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Screening of natural and synthetic compounds with antitumour activity and insights into a thioxanthone mechanism of action

Instituto Superior de Ciências da Saúde – Norte

April 2013

Screening of natural and synthetic compounds with antitumour activity and insights into a thioxanthone mechanism of action

Dissertation for Master Degree in Molecular Therapies presented to Instituto Superior de Ciências da Saúde – Norte.

Host Institution:

Centro de Investigação em Ciências da Saúde (CICS)

Instituto Superior de Ciências da Saúde – Norte

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This work is part of a scientific project that is under financial support provided from Fundação para a Ciência e a Tecnologia.

The results presented in this dissertation are part of the following publications and scientific communications:

Articles

Azevedo, C. M. G.; Afonso, C. M. M.; Sousa, D.; Lima, R. T.; Vasconcelos, M. H.; Pedro, M.; **Barbosa, J.**; Corrêa, A. G.; Reis, S.; Pinto, M. M. "Multidimensional Optimization of promising antitumor xanthone derivatives." Accepted for publication by BMC in March 2013. Online publication complete: 25-APR-2013, DOI: http://dx.doi.org/10.1016/j.bmc.2013.03.079

Azevedo, C. M. G.; Afonso, C. M. M; Soares, J. X.; Reis, S.; Sousa, D.; Lima, R. T.; Vasconcelos, M. H.; Pedro, M.; **Barbosa, J.**; Gales, L.; Pinto, M. M. "Pyranoxanthones: synthesis, growth inhibitory activity on human tumor cell lines and determination of their lipophilicity in two membrane models." Submitted to Eur J Med Chem in April 2013.

Almeida, A. P.; Cardoso, É.; Faria, A.; Serdeiro, M.; **Barbosa, J. F.**; Pinto, M.; Pedro, M. "The *in vitro* anticancer activity of the crude extract of the *Cecropia catarinensis.*" (in preparation)

Posters

Barbosa, J. F.; Sousa, D.; Lima, R. T.; Bousbaa, H.; Gomes, A. S.; Pinto, M. M.; Sousa, E.; Vasconcelos, M. H.; Pedro, M. "A potent small molecule that irreversibly induces cell death in MCF-7 breast cancer cells." XII Porto Cancer Meeting and III Porto-Bordeaux Joint Meeting. Porto. April, 2013.

Durães, F.; Cardoso É.; Cravo, S.; **Barbosa, J. F.**; Pedro, M.; Pinto, M.; Almeida, A. P. "*Cecropia catarinensis*: A plant with antitumoral potencial." IJUP'13 – 6º Encontro de Jovens Investigadores da Universidade do Porto. Porto. February, 2013.

Cardoso, É. C.; Serdeiro, M. T.; Durães, F.; **Barbosa, J. F.**; Pedro, M.; Pinto, M.; Almeida, A. P. “Antitumoral potencial of crude extract of *Cecropia catarinensis* for breast cancer and melanoma”. 1st Luso-Brazilian Symposium on Science. Vassouras, Brazil. October, 2012.

Agradecimentos

O meu profundo agradecimento à Professora Doutora Madalena Pedro não só pela oportunidade que me proporcionou para realizar este trabalho de mestrado, mas principalmente pela orientação e disponibilidade demonstradas durante todo o processo, e por me ter ajudado a crescer a nível profissional e pessoal.

Ao Professor Doutor Hassan Bousbaa por me ter acolhido no CICS, pela sua boa disposição e prontidão para ajudar, e ainda pela valiosa orientação nos ensaios de microscopia.

À Professora Doutora Roxana Falcão Moreira e ao Instituto Superior de Ciências da Saúde-Norte por me terem proporcionado esta experiência.

À Professora Doutora Madalena Pinto e ao CEQUIMED-UP pela oportunidade de fazer parte do grupo. De igual forma quero agradecer à Professora Doutora Emília Sousa, à Professora Ana Paula de Almeida, ao Professor Doutor Anake Kijjoa, e ao Professor Doutor Carlos Afonso por terem cedido os compostos que foram objecto de estudo deste trabalho.

