcome is the induction of apoptosis in hematopoietic cells expressing BCR-ABL without affecting normal cells (Druker et al. 1996). Imatinib proved to be an efficient treatment for myeloid leukemia since patients showed 85% of overall survival (Gibson et al. 2013).

Despite the specific mechanisms of action, around 33% of patients started to fail to achieve a complete cytogenetic response (CCyR), either because of the toxicity or (mostly) due to the appearance of a resistant phenotype over time (O'Brien et al. 2009, Bhamidipati et al.

2013). The hematologic and nonhematologic toxicities caused by imatinib include neutropenia, thrombocytopenia, gastrointestinal disturbances, edema, skin rashes and musculoskeletal complaints (Gibson et al. 2013). Furthermore, some patients present, after a few years with imatinib treatment, signals of cardiotoxicity with congestive heart failure (CHF) (O'Brien et al. 2009). This resistance may be due to a range of mechanisms, some are BCR-ABL-dependent and others are BCR-ABL-independent mechanisms. The BCR-ABL-dependent mechanisms include, most frequently, point mutations in the ABL tyrosine kinase domain (TKD) and gene amplification of ABL (Bhamidipati et al. 2013). An important mutation is one that results in the formation of amino acid substitutions in imatinib binding sites, frequently called “gatekeeper” mutations (e.g. T315I) (Gibson et al. 2013). The BCR-ABL-independent mechanisms of resistance involve: (1) decrease in drug uptake by expression of the human Organic Cation Transporter 1 (hOCT1) (Thomas et al. 2004); (2) improved drug efflux by overexpression of P-glycoprotein (P-gp) efflux pumps, reducing the amount of intracellular drug (Mahon et al. 2000); (3) increase of plasma protein α 1 acid glycoprotein (AGP), which binds to imatinib preventing the ABL kinase inhibition (Gambacorti-Passerini et al. 2000); (4) increase of prostaglandin-endoperoxide synthase 1 (cyclooxygenase 1), which plays an important role in imatinib metabolism (Villuendas et al. 2006).

In response to the emergence of imatinib resistance, the development of second-generation TKI began. These include nilotinib (Tasigna[™]) and dasatinib (Sprycel[™]). Both drugs achieved positive molecular responses, even acting with imatinib-associated kinase domain mutations, yet each drug induced its own mutations, and neither could inhibit BCR-ABL T315I (Gibson et al. 2013, Zhou and Xu 2015). A mutation in the ATP phosphate-binding loop (P-loop) is associated with poor prognosis and a high risk for progression (O'Brien et al. 2009, Mughal et al. 2013). Other types of TKI are also in development, such bosutinib and ponatinib, the latter presenting activity against the T351I mutation (Gibson et al. 2013).

2.2.1.1. Current strategies to enhance the therapeutic effect of imatinib

Resistance development in leukemia cells and the potential toxicity have been great limitations for imatinib application (Hu et al. 2009). The future of CML therapy passes to the development of new potent agents, perhaps in combinations with existing treatments in lower doses.

Nimmanapalli et al. (2003) showed that imatinib, when combined with suberoylanilide hydroxamic acid (SAHA), known as an inhibitor of histone deacetylases, can enhance the cytotoxicity effects in leukemia cells (K562 cell line) by up-regulation of p21 and p27 and down-regulation of BCR-ABL levels with induction of apoptosis in BCR-ABL-expressed cells. A decrease in phospho-AKT and Bcl-xL levels was also observed. After experiments in two CML murine models, Hu et al. (2009) concluded that a low dose of imatinib combined with bortezomib (proteasome inhibitor) or proteasome inhibitor might optimize the CML treatment. The results showed inhibition in Bcl-2, increase of cytochrome c and activation of caspases, along with inhibition of proteasomal degradation of protein phosphatase 2A (PP2A). The combination was proven to have an inhibitory effect on tyrosine kinase via suppression of NFkB. Lin et al. (2016) demonstrated that by down-regulating RanGTPase and activating protein 1 (RanGAP1) it is also possible to improve the imatinib efficiency because RanGAP1 can mediate BCR-ABL nuclear entrapment to activate the P73-dependent apoptosis pathway.

2.2.2. Anthracyclines

Anthracyclines are one of the most effective classes of anti-tumor antibiotics used for the treatment of numerous cancers, including breast, ovarian (Bellarosa et al. 2001), prostate, lung (Pratesi et al. 1998), and leukemia, amongst others (Hehlmann 2012). One of the first anthracyclines to be isolated was doxorubicin, isolated from *Streptomyces peucetius* var. *caesius* in the 1960s (Malla et al. 2010). Despite the wide use of this drug, its mechanism of action is not completely understood. However, several studies demonstrated its involvement in the inhibition of DNA and RNA synthesis, in the inhibition of topoisomerase II with subsequent formation of DNA double-strand breaks and the induction of cell death (Laroche-Clary et al. 2000, Park et al. 2005), and in the synthesis of free radicals (Lebrecht et al. 2004, Thorn et al. 2011).

Beside the broad anticancer activity, the action of doxorubicin has been related with several side effects such as non-targeted cytotoxicity and acquisition of multidrug resistance

(MDR) phenotype. In CML the main mechanisms that contribute for MDR involve the P-gp by sequestration of the drug into cytoplasmic vesicles, the expression of antiapoptotic protein Bcl-2 and the gene *BCR-ABL* (Misra and Sahoo 2011).

2.2.2.1. Current strategies to enhance the therapeutic effect of doxorubicin

Referring to the limitations of doxorubicin, such as drug resistance of cancer cells and the side-effects on normal cells, several strategies have been made to overcome these problems. One possibility to potentiate the action of doxorubicin is to combine this drug with synthetic or natural agents (Ghosh et al. 2014).

According to Misra and Sahoo (2011), the combination of doxorubicin with curcumin (a natural polyphenol) exhibited a synergistic inhibitory effect on growth of K562 cells. The synergistic growth inhibition was mediated through different mechanisms that involved the inhibition of p210^{BCR-ABL}. The authors suggest that the synergistic effect is clinically important and may provide combinatorial strategies in cancer therapy. Jang et al. (2013) reported the synergetic effect of the combination treatment of decursin (a natural compound present in *Angelica gigas*) and doxorubicin, in multiple myeloma cells, by enhancing apoptotic activity via mTOR. Also observed was the activation of caspase -9 and -3, the cleavage of poly (ADP-ribose) polymerase (PARP), increase of sub-G1 population, and decrease in the expression of cyclind-D1 and survivin amongst other effects in U266 cells. Ghosh et al. (2014) showed that extracts from the hot water in black tea have protective effects against chemotherapeutic drugs, such as daunorubicin (an anthracycline), in normal lymphocytes. This extract is able to inhibit drug-induced ROS generation and renew the mitochondrial membrane potential (MMP). It increases cellular viability, up-regulates endogenous antioxidants enzymes, inhibits mRNA expression of apoptotic genes, prevents caspase-3 activation and reduces DNA fragmentation.

3. Seaweed as a source of bioactive compounds

Several epidemiological studies have shown that some phytochemicals present in vegetables and fruits are able to reduce the risk of degenerative processes (Abubakar et al. 2012, Anand et al. 2008, Kim et al. 2010, Rengarajan et al. 2013). Marine organisms are a rich

source of bioactive compounds with several biological activities, and the interest by the scientific community has grown rapidly. In particular, seaweed, as photosynthetic organisms, are exposed to high amounts of light and oxygen which favors the formations of free radicals and other oxidative reagents. However, the absence of any serious photodynamic damage means that marine algae have the ability to generate bioactive components to protect themselves (Heo et al., 2008).

Marine algae can be divided into four main groups, according to the type of pigments, morphology, anatomy and reproductive structures: Chlorophyceae (green algae), Phaeophyceae (brown algae), Rhodophyceae (red algae) and Cyanophyceae (blue-green algae) (Kolanjinathan et al. 2014). Seaweed are multicellular eukaryotic and macroscopic organisms living in salty water and mostly belong to the green, brown and red algae. They have been integrated into the human diet in some countries, such as Japan, since ancient times. In line with such traditions, the consumption of this natural product around the world is increasing nowadays (Kolanjinathan et al. 2014). Seaweed are a rich source of polysaccharides, minerals, protein, vitamins, and low-fat carbohydrate (Cornish and Garbary 2010). High levels of bioactive compounds can be produced by a variety of marine macro and microalgae which provide several properties such as antibacterial, antiviral, immunosuppressant, antioxidant, antiproliferative and antitumor activity (Sharif et al. 2014).

The use of bioactive compounds in combination with conventional anticancer drugs is poorly explored but the strategy may improve the clinical performance in conventional chemotherapy by altering the biological disposition to minimize toxicity, maximize efficacy and possibly reduce the dose of therapy (Yamamoto et al. 2011, Ghosh et al. 2014).

3.1. Carotenoids

Seaweed pigments can be divided into three main groups: chlorophylls, carotenoids, and phycobiliproteins. Carotenoids, organic pigments found in chloroplasts and chromoplasts, exhibit a purple, red, orange and yellow color — actually, carotenoids are a class of tetraterpene pigments found in bacteria, fungi, algae, as well as in higher plants and animals (Chojnacka et al. 2012). Nowadays, more than 700 carotenoids have been found in nature (Dembitsky and Maoka 2007, Maoka 2011, Takaichi 2011). The intake of dietary carotenoids has been correlated with a lower incidence of cardiovascular and neurodegenerative diseases, cataract

3.1.2. Anticancer effect of fucoxanthin in leukemia

In vitro and in vivo research has shown the anticancer effects of fucoxanthin in leukemia. Fucoxanthin and fucoxanthinol (its metabolite) decrease the viability of HTLV-1 infected T-cell lines and ATL cells through the induction of G1 cell cycle arrest, associated with down-regulation of cyclin D1, cyclinD2, CDK4 and CDK6 expression, and up-regulation of GADD45 α , which will inhibit the entry of cells into S phase (Ishikawa et al. 2008). These authors also showed induction of apoptosis with activation of caspase-3, -8 and -9, and down-regulation of antiapoptotic protein expression, such as XIAP, cIAP2, Bcl-2 and survivin. In mice, fucoxanthinol suppresses tumor growth without adverse effects (Ishikawa et al. 2008).

Nakazawa et al. (2009) observed that fucoxanthin induces apoptosis in HL-60 leukemia cells and other cell lines with down-regulation of anti-apoptotic Bcl-2 proteins. Kim et al. (2010) also showed that the inhibitory growth of fucoxanthin in leukemia cell lines was due to apoptosis induction caused by the generation of ROS, along with the indication of cell cycle arrest in G1 stage. However, Kotake-Nara et al. (2005) demonstrated that apoptotic activity of fucoxanthin in promyelocytic cell lines is not caused by ROS. Besides the antiproliferative and proapoptotic effects of fucoxanthin in HL-60 leukemia cells, Ganesan et al. (2011) showed the anti-angiogenesis effects of fucoxanthin mediated by down-regulation of signal transduction by fibroblast growth factor 2 (FGF-2) and its receptor (FGFR-1) in human umbilical vein endothelial cells (Ganesan et al. 2013). However, information about the effects of fucoxanthin in CML is clearly missing.

3.2. Phlorotannins

Phlorotannins are tannin derivatives composed of phloroglucinol units linked to each other in different ways (Cornish and Garbary 2010, Thomas and Kim 2011). Phloroglucinol (1,3,5-trihydroxybenzene) is a phenolic compound isolated from *Ecklonia cava* that has three hydroxyl groups in the benzene ring (Figure 5). This class of polyphenols is unique in brown seaweed and several biological activities have been attributed to it, such as antioxidant, anti-inflammatory, antibacterial, anti-allergic and anticancer properties (Pádua et al. 2015, Corona et al. 2016).

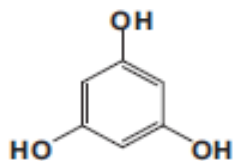


Figure 5 – Phloroglucinol structure.

3.2.1. Anticancer effect of phloroglucinol in leukemia

Phloroglucinol has demonstrated cytoprotective effects in normal cells from the toxicity found in cancer treatments, proving to be a good candidate to improve the quality of these treatments. The protective effect in mice intestinal cells was accomplished by blocking the activation of p53, downregulation of Bax and Bak-dependent pathway and up-regulation of the levels of Bcl-2 and Bcl-X_{SL} (Ha et al. 2013). According to Kang et al. (2010), phloroglucinol efficiently protected cells against radiation and extended the survival of mice exposed to lethal doses of radiation through the inhibition of mitogen-activated protein kinase kinase-4 (MKK4/SEK1), c-Jun NH₂-terminal kinase (JNK), activator protein-1 (AP-1) cascades and protected the depletion of reduced glutathione. Phloroglucinol also has a protective effect against lipid peroxidation and DNA damage induced by radiation (Kang et al. 2010).