À Madalena Silva pelo apoio que deu na obtenção dos extractos de origem natural; e ainda à Diana Sousa pelo apoio oferecido nos ensaios de avaliação da autofagia, à Professora Doutora Helena Vasconcelos e ao IPATIMUP.

De uma maneira especial quero agradecer à Joana por se ter tornado uma grande amiga, e por tudo aquilo que me ensinou durante este percurso. De igual forma quero agradecer à Juliana pela sua amizade, simpatia e sensatez. Obrigado às duas por terem acreditado em mim.

À Francisca e à Inês pelo companheirismo e amizade, e por terem estado sempre lá.

Não posso deixar de agradecer ainda à Rita, à Andrea, à Diana, e ao Miguel por terem tornado este percurso mais fácil.

Ao Jorge pelo companheirismo e amizade de longa data.

À Patrícia, à Vanessa e à Rita pela prontidão para ajudar que sempre demonstraram quando mais precisei.

Ao Manú pelo tempo que gentilmente me dispensou e pela ajuda com o tratamento de dados experimentais.

Ao Professor Doutor Joaquim Monteiro e ao Professor Doutor Luís Moutinho pelo auxílio na análise estatística dos dados experimentais.

Ao Professor Doutor César Portela pelos conselhos para a vida.

Por último, quero agradecer à Rita, aos meus Pais e aos meus Avós por todo o apoio oferecido ao longo deste percurso. A eles devo tudo.

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Abstract

Cancer is currently a leading cause of mortality and morbidity throughout the world and considering its increasing incidence the development of new cancer therapies and the improvement of the classic therapeutics, namely chemotherapy, become an urgent demand. Knowledge of the molecular targets for the antitumour agents is a crucial instrument for this achievement.

The present work was grounded on two main objectives. The first one was to evaluate the potential inhibitory effect on cell growth of 9 natural product-derived samples (2 extracts obtained from *Gelidium microdon*; 2 extracts and 2 compounds obtained from *Ulva rigida*; and 3 extracts obtained from *Cecropia catarinensis*) and 12 synthetic compounds (11 pyranoxanthones and 1 thioxanthone). Cell growth inhibitory activity was evaluated on different cell lines with different origins such as A375-C5 (melanoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and HeLa (cervical adenocarcinoma) cell lines. Several natural product-derived samples and some synthetic compounds revealed to successfully inhibit cancer cell growth.

A thioxanthone (TXA1.HCl) deserved particular interest since it has shown a potent cell growth inhibitory activity, and due to this fact the aim of the second objective of this work was the elucidation of its molecular mechanism of action in the breast adenocarcinoma cell line (MCF-7).

This compound successfully inhibited cell growth and decreased metabolic activity of the four previously referred cell lines. TXA1.HCl effect significantly decreased cell viability in a both time- and concentration-dependent manner, and it was also proven to be irreversible and to decrease MCF-7 cell ability to proliferate. Different assays pointed that apoptosis and autophagy are two cell death mechanisms through which this small molecule exerts its effects. Such characteristics make it a promising compound for further studies.

Resumo

O cancro é atualmente uma das principais causas de morte e morbilidade em todo o mundo, e tendo em consideração o aumento da sua incidência, o desenvolvimento de novas terapias e a melhoria das terapias clássicas, nomeadamente da quimioterapia, tornam-se um pedido urgente. O conhecimento dos alvos moleculares dos compostos antitumorais é um instrumento crucial para este fim.

Este trabalho baseou-se em dois principais objectivos. O primeiro consistiu na avaliação do potencial efeito inibidor do crescimento celular de 9 amostras derivadas de produtos naturais (2 extractos de *Gelidium microdon*; 2 extractos e 2 compostos obtidos de *Ulva rigida*; e 3 extractos obtidos de *Cecropia catarinensis*) e 12 compostos de origem sintética (11 xantonas e 1 tioxantona). A atividade inibidora do crescimento celular foi avaliada em diferentes linhas celulares tumorais de diferentes origens tais como A375-C5 (melanoma), MCF-7 (adenocarcinoma da mama), NCI-H460 (cancro de pulmão de células não-pequenas) e HeLa (adenocarcinoma cervical). Diversas amostras derivadas de produtos naturais e alguns compostos de origem sintética demonstraram inibir, com sucesso, o crescimento de células tumorais.