Besides the cytoprotective effects in normal cells, phloroglucinol also showed cytotoxic effects against cancer cells. Such effects in leukemia cell lines are poorly explored, however, some studies showed in vitro antiproliferative activity of phloroglucinol and of its derivatives in a variety of cancer cell lines including K562 cells. According to Liu et al. (2011) hyperforin, an abundant phloroglucinol-type, induced, in K562 cells, the dissipation of mitochondrial transmembrane potential caused by radiation, through the release of cytochrome c. Phloroglucinol induced the activation of the caspase-3, -8 and -9 cascade and consequently PARP cleavage. Cell cycle arrest was also observed with the increased expression levels of both p53 and p27^{kip1}. Quiney et al. (2006) presented the same results for other leukemia cells, e.g. B-cell chronic lymphocytic leukemia (B-CLL), where hyperforin induced apoptosis by disruption of the mitochondrial transmembrane potential, activation of caspase-3 and cleavage of the PARP. Furthermore, downregulation of Bcl-2 proteins, Mcl-1 (anti-apoptotic proteins) and nitric oxide (NO) synthase of type 2 were also observed. In this study, downregulation of the cell cycle inhibitor, p27^{kip1} was detected in contrast with the results from Liu et al. (2011). Using

chronic lymphocytic leukemia cells in *ex vivo* studies Merhi et al. (2012) showed that the mitochondrial pathway of caspase-dependent apoptosis was induced by up-regulation of Noxa (protein of Bcl-2 family). More studies are needed to properly understand the effects of phloroglucinol against leukemia and information about the effects in CML is clearly missing.

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CHAPTER II - OBJECTIVE

Objective

The objective proposed for this work was to evaluate the anticancer activity of two natural compounds — fucoxanthin, a marine carotenoid, and phloroglucinol, a phenolic compound — in vitro, using two cancer cell lines (K562 and TK6 cells) derived from CML-BC. The anticancer effects were evaluated either alone or in co-incubation with IC₃₀ of two anticancer drugs, to evaluate the ability of natural compounds to potentiate the existing cancer treatments. To accomplish this objective, the following assays were carried out:

- Effects on cell viability: After an exposure time of 24 h, the cellular density was determined by cell counting using a Neubauer chamber. This technique allows us to determine the short-term effects of the desired treatments on the cells, in other words the cytotoxicity of the desired treatment.
- Antiproliferative effects: After an exposure time of 24 h followed by a further 48 h in fresh medium, the cellular density was determined by cell counting using a Neubauer chamber. With this assay, it was possible to determine the ability of the cells to proliferate after short-term exposure to the desired treatments. The combinations that showed anticancer potential were selected for the next assays.
- Induction of DNA damage: After 24 h of incubation with the selected conditions, comet assay with and without the incubation of FPG enzyme was performed to determine the FPG-sensitive sites and strand breaks, respectively.
- Effects on cell death: After 48 h of treatment with the selected conditions, the nuclear condensation assay was performed to assess induction of cell death by apoptosis.

**CHAPTER III - BIOACTIVE COMPOUNDS FROM SEAWEED
WITH IN VITRO ANTI-LEUKEMIA ACTIVITY**

This Chapter compromises the draft of a manuscript to be submitted for publication:

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BIOACTIVE COMPOUNDS FUCOXANTHIN AND PHLOROGLUCINOL FROM SEAWEED WITH IN VITRO ANTI-LEUKEMIA ACTIVITY

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Abstract

Aim: The anticancer effects of fucoxanthin (Fx) and phloroglucinol (Ph), alone or co-incubated with anticancer drugs, doxorubicin (Dox) and imatinib (Imat), were evaluated in two cancer cell lines (K562 and TK6) representative of CML-BC.

Material and methods: To achieve this aim, cytotoxicity at short time (24 h), proliferation capability (24 h with treatment and a further 48 h in fresh medium) by cell counting using a Neubauer chamber, induction of DNA damage by comet assay, and induction of cell death by apoptosis observed by morphological changes, were evaluated in K562 and TK6 cancer cells.

Results and conclusions: Doxorubicin decreased cell proliferation in both cell lines increasing oxidative DNA damage and nuclear condensation in TK6 cells. Imatinib also inhibited cell proliferation in K562 cells. However, no effects were founded on DNA damage and nuclear condensation. Fucoxanthin demonstrated a decrease on cell proliferation without induction of DNA damage or nuclear condensation in both cell lines. In co-incubation increased the antiproliferative effects of both drugs in the cell lines tested, however no differences were obtained when compared the combinations with fucoxanthin alone. Phloroglucinol also presented anticancer capabilities, decreasing cell proliferation alone, and in co-incubation to potentiate imatinib action in TK6 cell lines, without induction of DNA damage or apoptosis levels. In conclusion, both natural compounds (fucoxanthin and phloroglucinol) exhibit antiproliferative effects in K562 (fucoxanthin) and TK6 (fucoxanthin and phloroglucinol) cells. When co-incubated with the drugs (doxorubicin and imatinib), fucoxanthin increased antiproliferative effects of doxorubicin (in K562 cells) and imatinib (in K562 and TK6 cells) while phloroglucinol increased the antiproliferative effects of imatinib mainly in TK6 cells. DNA damage or apoptosis seems not to be the main mechanisms behind the antiproliferative effects induced by fucoxanthin and phloroglucinol.

Significance and impact of the study: The inclusion of the studied natural compounds with the existing treatments could promote significant advances for cancer treatment, such as enhancement of the applied treatments and minimize side-effects mainly in more advanced stages of the disease. The findings call for a deeper understanding of the molecular mechanisms underlying the effects of the studied compounds.

Keywords: Doxorubicin · Fucoxanthin · Imatinib · Leukemia · Phloroglucinol

Abbreviations: Abelson murine leukemia (ABL); Accelerated phase (AP); Adenosine triphosphate (ATP); Alpha-1-acid glycoprotein (AGP); Blast crisis (BC); Breakpoint cluster region (BCR); Chronic phase (CP); Chronic myeloid leukemia (CML); 4',6-diamidino-2-phenylindole (DAPI); Dimethyl sulfoxide (DMSO); Doxorubicin (Dox); Fetal bovine serum (FBS); Formamidopyrimidine DNA glycosylase (FPG); Fucoxanthin (Fx); Human organic cation transporter 1 (hOCT1); Imatinib (Imat); Multidrug resistance (MDR); P-glycoprotein (P-gp); Paraformaldehyde (PFA); Phloroglucinol (Ph); Phosphate-binding loop (P-loop); Phosphate buffered saline (PBS); Roswell Park Memorial Institute Medium (RPMI); Tyrosine kinase inhibitors (TKI).

1. Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder which accounts for 15-20% of all adult leukemias (Yin et al. 2004, Gu et al. 2012). CML is characterized by uncontrolled proliferation of myeloid cells in the bone marrow and blood (Ghosh et al. 2014). The genetic hallmark of CML is the *Philadelphia chromosome*, an abnormality acquired from a chromosome translocation t(9;22) q(34;q11), resulting in the generation of the *BCR-ABL* fusion oncogene (Rowley 1973, Burke and Carroll 2010). BCR-ABL has high tyrosine kinase activity disrupting signaling pathways that regulate cell proliferation, apoptosis, differentiation, adhesion and genomic stability (Skorski 2002, Druker 2008). CML treatment includes several conventional chemotherapeutic agents, such as busulfan and hydroxyurea, however the incomplete cytogenetic response and the side-effects may limit the use of this drug as a clinical treatment (Henkes et al. 2008). The need to develop more potent and specific agents using knowledge of the molecular mechanisms underlying CML lead to the development of tyrosine kinase inhibitors (TKI) (Zhang et al. 2008).

Imatinib mesylate (Gleevec) is the first generation of BCR-ABL TKI that was clearly a great achievement for CML treatment, since this drug acts in a specific way, inhibiting the activity of BCR-ABL. Imatinib interacts with BCR-ABL adenosine triphosphate (ATP) binding sites and consequently inhibits the phosphorylation of proteins related with BCR-ABL signaling pathways (Mahon et al. 2000, Gibson et al. 2013). However, around 33% of patients have relapsed due to the appearance of a set of mutations, mainly T315I mutation, leading to the appearance of resistant phenotype overtime (O'Brien et al. 2009, Bhamidipati et al. 2013, Gibson et al. 2013). These mutations represented a new challenge for CML treatments, which promoted the development of the second generation of TKI, including nilotinib (TasignaTM) and dasatinib (SprycelTM), yet other mutations in the ATP phosphate-binding loop (P-loop) also occur (O'Brien et al. 2009). Alternative strategies to overcome the T315I mutation and effectively kill leukemia cells in a more advanced stage such as blast crisis are applied, for instance, anthracyclines (O'Brien et al. 2009). One of the first anthracyclines to be isolated was doxorubicin, isolated from *Streptomyces peucetius* var. *caesius* in the 1960s (Malla et al. 2010). The main mechanisms of action include: (i) inhibition of DNA and RNA synthesis; (ii) inhibition of topoisomerase II with subsequent formation of DNA double-strand breaks and induction of cell death; and (iii) free radical synthesis (Laroche-Clary et al. 2000, Lebrecht et al. 2004, Park et al. 2005, Thorn et al. 2011). However, the use of doxorubicin has been linked with some

adverse side-effects including cytotoxicity in normal cells (mainly in cardiomyocytes) and acquisition of multidrug resistance (MDR) phenotype. MDR is a multifactorial process amongst which the well-known are drug efflux and influx, drug detoxification, mechanisms of DNA repair and resistance to apoptosis, all contributing to chemotherapy failure (Misra and Sahoo 2011).

Since ancient times, seaweed have played an important part in the diet in Asian countries, and in the last decades their use as a food underwent a great expansion in the western world as well. Seaweed are rich in minerals, vitamins, polyunsaturated fatty acids, polysaccharides, carotenoids and phenolic compounds (e.g. phlorotannis) (Cornish and Garbary 2010, Chojnacka et al. 2012, Mohamed et al. 2012). Due to the presence of a high content of bioactive molecules, several biological activities have been attributed to seaweed, namely antioxidant, anti-inflammatory, antiestrogenic, anticancer, antidiabetic, antiobesity, antihypertensive, and antihyperlipidemic activity, protections against neurodegenerative diseases, healing, antiviral, antifungal, antibacterial, and larvicide activity, amongst others (Mohamed et al. 2012, Fernandes et al. 2014, Kolanjinathan et al. 2014, Pádua et al. 2015). Fucoxanthin is one of the most abundant carotenoids found in the marine environment, representing more than 10% of the total carotenoid production, and it is widely distributed in brown algae (Phaeophyceae) and diatoms (Bacillariophyta) (Rengarajan et al. 2013). Fucoxanthin has preventive and therapeutic effects against several types of cancer due to its mechanisms of action, which include antiproliferation, cell cycle arrest, apoptosis induction and suppression of angiogenesis (Anand et al. 2008, Wang et al. 2012, Rengarajan et al. 2013). Furthermore, the anticancer effects of fucoxanthin seem to be, in some cases, specific for cancer cells without cytotoxic effects against normal cell lines (Ishikawa et al. 2008). Phloroglucinol (1,3,5-trihydroxybenzene), a phenolic compound present mainly in brown seaweed, has anticancer activity by acting as an antioxidant, antiproliferative, proapoptotic, antiangiogenic and antimetastatic agent (Pádua et al. 2015, Corona et al. 2016).

Anticancer activity of seaweed extracts and isolated compounds has been reported in several studies. However, effects in CML and its interaction with the conventional anticancer drugs has been less explored. In the present study, we intended to analyze the *in vitro* anticancer effects of two seaweed compounds (fucoxanthin and phloroglucinol) in leukemia cell lines, either alone or in combination with two conventional chemotherapeutic drugs used in leukemia treatment (imatinib and doxorubicin). The search for natural compounds with biological activity, namely anticancer activity and/or improvement of the actions of drugs

against cancer cells, is a promising field that can improve several cancer treatments. In this quest, reducing the anticancer drugs' cytotoxicity against normal cells is also a key target.

2. Materials and methods

2.1. *Chemicals and reagents*

Roswell Park Memorial Institute medium (RPMI-1640), doxorubicin, imatinib, sodium pyruvate, sodium bicarbonate, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), penicillin/streptomycin, 4,6-diamidino-2-phenylindole (DAPI), fucoxanthin and phloroglucinol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was acquired from Biochrom KG (Berlin, Germany). Dimethyl sulfoxide (DMSO) was bought from AMRESCO LLC (Solon, SO, USA) and RO 19-8022 was generously provided by Hoffmann-La Roche and FPG enzyme was obtained from NEW ENGLAND BIOLABS U.S.A. All other reagents and chemicals used were of analytical grade.

Stock solutions of doxorubicin, imatinib, fucoxanthin, and phloroglucinol were prepared in DMSO and aliquots kept at -20 °C. For the use in each experiment, compounds and drugs were diluted in fresh culture medium and the final concentration of DMSO in the medium was 0.5% (v/v). Negative controls were subjected only to culture medium with 0.5% of DMSO (v/v), to assess the effect of the solvent.

2.2. *Cell cultures*

Two human cancer cell lines were used to perform all the assays. K562 cell line, derived from blood of a patient with chronic myeloid leukemia (CML) in terminal blast crisis, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and TK6 cell line, a human-derived lymphoblastoid cell line, was acquired from the American Type Culture Collection (ATCC). Both cell lines were maintained as suspension cultures in RPMI-1640 supplemented with 10% FBS and 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 0.1 mM sodium pyruvate and 10 mM HEPES under an atmosphere of 5% CO₂ at 37 °C.

2.3. *Cytotoxicity assay*

An important initial step is the determination of the optimal cell density growth for each cell line to perform the following assays. For this, cells were plated at different densities between 1×10^4 cells/ml and 4×10^5 cells/ml. The cells were counted at 24, 48 and 72 h after plating, using the Neubauer chamber on an inverted contrast phase microscope (Olympus CKX41, Japan), mixing the cell suspension with trypan blue (1:1) to allow standard counting.

To evaluate the cytotoxic effect of the anticancer drugs (imatinib and doxorubicin) alone, cells were plated, K562 (0.3×10^6 cells/ml) and TK6 (0.2×10^6 cells/ml), in 24-multiwell culture plates, and incubated with different concentrations of imatinib (0 - 10 μ m) and doxorubicin (0 - 1 μ m). Twenty-four hours after plating, cells were counted as described previously and cell density determined. The results are expressed as the survival percentage of cell viability that was calculated taking into consideration that the negative control (treated with 0.5% DMSO) had a 100% of survival.

For the combination assays, cells were plated as referred above and incubated with the drug at IC_{30} concentration (concentration that inhibits cell viability in 30%) combined with different concentrations of fucoxanthin (0 - 10 μ m) or phloroglucinol (0 - 300 μ m). The cell density was determined after 24 h, as already described. Cells treated solely with either drug or with fucoxanthin or with phloroglucinol were also tested.

2.4. *Antiproliferation assay*

To assess the proliferation effects, 100 μ l of cell suspension provided from the cytotoxicity assay were centrifuged, to remove the treatment, and plated again in fresh medium for more 48 h. After the incubation time, the cells were counted and cell density calculated. The results are expressed in relative suspension growth (RSG) that was the ratio of the total suspension growth (TSG) of each condition and the TSG of the negative control (treated with 0.5% DMSO). TSG corresponds to the ratio between the number of cells seeded and the number of cells at the end of the assay (Azqueta et al. 2013).

Only the conditions that decreased cell proliferation according to the data obtained in the respective assays were selected for the following assays.

2.5. Comet assay

To determine the genotoxic effect of the conditions selected, cells were plated – K562 at 0.3×10^6 cells/ml and TK6 at 0.2×10^6 cells/ml – in 6-multiwell culture plates and incubated with the desired treatment at 37 °C in an incubator with 5% of CO₂. After the incubation time (24 h), DNA damage (strand breaks and alkali-labile sites) was assessed by the alkaline version of the single-cell gel electrophoresis (SCGE) or comet assay (Collins et al. 2008, Ramos et al. 2010). Briefly, cells were washed, centrifuged and the pellet suspended in 0.5% low melting point agarose at 37 °C. About 2×10^4 cells/gel were spread on a microscopic slide pre-coated with 1% normal melting point agarose. For each condition three slides (lysis, buffer F, and FPG) were performed and in each slide two duplicated gels. Slides were incubated in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris Base, pH 10 plus 1% (v/v) Triton X-100) for 1 h at 4 °C. After lysis, the slides for buffer F and FPG were removed, washed three times with buffer F solution and then incubated with the respective solution (buffer F or FPG) for 30 min at 37 °C. Followed by place all the slides (lysis, buffer F and FPG) in a horizontal electrophoresis chamber with electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH >13), for 40 min at 4 °C, allowing the DNA to unwind. Then run electrical field was applied, 300 mA, 25 V (1 V/cm) for 20 min at 4 °C to draw the negatively charged DNA toward an anode. After the electrophoresis, slides were washed twice with PBS and distilled water, and at the end dried at room temperature overnight.

As a positive control for oxidative damage both cell lines were exposed to the photosensitizer Ro 19-8022 at three concentrations (1, 3 and 9 µm) (Azqueta et al. 2013). The cells were seeded at 1×10^6 cells/ml and exposed for 5 min to Ro 19-8022 on ice under a halogen lamp (33 cm of distance). After the collection, cells of positive control were submitted at the same procedure that was mentioned above.

For the analysis of the comet images, slides were stained with 30 µl of DAPI solution (1 µg/ml), covered with coverslips and comets were visualized in a fluorescence microscope (Olympus IX71, Japan). At least fifty comets were captured randomly per gel. The percentage of tail intensity was measured using OpenComet software (Gyori et al. 2014). Three independent assays were performed with two replicates per condition.

2.6. Nuclear condensation

To assess the involvement of cell death as a possible mechanism of anticancer activity, cells were plated in 24-multiwell culture plates – K562 at 0.3×10^6 cells/ml and TK6 at 0.2×10^6 cells/ml – and incubated (alone or in co-incubation) with the previously selected test compounds. After 48 h of incubation, nuclear condensation was assessed by observation in the fluorescence microscope (Olympus IX 71, Japan). Briefly, cells were collected, washed, centrifuged, and fixed with 4% paraformaldehyde (w/v) for 20 min at 37 °C, and then attached to a poly-L-Lysine-covered slide using a Cytospin Cytocentrifuge (Thermo Scientific, USA). Once dried, slides were incubated with DAPI (1 µg/ml) for nuclei staining. The percentage of cells with condensed nuclei was calculated from the ratio between cells with nuclear condensation and the total number of cells. At least 300 cells were counted per sample (Ramos et al. 2016).

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA), being the results expressed as mean \pm standard deviation (SD) from at least 2 independent experiments. The results were evaluated using one-way or two-way ANOVA to assess significant differences ($p \leq 0.05$) between treatment conditions and the negative control, followed by a *post hoc* multiple comparisons using the Bonferroni's test or Dunnett's test as appropriate. The assumptions of normality and of homogeneity of variances were not tested due to the low "n", but they were assumed to occur, in line with the cell culture responses as detected in many resembling published studies and with the general robustness of the parametric tests from departures of the basic assumptions (Rasch and Guiard 2004, Schmider et al. 2010, Mckillup 2011).

3. Results

3.1. Effects of fucoxanthin and phloroglucinol alone and in co-incubation with doxorubicin or imatinib on cell viability in leukemia cell lines

In vitro anticancer effects of fucoxanthin and phloroglucinol, alone or in combination with anticancer drugs (doxorubicin or imatinib), in two cancer cell lines (K562 and TK6 cells) representative of CML-BC were tested. First, the optimal growth density for each cell line was determined by a density assay. The results showed that the optimal density for K562 cells was 0.3×10^6 cells/ml and for TK6 was 0.2×10^6 cells/ml. All the followed assays were performing using the optimal density growth. Two different assays were performed the cytotoxic assay and the proliferation assay. The cytotoxic assay was used to determine the ability of the applied treatment to kill the cells in the short-term, and the proliferation assay was used to determine the ability of the cells, after treatment removal, to recover the damage and proliferate. Initially both cell lines were incubated with different concentrations of doxorubicin or imatinib to determine the IC_{30} for each cell line based on the results from the proliferation assay. The anticancer effects of fucoxanthin and phloroglucinol were evaluated either alone or in co-incubation with IC_{30} for each drug, to assess their ability to potentiate the effects of the anticancer drugs tested.

Effects of imatinib and doxorubicin on cell viability and proliferation in leukemia cell lines

To assess cytotoxic effects of doxorubicin (0-1 μ m) and imatinib (0-10 μ m), K562 and TK6 cell lines were incubated for 24 h with each drug. Cell viability was significantly reduced in both cell lines when treated with doxorubicin in a dose-dependent manner. The decrease of cell viability was more pronounced in TK6 cell line with an inhibition of 87% at 1 μ m (Figure 1c). Doxorubicin at all tested concentrations showed a significant cytotoxicity in TK6 cells. Imatinib alone, at the tested conditions, did not affect cell viability in both cell lines.

Inhibition of cell proliferation was determined after 24 h of treatment, as in cytotoxic assay, followed by 48 h in new and fresh medium. The results showed that in K562 cell line, both drugs (doxorubicin and imatinib) inhibit the cellular growth, being almost 100% in the highest concentration tested (Figure 2a and b). Regarding TK6 cell line, doxorubicin clearly

showed to be more efficient, reaching 100% of inhibition with 0.01 μM of doxorubicin (Figure 2c), while imatinib did not show any inhibitory effect (Figure 2d). IC_{30} values of doxorubicin and imatinib alone for each cell line were determined using the data from the proliferation assay to perform the co-incubation assays with the natural compounds (fucoxanthin and phloroglucinol).

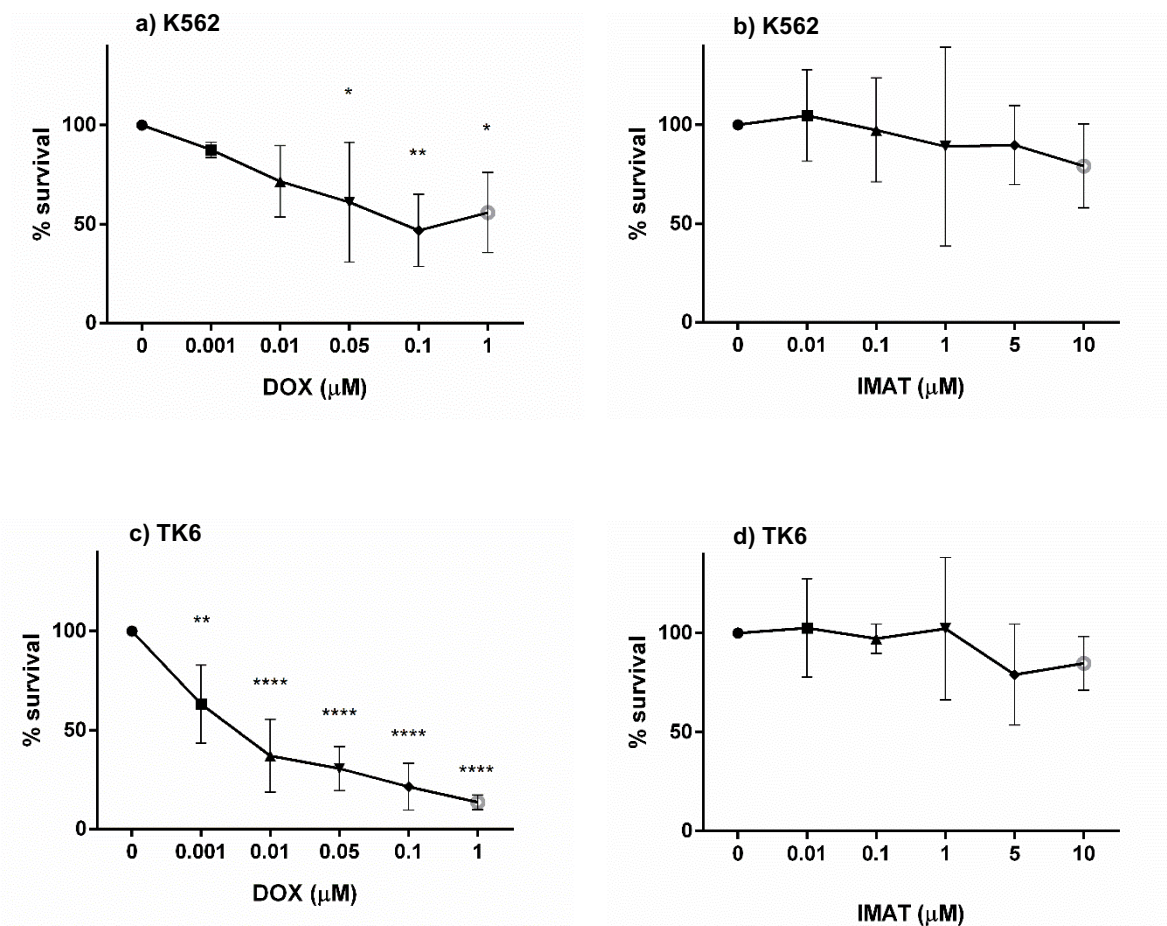


Figure 1 – Dose-response effects on cell survival, after 24 h of incubation with (a) doxorubicin (Dox), (b) imatinib (Imat), in K562 cell line, (c) Dox and (d) Imat, in TK6 cell line, evaluated by cell counting with a Neubauer chamber. Results are expressed as mean \pm standard deviation (SD) of at least four independent experiments. Significant differences (* p \leq 0.05; ** p \leq 0.01; **** p \leq 0.0001), when compared with those of the control, were tested by one-way ANOVA, followed by post-hoc Dunnett's tests.