Uma tioxantona (TXA1.HCl) mereceu particular interesse uma vez que demonstrou elevada atividade inibidora do crescimento celular, o que a levou a ser o alvo de estudo do segundo objectivo deste trabalho que consistiu na elucidação do seu mecanismo de ação molecular na linha celular de adenocarcinoma da mama (MCF-7).

Este composto inibiu, com sucesso, o crescimento celular e diminuiu a atividade metabólica das quatro linhas celulares referidas anteriormente. O efeito da TXA1.HCl diminuiu significativamente a viabilidade celular de forma dependente do tempo de exposição e da concentração, e demonstrou ainda ser irreversível e capaz de diminuir a capacidade de proliferação das células MCF-7. Diferentes ensaios indicaram que a apoptose e a autofagia são dois mecanismos de morte celular através dos quais esta pequena molécula exerce os seus efeitos. Estas características fazem deste um composto promissor para futuros estudos.

Abbreviations

Abs	absorbance
ACS	American Cancer Society
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
BH	BCL-2 homology
CEQUIMED-UP	Centro de Química Medicinal da Universidade do Porto
CO_2	carbon dioxide
COX	cyclooxygenase
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DED	death effector domain
D-MEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DMXAA	5,6-dimethylxanthenone-4-acetic acid
dUTP	2'-deoxyuridine, 5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
FADD	FAS-associated protein with a death domain
FBS	fetal bovine serum
G-CSF	granulocyte colony-stimulating factor
GI	growth inhibition
GTP	guanosine-5'-triphosphate
IC_{50}	half maximal inhibitory concentration
LC3	microtubule-associated protein 1 light chain 3
µg	microgram
µl	microliters
µM	micromolar
ml	millilitres
MAPK	mitogen-activated protein kinases

MDC	monodansylcadaverine
MOMP	mitochondrial outer membrane permeabilization
MMP	metalloproteinase
MTT	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide
MRP	multidrug resistant protein
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NCI	National Cancer Institute
nm	nanometers
nM	nanomolar
NSCLC	non-small cell lung cancer
PBS	phosphate-buffered saline
PGE2	prostaglandine E2
P-gp	P glycoprotein
PKC	protein Kinase C
PI3K	phosphatidylinositide 3-kinases
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
rTdT	recombinant terminal deoxynucleotidyl transferase
SD	standard deviation
SRB	sulforhodamine B
TCA	trichloroacetic acid
TNF	tumour Necrosis Factor
TRAIL	TNF-related apoptosis-induced ligand
Tris	tris(hydroxymethyl)aminomethane
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
v/v	volume/volume
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
w/v	weight/volume

Thesis Organization

Chapter I. Introduction

This thesis starts with a detailed introduction to the main topics addressed in this work. It consists on a review of the current anticancer therapeutic approaches, as well as some chemotherapeutic targets and chemical families with therapeutic value of interest to this work.

Chapter II. Objectives

This chapter describes the objectives of this scientific work.

Chapter III. Materials and Methods

This chapter describes all materials and methods used in the experimental part of this work.

Chapter IV. Results and Discussion

In this chapter, all results achieved are stated and discussed. Two sub-sections that resemble the two main objectives of this work organize this section in:

- Part I - Screening of antitumour activity on compounds from natural and synthetic origin;
- Part II – Insights into a thioxanthone mechanism of action.

Chapter V. Conclusions

This chapter sums up the main findings of this work.

Chapter VI. Future Perspectives

Future perspectives related to this work are stated in this chapter.

Chapter VII. References

This chapter ends this thesis, and consists on a list of references of all the consulted literature. Most references were retrieved through NCBI's PubMed.