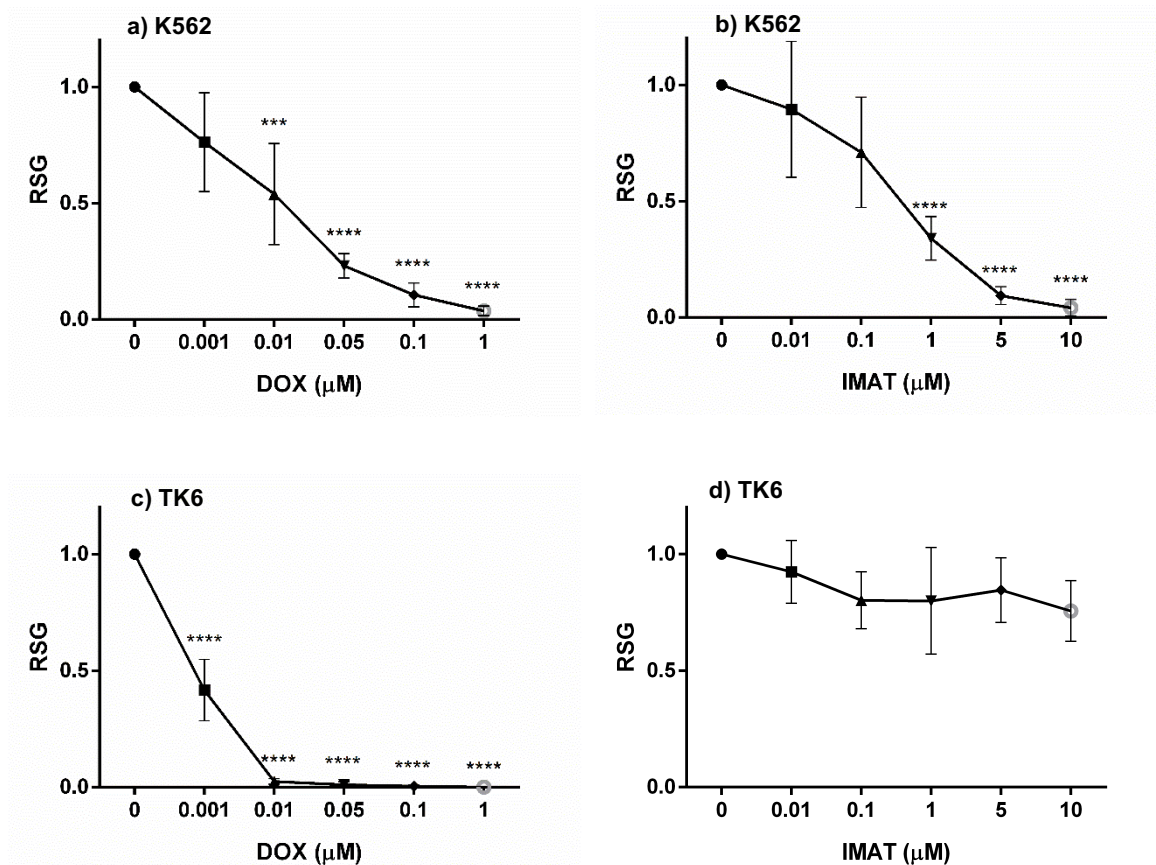


Figure 2 – Dose-response effects on cell proliferation via Relative Suspension Growth (RSG), after 24 h of incubation plus 48 h in new medium with (a) doxorubicin (Dox) and (b) imatinib (Imat), in K562 cell line; (c) dox and (d) imat in TK6 cell line, evaluated by cell counting with a Neubauer chamber. Results are expressed as mean \pm standard deviation (SD) of at least four independent experiments. Significant differences (***) $p \leq 0.001$; **** $p \leq 0.0001$) when compared with the control were tested by one-way ANOVA, followed by post-hoc Dunnett's tests.

Effects of fucoxanthin and phloroglucinol alone or combined with imatinib / doxorubicin on cell viability and proliferation in leukemia cell lines

Cells were incubated with fucoxanthin (0-10 μM) or phloroglucinol (0-300 μM) alone and in combination with IC_{30} of doxorubicin or imatinib for 24 h (cytotoxic assay). The results showed that fucoxanthin at 10 μM alone inhibits cell viability by 30% but only in K562 cells (Figure 3a). In TK6 cell line, fucoxanthin did not induce cytotoxic effects when compared with the respective control (Figure 3c and d). In combination, fucoxanthin did not potentiate the

cytotoxic activity of both drugs in the cell lines tested. An exception was the combination (10 μM Fx + 0.05 μM Imat) in K562 cells that significantly decreases cell viability by 29% when compared with imatinib alone, but there are no differences when related with fucoxanthin alone (Figure 3b).

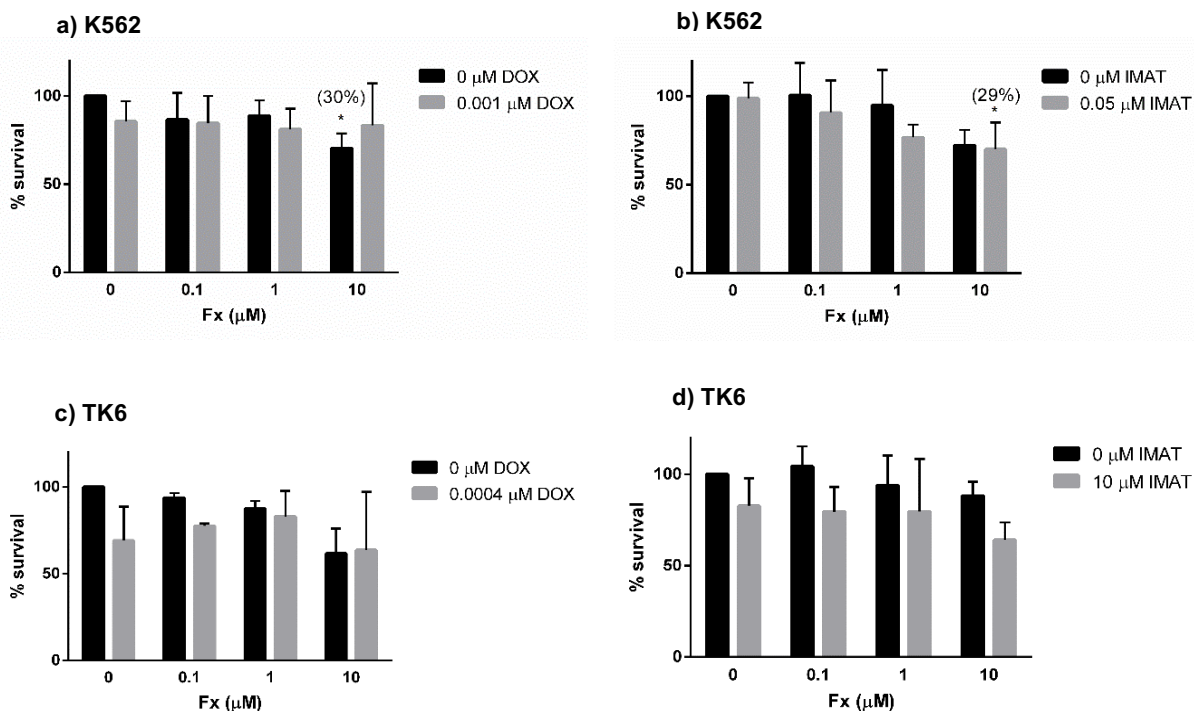


Figure 3 - Effect of fucoxanthin (Fx) alone (black bars) or in combination (gray bars) with doxorubicin (Dox) and imatinib (Imat) (at IC_{30}) on cell cytotoxicity after 24 h of treatment and assessed by cell counting. Fx combined with (a) Dox or (b) Imat in K562 cell line; Fx combined with (c) Dox or (d) Imat in TK6 cell line. Results are expressed as mean \pm standard deviation (SD) of at least four independent experiments. Significant differences (* $p \leq 0.05$) among groups per situation of exposure were tested by two-way ANOVA, followed by post-hoc Bonferroni multiple comparison test. Percentages in brackets showed the decrease on cell viability in relation to respective control.

Phloroglucinol alone, at the conditions tested, did not affect cell viability of both cell lines. Also, the combination of phloroglucinol with doxorubicin or imatinib did not increase the cytotoxicity of the drugs in the tested cell lines (Figure 4).

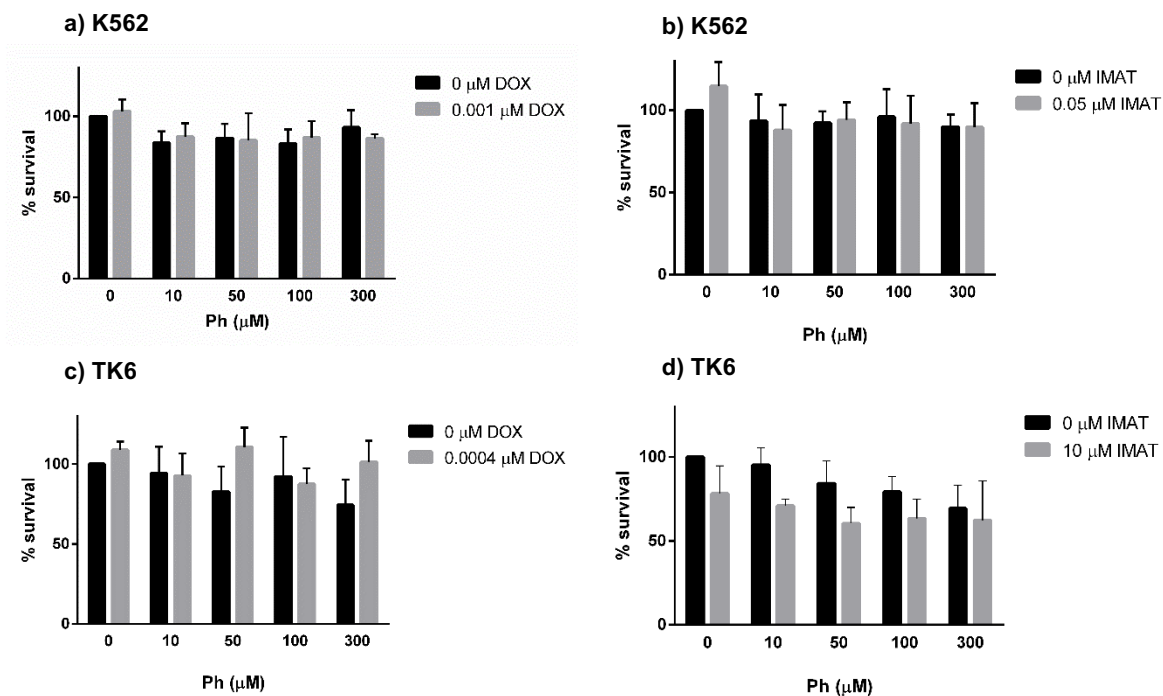


Figure 4 - Effect of phloroglucinol (Ph) alone (black bars) or in combination (gray bars) with doxorubicin (Dox) and imatinib (Imat) (at IC₃₀) on cell cytotoxicity after 24 h of treatment and assessed by cell counting. Ph combined with (a) Dox or (b) Imat in K562 cell line; Ph combined with (c) Dox or (d) Imat in TK6 cell line. Results are expressed as mean ± standard deviation (SD) of at least four independent experiments.

Attending to the results from the proliferation assay, in general, the inhibitory effects were more evident than in the cytotoxic assay. Fucoxanthin, in both cell lines, decreased cell proliferation, showing an inhibition around 50% for the highest concentration tested (10 μm) for both cell lines (Figure 5). In combination, fucoxanthin at 10 μm increased the antiproliferative effects of doxorubicin and imatinib in 36% and 55%, respectively, in K562 cells (Figure 5a and b). In TK6 cell line, only the co-incubation of 10 μm Fx with imatinib potentiated the antiproliferative effects of the drug by 41%, when compared with the drug alone. However, no significant differences were obtained when compared with the fucoxanthin alone in both cell lines tested. The combination 0.1 μm Fx + 10 μm Imat inhibited cell proliferation of TK6 cells in 48% when compared with fucoxanthin alone but did not show differences regarding the drug alone (Figure 5d).

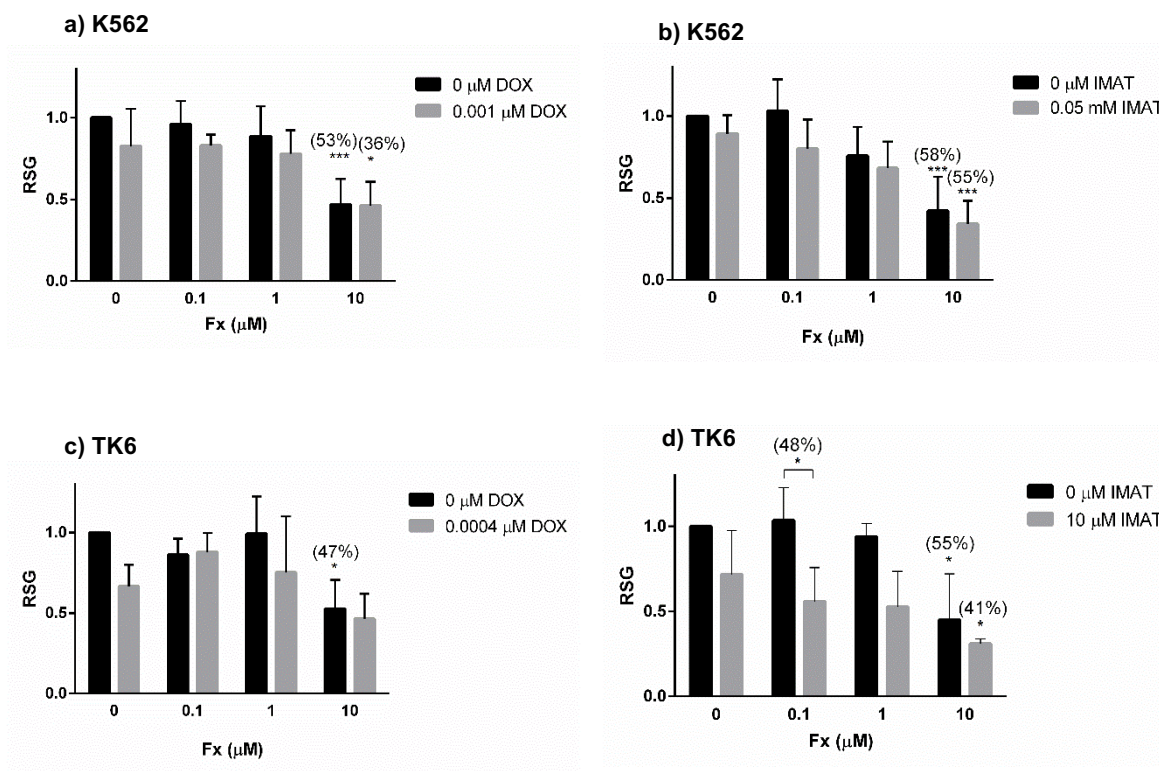


Figure 5 - Effect of fucoxanthin (Fx) alone (black bars) or in combination (gray bars) with doxorubicin (Dox) and imatinib (Imat) (at IC₃₀) on cell proliferation via Relative Suspension Growth (RSG) after 24 h of treatment plus 48 h in new medium, assessed by cell counting. Fx combined with (a) Dox or (b) Imat in K562 cell line; Fx combined with (c) Dox or (d) Imat in TK6 cell line. Results are expressed as mean ± standard deviation (SD) of at least four independent experiments. Significant differences (* p ≤ 0.05; *** p ≤ 0.001) among groups per situation of exposure were tested by two-way ANOVA, followed by post-hoc Bonferroni multiple comparison test. Percentages in brackets showed the decrease on cell proliferation in relation to respective control.

Phloroglucinol alone did not show any significant decrease in cell proliferation in K562 cells (Figure 6a). In co-incubation, only (300 μm Ph + 0.05 μm Imat) decreased cell proliferation by 34% when compared with imatinib alone (Figure 6b). In TK6 cells, phloroglucinol caused a decrease in cell proliferation in a dose-dependent manner reaching, at the highest concentration (300 μm of Ph), around 60% of inhibition when compared with the respective control. In combination with doxorubicin, phloroglucinol did not affect cell proliferation in TK6 cells. However, a significant increase of the proliferation capability (46%) was observed in the combination (50 μm Ph + 0.0004 μm Dox) when compared with phloroglucinol alone (Figure 6c). Contrary, the combination of phloroglucinol with imatinib in TK6 cells at 50 and 300 μm significantly decreased cell proliferation when compared with imatinib alone, 28% and 44%, respectively. The inhibition induced by the combination 50 μm of Ph + Imat was also

significantly different when compared with phloroglucinol alone at the same concentration (Figure 6d). In resume, phloroglucinol seems to potentiate the antiproliferative activity of imatinib in TK6 cells.

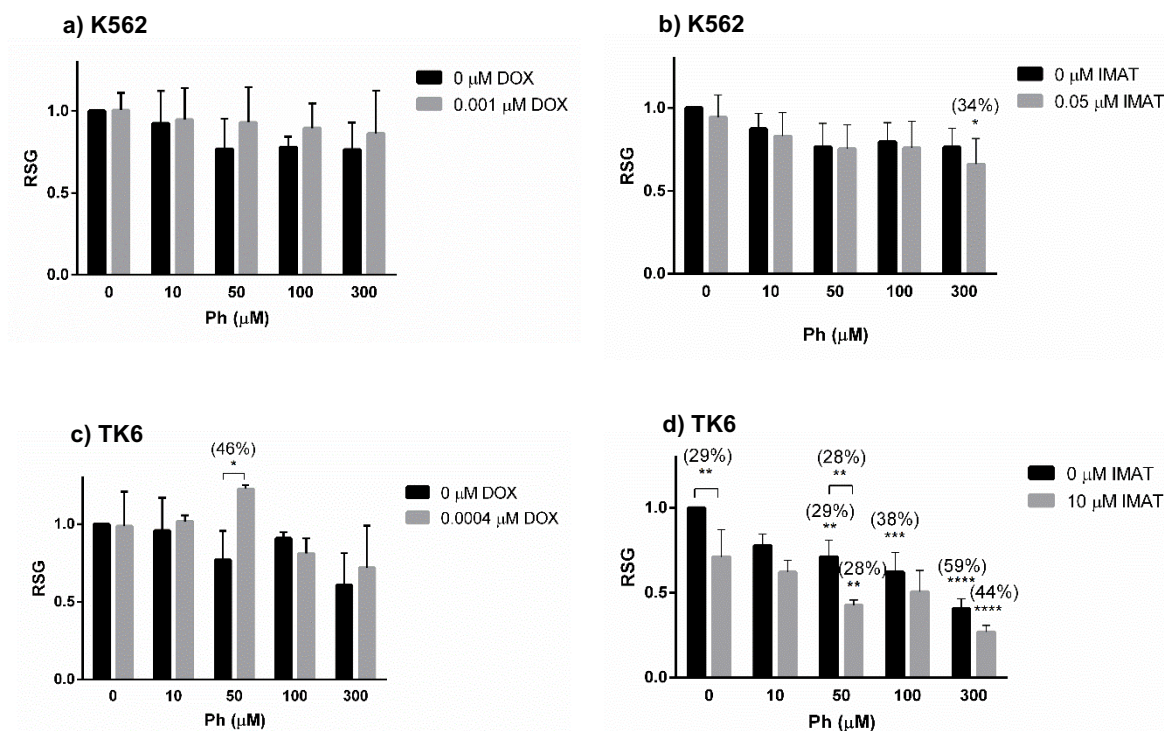


Figure 6 - Effect of phloroglucinol (Ph) alone (black bars) or in combination (gray bars) with doxorubicin (Dox) and imatinib (Imat) (at IC₃₀) on cell proliferation via Relative Suspension Growth (RSG) after 24 h of treatment plus 48 h in new medium, assessed by cell counting. Ph combined with (a) Dox or (b) Imat in K562 cell line; Ph combined with (c) Dox or (d) Imat in TK6 cell line. Results are expressed as mean ± standard deviation (SD) of at least four independent experiments. Significant differences (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001) among groups per situation of exposure were tested by two-way ANOVA, followed by post-hoc Bonferroni multiple comparison test. Percentages in brackets show the decrease or increase on cell proliferation in relation to respective control.

The antiproliferative effects of fucoxanthin and phloroglucinol alone or in co-incubation with anticancer drugs (doxorubicin and imatinib) were confirmed in some combinations by the decrease of cell density and by alterations in cell morphology using a phase contrast microscope (data not shown) in K562 and TK6 cell lines. To evaluate the specific mechanisms behind these antiproliferative effects, some conditions that showed statistically significant differences when compared with the controls were selected for the following assays.

3.2. Effects of fucoxanthin and phloroglucinol alone and in co-incubation with doxorubicin and imatinib on DNA damage by comet assay in leukemia cell lines

To assess the genotoxic effects of the natural compounds alone or in combination with these drugs, cells were incubated with selected conditions for 24 h and DNA damage (strand breaks and the FPG-sensitive sites) assessed by alkaline comet assay.

As a positive control, K562 and TK6 cell lines were exposed to oxidative stress-inducing photosensitizer Ro 19-8022 and an increase in the DNA damage recognized by FPG was observed in all the concentrations tested (data not shown). In relation to the selected conditions, the results showed that none of the tested conditions increased the DNA damage detected by comet assay in K562 cells (Figure 7a). In TK6 cells, only doxorubicin significantly increased DNA damage recognized by FPG, with an increase of 16% in relation to the negative control (cells treated with 0.5% DMSO). These are preliminary results since it was not possible to quantify all the data from the comet assay.

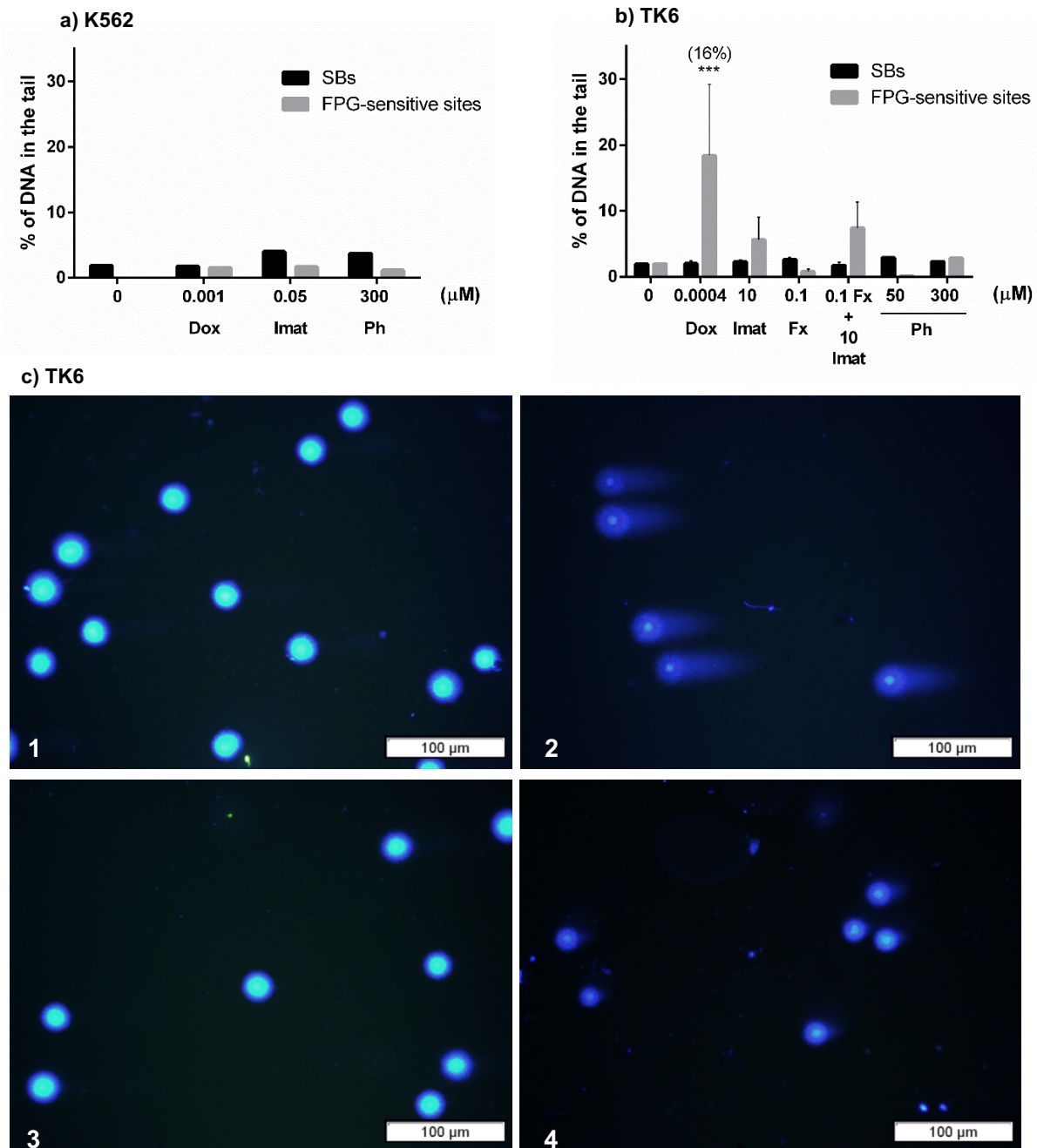


Figure 7 – Effect of natural compounds, fucoxanthin (Fx) and/or phloroglucinol (Ph) alone or in combination with doxorubicin (Dox) or imatinib (Imat) on DNA damage (strand breaks – SBs; FPG-sensitive sites) after 24 h in (a) K562 cell line and (b) TK6 cell line, assessed by comet assay, (c) Images from comet assay in TK6 cell line: 1 – SBs of negative control; 2 – FPG-sensitive sites of positive control; and 3 – SBs and 4 – FPG-sensitive sites in 0.0004 μM of Dox. Results are expressed as mean ± standard deviation (SD) of at least two independent experiments. Significant differences (***) $p \leq 0.001$ among groups per situation of exposure were tested by one-way ANOVA, followed by post-hoc Bonferroni multiple comparison test. Percentages in brackets show the increase in relation to respective control. In the case of K562 cells, the results are exploratory as they correspond at only one independent experiment (ongoing work). Scale bar – 100 μm.

3.3. Effects of fucoxanthin and phloroglucinol alone and in co-incubation with doxorubicin on cell death by nuclear condensation in leukemia cell lines

To evaluate if the inhibition of cell proliferation was caused by induction of cell death, the cells (K562 and TK6) were incubated, with the conditions previously selected, for 48 h and nuclear chromatin condensation was performed. As shown in Figure 8a, none of the experimental conditions tested increased the percentage of condensed nuclei in K562 cells. In TK6 cell line, only 0.0004 μm of doxorubicin induced an increase of 10% in the condensed nuclei when compared with the control. Fucoxanthin at 10 μm in combination with doxorubicin significantly decreased the condensed nuclei by 14% when compared with doxorubicin (Figure 8b). Phloroglucinol was tested only in TK6 cell line and the results did not show any significant increase in the percentage of condensed nuclei (Figure 8c).

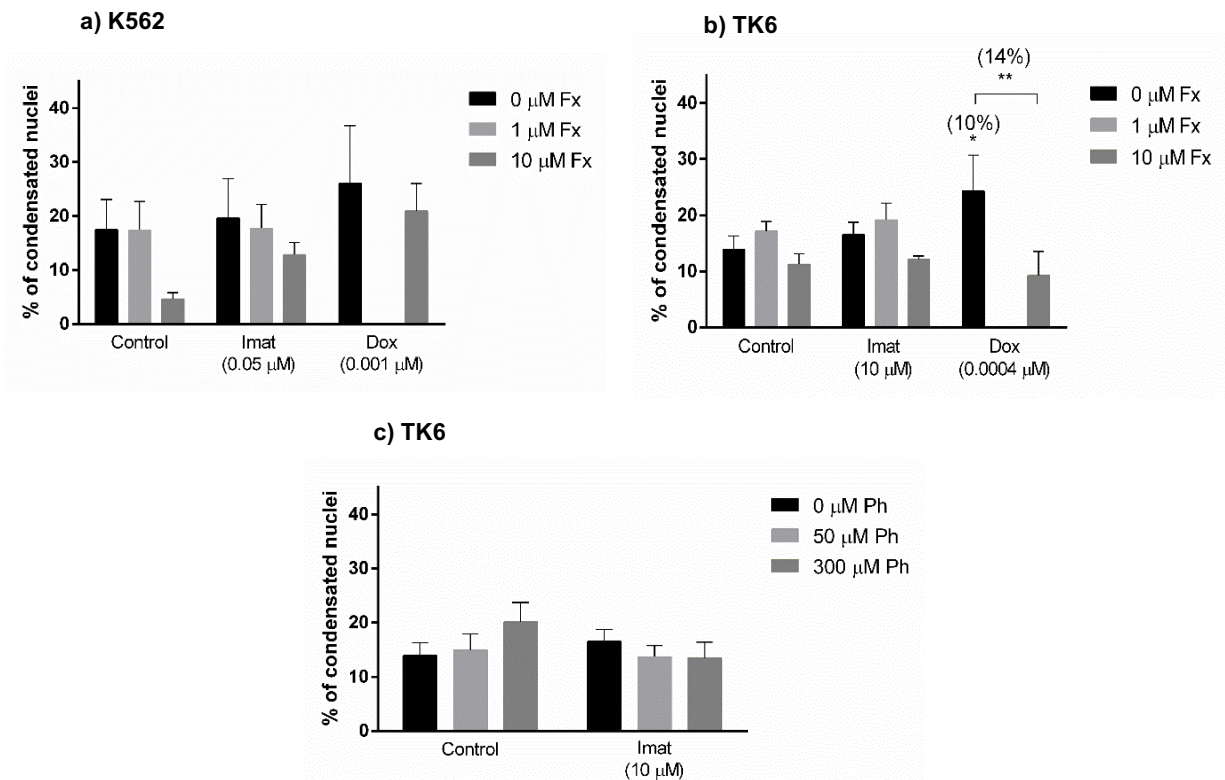


Figure 8 – Effect of fucoxanthin alone or co-incubated with doxorubicin (Dox) or imatinib (Imat) (IC₃₀) on the induction of nuclear chromatin condensation in (a) K562 cell line and (b) TK6 cell line, and (c) phloroglucinol (Ph) in TK6 cell line, evaluated by the nuclear condensation assay after 48 h of incubation. Results are expressed as mean ± standard deviation (SD) of at least four independent experiments.

4. Discussion

CML is a myeloproliferative disorder characterized by the presence of a new gene (*BCR-ABL*). Imatinib, a specific inhibitor of BCR-ABL protein kinase, is the first line of treatment for CML-CP, and has a great efficacy (Druke et al. 2006). However, in advanced stages of the disease (CML-BC) more aggressive treatments are applied. The search for new effective therapies with less side-effects are needed.

Marine organisms are a rich source of bioactive compounds with several biological activities (Cornish and Garbary 2010, Sharif et al. 2014). Previous studies demonstrated that fucoxanthin, the most abundant marine carotenoid, and phloroglucinol, a phlorotannin, have in vitro cytotoxic effects against cancer cell lines (Kim et al. 2005, Kotake-Nara et al. 2005, Ganesan et al. 2011, Liu et al. 2011). The current work addresses – to our knowledge for the

first time – the in vitro anticancer activity of fucoxanthin and phloroglucinol alone or co-incubated with anticancer drugs (imatinib and doxorubicin) in two human cancer cell lines representative of more advanced stages of CML (K562 and TK6 cell lines). For this, (i) short time cytotoxicity; (ii) proliferation capability; (iii) DNA damage induction; and (iv) cell death induction by apoptosis, were evaluated.

Our results showed that doxorubicin seems to be more effective than imatinib in decrease cell viability and proliferation since it caused inhibitory effects at lower concentrations in both cell lines. However, imatinib proved to be more specific to K562 cell line, as expected. The efforts to eliminate cancer cells requires the knowledge of the cellular responses that are induced by anticancer drugs. Imatinib, a TKI, allows a faster reduction of *BCR-ABL* transcript levels when compared with doxorubicin which has a large range of cellular targets (Czyz et al. 2008, Hanfstein et al. 2012, Minotti et al. 2004, Zhou and Xu 2015). Furthermore, the understanding of the characteristics of each cell line is also an important point. K562 cells are derived from a patient in blast crisis, being normally used as a cellular model for advanced stage of CML to investigate the cellular response to cytotoxic drugs. While TK6 cells were established from a patient with T-cell lineage blast crisis of CML and further complicated by hypercalcemia (Watanabe et al. 1995, Naumann et al. 2001).

In a general way, the inhibitions obtained in the proliferation assay are higher than in the cytotoxic assay, which could mean that the effects are not related with direct cytotoxicity of the compounds, but are mediated by effects on different signaling pathways. Fucoxanthin decreased cell viability (in K562 cells) and proliferation in both cell lines tested. When co-incubated with doxorubicin at IC_{30} , fucoxanthin did not affect cell viability of both cell lines. However, the co-incubation with imatinib increased the cytotoxic effect of imatinib in K562 cells. In co-incubation, fucoxanthin plus doxorubicin or imatinib inhibited cell proliferation in K562 cells. In TK6 cells, only fucoxanthin plus imatinib decreased cell proliferation when in combination. However, no significant differences were obtained when compared with the inhibition caused by fucoxanthin alone. Previous studies reported the antiproliferative effects of fucoxanthin on other leukemia cell lines, such as the HL-60 (Kotake-Nara et al. 2005, Ganesan et al. 2011). Furthermore, Ishikawa et al. (2008) reported that fucoxanthin was not cytotoxic to normal peripheral blood mononuclear cells supporting the specificity action of this natural compound on cancer cell lines.

Phloroglucinol showed ability to decrease cell proliferation only in TK6 cell line, starting to induce a slight inhibition of proliferation at 50 μm and reaches 50% of inhibition with the

highest concentration (300 μm). In co-incubation with doxorubicin (at IC_{30}) the proliferation was not affected, however, one condition revealed an increase of cell proliferation (e.g. 50 μm Ph + 0.0004 μm Dox in TK6 cells). The same results were observed in our laboratory by other colleges, where phloroglucinol at low concentrations seems to protect cancer cell lines (HT-29 and HCT116). Contrary, the combination of phloroglucinol with imatinib showed a great potential in TK6 cells. In this vein, the potentiation of the antiproliferative effects of imatinib was higher in TK6 cells, reaching almost 75% of inhibition when compared with control (cells treated with 0.5% DMSO). These data are in line with other studies that reported antiproliferative effects of phloroglucinol in several cell lines (Hostanska et al. 2003, Liu et al. 2011, Sun et al. 2011, Wijesinghe et al. 2013). The antiproliferative effects of fucoxanthin and phloroglucinol alone or in co-incubation with doxorubicin and imatinib were confirmed in this study in some combinations, either by the reduction of cell density or by alterations in cell morphology.

The decrease in cell proliferation could be due to several mechanisms, such as increase of cell death and/or delayed mitosis. In the present work, we showed that decrease of cell proliferation induced by doxorubicin could be partially due to induction of cell death by apoptosis in TK6 cells; because a slight increase of nuclear condensation was observed. However, induction of apoptosis seems not to be the main mechanism behind the antiproliferative effect. In K562 cells, inhibition of cell proliferation induced by doxorubicin was not related with changes in the percentage of cells with nuclear condensation suggesting induction of other pathways. Different values of nuclear condensation between cell lines can be explained by the lack of a functional p53 protein observed in K562 cell line, which allow them to be more resistant to apoptosis, while TK6 cells express a stable p53 (Kuzelova et al. 2005, Schwartz et al. 2004). Other studies reported the same results, and the apoptosis reluctance, in both cell lines, can be explained due to the presence of deregulated tyrosine kinase activity of BCR-ABL protein. BCR-ABL protein phosphorylates substrate proteins which stimulate several signal transduction pathways (such as Ras, STAT5 and PI-3k) resulting in the increase of proliferation rate and resistance to apoptosis (Kuzelova et al., 2005, Laroche-Clary et al. 2000, Watanabe et al. 1995). Even using imatinib, that inhibits BCR-ABL kinase activity, the inhibition may not have been complete, as so low levels of activity allowed cells to survive but not proliferate (Druker et al. 2006). Others mechanisms that have been described, could explain the low levels of apoptosis in the tested cell lines, such as *BCR-ABL* gene amplification and mutations, increase of drug efflux by overexpression of the multidrug resistant P-glycoprotein (P-gp) and low activity of drug influx transporter (hOCT1). In particular to imatinib, the apoptosis resistance could be also influenced by the increase of plasma protein α 1 acid glycoprotein (AGP) that

inhibit imatinib action, and the prostaglandin-endoperoxide synthase 1 involved in imatinib metabolism (Apperley 2015, Druker et al. 2006, Gambacorti-Passerini et al. 2000, Mahon et al. 2000, Villuendas et al. 2006). Czyz and collaborators demonstrated the induction of other pathways, such as senescence or differentiation by doxorubicin and imatinib in leukemic cells (Czyz et al. 2008).

Our results suggest that the antiproliferative effect of fucoxanthin and phloroglucinol seems to be not related to induction of cell death since no significant increase of nuclear condensation was observed in both cell lines. However, fucoxanthin at 10 μm when co-incubated with doxorubicin in TK6 cell line decreased the levels of nuclear condensation when compared with the drug alone. Ganesan et al. (2011) also reported low levels of apoptosis induced by fucoxanthin, which can indicate that the reduction in cell viability can be due to other mechanisms. Ishikawa et al. (2008) showed the ability of fucoxanthin to induce cell cycle arrest but also apoptosis, in agreement with other authors that reported the ability of fucoxanthin to induce apoptosis in leukemia cell lines, in opposite to our results (Kotake-Nara et al. 2005, Kim et al. 2010). An important characteristic of fucoxanthin to have in consideration for further utilization in cancer therapy is their ability to protect normal cell lines by inhibition of DNA damage and apoptosis (Heo et al. 2008). Regarding phloroglucinol, no significant changes were observed in the nuclear condensation when cells were treated with the selected combinations. In opposition to our results, induction of apoptosis by phloroglucinol in leukemia cell lines was reported by other authors (Hostanska et al. 2003, Liu et al. 2011, Sun et al. 2011). As already noted by other authors and the same was found in our work, apoptosis induction was not the major mechanism for the reduction in cell proliferation after exposure to doxorubicin or imatinib (IC_{30}) alone or in combination with the natural compounds (Castañeda et al. 2012). However, the analysis of nuclear condensation after longer incubation periods and other assays, such as TUNEL, Annexin V, cytochrome release or measure the levels of caspases, are needed to verify the induction of apoptosis. Other mechanisms such as cell cycle arrest or another type of cell death could be involved in the antiproliferative effects of the tested compounds and should be evaluated.

DNA damage was assessed here since chemotherapeutics are known to induce it. DNA damage if not correctly repaired will accumulate in the cell and can cause either cell cycle arrest or apoptotic cell death (Bellarosa et al. 2001, Abubakar et al. 2012). Regarding our preliminary results, in K562 cell line none of the tested compounds induced DNA damage recognized by comet assay. In TK6 cell line, DNA strand breaks remained very low after cell exposure at

selected conditions. These results are supported by the presence of deregulated tyrosine kinase activity of BCR-ABL protein, as explained before, some pathways such as STAT5 can enhance the global levels of DNA repair mechanisms. However, doxorubicin in the tested conditions proved to be able to increase DNA damage recognized by FPG enzyme (recognize oxidative damage). Other authors already reported that, doxorubicin (Ferreira et al. 2007, Manjanatha et al. 2014) and imatinib (Koptyra et al. 2006) can increase FPG-sensitive sites in different cell types. Our preliminary results seem to indicate that doxorubicin can induce oxidative DNA damage that could culminate in a decrease of cell proliferation. This was an expected effect because the anticancer activity of many chemotherapeutic drugs is mainly due to their ability to induce DNA damage (Skorski 2002). However, the precise response to DNA damage should be further assessed.

In conclusion, the results presented here indicate that both natural compounds alone showed antiproliferative effects against K562 (fucoxanthin) and TK6 (fucoxanthin and phloroglucinol) cells. Fucoxanthin increased the cytotoxic effects of doxorubicin (in K562 cells) and imatinib in both cells lines. However, this enhancement seems to be due to fucoxanthin alone and not due to the combination with the drug. Phloroglucinol increases the antiproliferative effects of imatinib in both cell lines but with more extension in TK6 cells. In this study, the antiproliferative effects of both compounds seem not to be due to cell death caused by apoptosis. However, more research is needed to confirm our results and to demonstrate the real potential of fucoxanthin and phloroglucinol against the cell lines tested.

Conflict of interest

There are no conflicts of interest to report.

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CHAPTER IV – CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions and future perspectives

The purpose of this study was to evaluate the anticancer activity of two natural compounds present in seaweed – fucoxanthin and phloroglucinol – alone or in combination with anticancer drugs (doxorubicin and imatinib), on two human cancer cell lines (K562 and TK6 cells) representative of CML-BC.

In vitro anticancer activity was evaluated by the decrease of cell viability and cell proliferation after a short-term exposure when compared with negative control (cells treated with 0.5% DMSO). Our results showed, as expected, that doxorubicin is more effective in the decrease of cell viability when compared with imatinib in both cell lines. K562 cells proliferation were affected in the same way by both drugs, yet TK6 cells proliferation was inhibited only by doxorubicin. Our study confirmed the specificity of imatinib to K562 cell line that can be explained by the specific mechanism of action of imatinib which binds to BCR-ABL specific-sites. Different genetic characteristics are reported in the literature for each cell line, which could explain the differences attained in the results. Analyzing the data obtained by the natural compounds, fucoxanthin alone decreases cell viability in K562 and proliferation in both cell lines at 10 μm . In co-incubation with doxorubicin or imatinib (at IC_{30}), fucoxanthin enhances the antiproliferative effects of the drugs in both cell lines. Phloroglucinol alone did not induce direct cytotoxicity at short term neither when co-incubated with the drugs. Cell proliferation seems to be affected by phloroglucinol, reaching 50% at the highest concentration tested in TK6 cell line. In co-incubation with imatinib (at IC_{30}) showed an antiproliferative effect in both cell lines, higher in TK6 cells with inhibition reaching 75% when compared with the control.

The decrease in cell proliferation could be due to increased cell death and/or decrease of mitosis rate. In our study, the decrease of cell viability and cell proliferation of doxorubicin could be partially explained by increase of oxidative DNA damage and apoptotic levels. However, imatinib, fucoxanthin and phloroglucinol did not show ability to induce DNA damage and cell death through apoptosis. Previous studies already demonstrated that induction of apoptosis is one of the main mechanisms behind the anticancer activity of both natural compounds. However, a very important characteristic of both cell lines is their apoptosis reluctance, which can explain the low levels of cell death (detected by nuclear condensation assay) in this work. The antiproliferative effects of fucoxanthin and phloroglucinol could possibly

not be related with induction of DNA damage since no effects were observed by comet assay. However, the presented data are not enough to draw a definitive conclusion.

Even with the excellent advances in CML treatments, patients (mainly in blast crisis) remain without adequate therapies, so alternative treatments are necessary. As future perspectives, and having in consideration our results, since fucoxanthin and phloroglucinol appear to have some anticancer activity in the tested cell lines it will be interesting to understand how these natural compound act. First, is necessary to finish the analysis of the data from comet assay to determine the real levels of DNA damage. The next step is to use other assays to confirm the apoptosis induction (e.g. TUNEL, annexin V, cytochrome c release and caspases activation) using longer exposure times. Then other types of assays can be used to explore the exact mechanisms involved in the antiproliferative effects (e.g. assessment of mitochondrial membrane potential, cell cycle arrest, determination of reactive oxygen species (ROS) generation, selective gene expression and signaling pathways activation related with cell proliferation). It would be of interest to further investigate the action of these compounds in normal cell lines to determine the anticancer specificity and if possible include primary cells in the study.

The search for natural compounds with an anticancer activity that could work alone or co-incubated with existing anticancer drugs is one important field to investigate. A deep understanding of the molecular mechanisms involved are needed to develop targeted and selective therapies. Here, we reported for the first time the potential of fucoxanthin to enhance the efficacy of anticancer drugs such as doxorubicin (in K562 cells) and imatinib (K562 and TK6 cells) while phloroglucinol increase the effect of imatinib in both cell lines. Increase the therapeutic efficiency and minimize the toxicity against normal cells may bring huge benefits to patient so all the efforts to address these goals are welcome.

APPENDICES

Principle

The technique allows the cells to grow and be maintained outside the body, this provides us an opportunity to study the biochemistry and molecular biology associated with both normal and cancer cell lines. George Gey developed, in 1951, the first continuous human cancer cell line (HeLa). The great advantage of cell lines is their ability to provide a renewable source of cell material that can be used in several types of studies. Yet sometimes the in vitro culture does not reflect most of the clinical cases. Cells can either grow adherent or in suspension, the majority are adherent to a “substrate” such as plastic or glass and the growth is in monolayer. Regarding the neoplastic cells, they are initially extracted from tumors and cultivate in appropriate medium with certain additives for growth. The maintenance of some physical environmental conditions is required for the optimal cell growth and to maintain durable cultures, such as the temperature at $36.5\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$, pH ideally around 7.4, CO_2 atmosphere frequently at 5 % and control O_2 tension (Langdon 2004, Cree 2011).

In an initial phase, after defrosting, the cells should be only maintained, being subjected to successive passages/dilutions only when the confluence is reached (during at least two weeks), to ensure the stabilization of the growth. After that, the cells can be used to perform the desired experimental assays. All the cells pass through some well-defined stages of growth, the “lag phase” characterized for a relatively slower growth right after a passage. Then the culture will reach the most rapid phase of growth, called “log phase”. Finally, with the high cell density, space starts to be less and the medium poor, the growth rate decreases again and this is referred as “plateau phase” (Langdon 2004).

The culture should be regularly checked (daily if possible) macroscopically and microscopically. Regarding the microscope observation, the morphology and cell density is evaluated, and the possible presence of fungus and bacteria in the culture. Macroscopically, the color and turbidity are the most important aspect to be considered. The color will indicate a change in the pH of the medium (in the present of phenol red). So, the medium should be renewed frequently to avoid the lack of specific nutrients or the acidification of the medium, the frequency of the medium renewal will depend on the cell lines and their growth rate. The increase of turbidity could indicate the presence of contamination (Langdon 2004, Freshney 2006).

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Materials

- Humidified, controlled temperature/CO₂ incubator
- Inverted phase contrast microscope (e.g. OLYMPUS SKX41, Japan)
- Laminar flow hood
- Neubauer chamber
- T25 cell culture flasks

Assay solutions and reagents

- Antibiotics (100U/ml penicillin and 100 µg/ml streptomycin)
- Cell culture medium (e.g. RPMI)
- Fetal Bovine Serum (FBS)

Work solutions preparation

(1) Cell culture medium (RPMI)

	Final concentration	Weight / Volume
RPMI	10.4 g/l	5.2 g
Sodium bicarbonate	2 g/l	1 g
Sodium pyruvate	110 mg/l	55 mg
HEPES	10 mM	1.192 g
FBS	10%	50 ml
Penicillin / Streptomycin	1%	5 ml

Weight and dissolve the reagents in ultrapure water to a final volume of 445 ml and mix until completely dissolved. Adjust pH to 7.4. Inside the laminar flow hood, filtrate the solution using a filter with 0.2 µm pore. Add FBS and antibiotics (penicillin/streptomycin), seal properly and store at 4 °C. Prepare one aliquot of 50 ml for each cell line to prevent the contamination of the stock solution of medium. All the mediums should be daily checked to avoid contamination.

General guidelines

Starting a new culture:

1. To start a new culture of cells, thaw an aliquot of frozen cells by mixing them with the appropriate cell culture medium. This process must be fast in order to avoid cytotoxicity from the DMSO used in the freezing medium.
2. Pipette the cells into a sterile centrifuge tube.
3. Add more medium into the centrifuge tube (approx. 10 ml in total).
4. Centrifuge the cells at 1300 rpm for 5 min.

5. Discard the medium with DMSO and add 5 ml of fresh and warm medium. Resuspend the pellet and transfer to a sterile cell culture flask.
6. Incubate at 37 °C and 5% CO₂ in a humidified incubator. The flask should be always standing vertically (for non-adherent cells).

Routine feeding and maintenance for non-adherent cells:

1. Medium containing cells must be centrifuged in a sterile manner, at 1300 rpm for 5 min.
2. Resuspended the pellet in fresh medium and transfer to a sterile cell culture flask.

Subculture of non-adherent cells:

1. Cell suspension should be checked to ensure that the cells are in late log/early plateau phase and to confirm that the cells are healthy and free of contamination.
2. The cell suspension can then either be counted if an accurate cell density is required, or the suspension can simply be “split” into an appropriate ratio (1/5 – 1/10).
3. Cell culture flasks are then returned to the CO₂ incubator. After 24 h, the culture should be checked to ensure that the pH of the medium is approx. pH 7.4.

P2. Viability assay

Principle

There is a wide variety of assays designed to evaluate cellular drug sensitivity. These assays are mainly divided into two groups: The measurement only of cytotoxicity and those that measure the cell proliferation. Cytotoxicity assays at short-term allow assessing the direct cytotoxic effect of the treatment. The proliferation assays require exposure for long-term to assess effects on the cell ability to proliferate (Langdon 2004). The cytotoxic and proliferative effects can be assessed through cell viability assays such as trypan blue dye exclusion (Langdon 2004). However, other types of assays are available to assess cell viability.

References

Langdon, S. P. (2004). "Basic principles of cancer cell culture." Cancer Cell Culture: Methods and Protocols: 3-15.

Materials

- 24-multiwell culture plate
- Inverted phase contrast microscope (e.g. Olympus SKX41, Japan)
- Microcentrifuge tube
- Neubauer chamber

Assay solutions and reagents

- Cell culture medium (e.g. RPMI)
- Trypan blue

General guidelines

Cell preparation:

1. In sterile conditions, plate 500 µl of cells in a 24-multiwell culture plate with the appropriate cellular density (K562: 0.3×10^6 cell/ml and TK6: 0.2×10^6 cell/ml) and incubate with the desired treatments.
2. Cells should be incubated for 24 h in a humidified incubator at 37 °C and 5% CO₂.
3. When the incubation time is complete, the content of each well is transferred to a microcentrifuge tube and centrifuged for 5 min at 1800 rpm.
4. Discard the supernatant and resuspend the pellet with 500 µl of fresh medium. Transfer 100 µl of each condition to a new 24-multiwell culture plate and fill until 500 µl with fresh medium. Incubate more 48 h in a humidified incubator at 37 °C and 5% CO₂.
5. The remaining cellular suspension is used to count the cellular density after 24 h of treatment using 10 µl of trypan blue in the Neubauer chamber (1:1), where live cells with intact cell membranes remain unstained. For each condition were used the mean of four squares counted in the Neubauer chamber – Cytotoxicity assay.
6. At the end of 48 h the cellular density was also determined by the same method – Proliferation assay.

Calculations:

- **Cytotoxicity assay:** The percentage of cellular viability for each condition was calculated based on the final control (t = 24 h), using the following equation:

$$\% \text{ viability} = \frac{\text{cel/ml sample} \times 100}{\text{cel/ml control}}$$

- **Proliferation assay:** The first step is to calculate the total suspension growth (TSG) using the density plated (t=0) and the cell density at the 72 h. Then is calculated the relative suspension growth that has in consideration the control. The respective equations are represented bellow:

$$\text{TSG} = \frac{\text{cel/ml (72h)}}{\text{cel/(ml)(0 h)}}$$

$$\text{RSG} = \frac{\text{TSG sample}}{\text{TSG control}}$$

Principle

The alkaline comet assay, also known as single cell gel electrophoresis, is a widely used method to measure in vitro and in vivo DNA damage (Neri et al. 2015). It detects strand breaks (SBs) and alkali-labile sites since a few hundred to several thousand breaks per cell. This technique allows to measure from low endogenous damage levels to a considerable amount of damage that can be caused experimentally without killing cells. The digestion of some nucleoids, after lysis, which present certain lesion-specific repair endonucleases enable the measurement of others damage than SBs, e.g. formamidopyrimidine DNA glycosylase (FPG) has been widely used to detect altered purines, which are converted to breaks by the enzyme (Azqueta and Dusinska 2015, Langie et al. 2015). The DNA damage or, in other words, the percentage of DNA in the tail, estimated by fluorescence staining using DAPI and observation under a fluorescence microscope reflects the amount of DNA breaks (Azqueta et al. 2011).

References

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Materials

- 6-multiwell culture plate
- Centrifuge tubes
- Coplin jar
- Cover slides (20 x 20 mm) and cover slides (22 x 22 mm)
- Electric power supply
- Fluorescence microscope (e.g. Olympus IX71, Japan)
- Horizontal electrophoresis tank
- Microwaves
- Metal tray
- Microcentrifuge tube
- Microscope slides
- Thermoblock

Assay solutions and reagents

- 0.5% (w/v) Low melting point (LMP) Agarose
- 1% (w/v) Normal melting point (NMP) Agarose
- Phosphate buffered saline (PBS)
- Buffer F (pH 8)
- DAPI staining solution (1 $\mu\text{g}/\text{ml}$)
- Electrophoresis buffer (pH 13)
- Lysis solution (pH 10)

Work solutions preparation

(1) Phosphate Buffered Saline (PBS) – 1L

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na ₂ PO ₄	10 mM	1.44 g
KH ₂ PO ₄	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure water. Adjust pH to 7.4. Add necessary water for a final volume of 1 L. If necessary, autoclave the solution to sterilize and store at 4 °C.

(2) 1 % NMP Agarose

To prepare 200 ml of NMP agarose (necessary volume to prepare between 100 and 150 slides) weight 2 g of agarose and add 200 ml of distillate water. Warm in microwaves to dissolve the powder, and keep the solution in a bath at 60 °C.

(3) 0.5% Low Melting Agarose

Weight 1 g of LMP agarose and add 200 ml of PBS (without calcium and magnesium). Warm in microwaves to dissolve, make aliquots of 3 ml and store at 4 °C.

(4) Lysis buffer, pH 10 – 1 L

	Final concentration	Weight
NaCl	2.5 M	146.1 g
Na ₂ EDTA	100 mM	37.22 g
Tris Base	10 mM	1.211 g

Dissolve the reagents listed above in 800 ml of ultrapure water. Add approximately 7 g of NaOH to help dissolve. When all the reagents are totally dissolved, adjust the pH to 10 with NaOH. Add ultrapure water until a final volume of 1 L and store at 4 °C.

Add immediately prior to use: TRITON X-100 1% (v/v) (e.g. 1 ml per 100 ml)

(5) Buffer F (stock solution 10X) – 1L

	Final concentration	Weight
HEPES	40 mM	95.32 g
KCl	0.1 M	74.56 g
EDTA	0.5 mM	1.86 g
BSA	0.2 mg/ml	2.0 g

Weight all the reagents and dissolve in approximately 600 ml of distillate water. Adjust the pH to 8.0 with KOH 10 M and fill with distillate water until 1 L. Prepare aliquots of 50 ml and keep them at -20 °C.

(6) Electrophoresis buffer - 1L

	Final concentration	Weight
NaOH	0.3 M	12 g
EDTA	1 mM	0.37 g

Dissolve the powder in distillate water and adjust the pH to 13. Store at 4 °C, maximum 1 week.

General guidelines

Slide preparation:

Prepare the slides to submerge in 1% of NMP agarose at 60 °C, clean the back side and let it dry overnight horizontally. The next day it should be marked with a pencil the side with agarose. The slides with agarose can be stored during months at room temperature and protected from light and dust.

Cells preparation:

1. Plate the cells (K562: 0.3×10^6 cell/ml and TK6: 0.2×10^6 cell/ml) with the desired treatments in a 6-multiwell culture plate and incubate in a humidified incubator at 37 °C and 5% CO₂ for 24 h. Note: In this case, the protocol is described for non-adherent cells. For adherent cells after plating is necessary a period for cell adhesion before add the treatments.
2. After the incubation time, transfer the content of each well to a centrifuge tube previously identified. Wash each well with 1 ml of PBS to ensure the passage of all the cells and transfer again to the respective centrifuge tube.
3. Centrifuge to remove the treatment at 1200 rpm for 5 min at 4 °C (from now on the cells should be kept on ice).
4. Remove the supernatant and add 500 µl of ice-cold PBS in each centrifuge tube.

5. Centrifuge at 1500 rpm for 5 min at 4 °C.
6. Remove the supernatant and resuspend the pellet, count the cells and calculate the cell density for each condition.
7. Adjust cell density to 1×10^6 cell/ml using ice-cold PBS.

Inclusion of the cells in agarose:

1. Warm in microwaves the LMP agarose tubes (previously prepared) submerged in water (always avoiding the evaporations).
2. Once in the liquid stage, transfer the tubes to a thermoblock to keep the temperature at 37 °C.
3. Prepare a metal tray on ice to dispose of the slides after preparing the gels.
4. Identified all the slides, each condition have three slides: "lysis", "FPG" and "buffer F".
5. In a microcentrifuge tube, add 90 μ l of cellular suspension plus 420 μ l of LMP agarose and resuspend with circular movements.
6. Apply a drop of 70 μ l in each slide (2 gels/slide) and immediately cover each drop with a 20 x 20 mm cover slide.
7. Transfer the slides to the metal tray and when the gels start to have an opaque color (around 10 min after), means that the gel is solidified, remove the cover slides by sliding to one side.

Nucleoid obtaining:

1. Submerge the slides in coplin jar with lysis solution (previously prepare with TRITON X-100 and stored at 4 °C) during 1 h. Keep the slides for lysis separated from the ones for "FPG" and "buffer F".

Enzymatic treatment:

1. Transfer the slides for "FPG" and "buffer F" to a coplin jar with buffer F at 4 °C for 5 min. Repeat the washes three times.
2. Meanwhile, defrost the FPG and dilute them to the desired concentration using buffer F. Keep the enzyme always on ice.

3. After the washes, put the slides in a metal tray on ice (certify that the tray is completely horizontal). Add 45 μ l of FPG in the slides for “FPG” and 45 μ l of buffer F in the slides for “buffer F” and cover them with a 22 x 22 mm cover slide.
4. Transfer the metal tray to a container with warm water and incubate at 37 °C for 30 min.
5. After the incubation time with the enzyme, put the tray again on ice to stop the enzymatic reactions.
6. Remove the cover slides from the slides with FPG and buffer F sliding to one side.
7. Put all the slides (from lysis, FPG, and buffer F) in the same way into the electrophoresis tank with electrophoresis buffer (1 L) at 4 °C (be sure that the tank is leveled) for the alkaline treatment.
8. Incubate for 40 min at 4 °C to unwind the DNA.

Electrophoresis:

1. After the alkaline treatment, turn on the electrophoresis and run the gels for 20 min at 25 V (1 V/cm).
2. Wash the slides with ice-cold PBS using the coplin jar for 10 min at 4 °C.
3. Wash again the slides, this time with ice-cold distillate water for 10 min at 4 °C.
4. Dispose the slides on a horizontal surface at room temperature until the next day to dry.

Microscope:

1. With the lights turned off, add 30 μ l of DAPI staining solution at 1 μ g/ml to each gel and apply a 22 x 22 mm cover slide. Incubate for 20 min at room temperature.
2. Turn on the computer and the fluorescence microscope (Olympus IX71, Japan).
3. Put the slide in the microscope (20x) and turn on the fluorescence lamp.
4. Count 50 comets in each gel, preferentially in the middle of the gel. The percentage of tail intensity was measured using OpenComet software (Gyori et al. 2014).

P4. Nuclear condensation

Principle

Apoptosis is a type of cell death that present specific morphological features, such as rounding-up of the cell, retraction of pseudopods, reduction nuclear volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing, amongst others (Kroemer et al. 2009). During apoptosis, the DNA that normally already contains some condensation, is strongly condensed and in the late stage occurs the formation of the “apoptotic bodies” (Toné et al. 2007).

References

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Materials

- 24-multiwell culture plate
- Centrifuge tubes
- Cover slides (22 x 22 mm)
- Cytocentrifuge filters
- Cytospin Cytocentrifuge (Thermo Scientific, USA) Hydrophobic barrier pen
- Fluorescence microscope (e.g. Olympus IX71, Japan)
- Microcentrifuge tubes
- Poly-L-lysine microscope slides

Assay solutions and reagents

- 4% Paraformaldehyde
- 50% Glycerol solution
- DAPI staining solution
- Phosphate Buffered Saline (PBS)

Work solution preparation

(1) 4% Paraformaldehyde (PFA)

	Final concentration	Weight
Paraformaldehyde (PFA)	4%	4 g

Add 4 g of PFA in 100 ml of PBS. Place the solution in a 60 °C water bath. Be careful with the vapors, check the solution frequently. When completely dissolve, wait until the solution cold down and adjust the pH to 7.4. Store at -20 °C in aliquots of 50 ml.

(2) DAPI staining solution (1 µg/ml)

Dilute the stock solution of 100 µg/ml in distilled water. Store at -20 °C, protected from the light.

(3) 50% Glycerol solution

Add 5 ml of pure glycerol in 5 ml of PBS (1x). The glycerol is very viscous, vortex if needed. Store at 4 °C.

General Guidelines

Cell preparation:

1. Plate the cells (K562: 0.15×10^6 cell/ml and TK6: 0.1×10^6 cell/ml) and incubate with the desired treatments in a 24-multiwell culture plate for 48 h at 37 °C and 5% CO₂ (Note this protocol was done in non-adherent cells).
2. Transfer the content of each well into a centrifuge tube labeled (resuspend in the well to ensure the passage of all the cells).
3. Wash, each well, with 1 ml of PBS, and recover the PBS into the respective tube.
4. Centrifuge the tubes at 1300 rpm for 10 min.
5. Discard the supernatant until 500 µl and resuspend the remaining volume with 3 ml of PBS.
6. Centrifuge the tubes at 1300 rpm for 10 min.
7. Discard the supernatant until 500 µl and resuspend the remaining volume with 2 ml of 4% PFA (previously defrosted in a bath at 37 °C) and incubate for 20 min at 37 °C.
8. Add more 4 ml of PBS above the PFA.
9. Centrifuge the tubes at 1300 rpm for 10 min.
10. Discard the supernatant until 500 µl, resuspend and collect the remaining volume into labeled microcentrifuge tubes.
11. Store at 4 °C. The samples can be stored for a few months.

Nuclear condensation:

1. Label poly-L-lysine microscope slides (two samples per slide), put them in the Cytospin Cytocentrifuge frames with the filters.
2. Pipette 100 µl of cell sample into the respective cytocentrifuge tube.
3. Run the Cytospin Cytocentrifuge at 500 rpm for 5 min.
4. Disassemble the frames and flip each slide to put another sample.
5. Pipette between 100 - 50 µl of cell sample into the respective Cytospin Cytocentrifuge tube.
6. Run the Cytospin cytocentrifuge at 500 rpm for 5 min.
7. Circle the samples with a hydrophobic barrier pen and leave them to air dry.
8. Wash the slides three times with PBS, for 5 min each turn.

9. Incubate 20 μ l of DAPI staining solution for 10 min in the dark.
10. Add a drop (6 μ l) of 50% glycerol to each sample and cover with a 22 x 22 mm coverslip.
11. Store the slides at -20 °C, protected from the light until the analysis.

Analysis of nuclear condensation:

Observe the slides under a fluorescence microscope. Count at least 300 cells, (at least three microscopic fields) for each sample. Register the total number of cells and the number of cells that present apoptotic signals (condensed nuclei). The percentage of cells with condensed nuclei is determined according to the ratio between the number of cells showing condensed nuclei and the total number of cells.