Chapter I.
Introduction

General Remarks About Cancer

Cancer is a leading cause of morbidity and mortality worldwide, accounting for about 12.7 million new cancer cases and 7.6 million deaths (around 13% of all deaths) in 2008, and this number is projected to continue rising, with an estimation of 13.1 million deaths for 2030 (WHO, 2012a).

This projection is based on current demographic trends, such as population ageing and the increase in life expectancy, and on behavioural and lifestyle habits that predispose the individual to cancer development: unhealthy diet and obesity, sedentarism, tobacco and alcohol consumption, increased exposure to pollutants and to industrial and agricultural carcinogens (Jemal et al., 2010, NCI, 2006, Pinheiro et al., 2003, WHO, 2012b). In particular, smoking, dietary factors and reproductive patterns are among the major risk factors for cancer in western countries, while infectious agents are the main responsible for cancer – such as cervical, liver and stomach cancers – in developing countries (Jemal et al., 2010, Pinheiro et al., 2003).

While cancer rates may stabilize in developed, industrialized countries in the next few years, its incidence is increasing in economically developing countries due to the adoption of western lifestyle patterns. In these countries, lung, colon and breast cancers, formerly known as diseases of the industrialized world, are currently common (Jemal et al., 2010).

As far as Europe is concerned, there were an estimated 3.2 million newly-diagnosed cases in 2008 (Ferlay et al., 2010). The most common were colorectal cancer (13.6%), breast cancer (13.1%), lung cancer (12.2%), and prostate cancer (11.9%) (Ferlay et al., 2010).

Lung cancer was the major responsible for cancer deaths in Europe, accounting for 19.9% of the total, followed by colorectal cancer (12.3%), breast cancer (7.5%) and stomach cancer (6.8%) (Ferlay et al., 2010).

In the last decades, cancer incidence and mortality have increased progressively in Portugal. For instance, while cancer accounted for about 9% of deaths in 1960, this number has risen to 20% in 1998, killing a larger proportion of males (22%) than females (17%) (Pinheiro et al., 2003).

Studies done in Portugal showed that there is a similar trend concerning cancer incidence rates. In 2000, there were a total of 19 880 and 17 000 new male

and female cancer patients, respectively. Colorectal cancer is the most frequent among Portuguese men, followed by prostate, lung, stomach and urinary bladder, while breast cancer is the most common among women, followed by colorectal, stomach and cervical cancer (Pinheiro et al., 2003). Recent data show that cancer incidence rates are increasing; every year, 40 000 new cases are diagnosed, from which 50% eventually lead to the patient's death (SOL, 2012). Considering lung cancer, it has killed 3579 people in 2005, while 3833 patients have died as a result of it in 2009 (ONDR, 2011)

Considering the increasing cancer incidence and mortality worldwide, the need for the development of new cancer therapies becomes clear and urgent (Chang, 2011). Cancer investigation is currently a wide area that comprises the identification of cancer causes and the establishment of preventive, diagnostic and therapeutic approaches (Suh et al., 2011). It has always been a particularly challenging and rewarding field because, besides being very prevalent, cancer is, in most cases, a very disabling and painful disease, with a considerable likeliness to relapse. Molecular biology advances have significantly helped to improve knowledge about tumour cell development and to identify novel molecular targets, while biotechnology progresses have enabled the design of new, targeted therapies (Chang, 2011).

Accordingly, current new anticancer strategies are based on the identification of tumour causes at the molecular level, so that tailored therapies can be designed and implemented according to the tumour type and to the patient's features, aiming to minimize side effects and to improve selectivity against cancer cells (Chang, 2011). In fact, effects on healthy cells and lack of specificity are the main issues that current anti-cancer research aims to overcome, thereby setting the ground for the development of new therapeutic approaches and for the improvement of the pre-existent ones, often used in combination in order to optimize therapeutic efficacy (Chang, 2011). However, classical chemotherapy remains as a rewarding tool against cancer, and still has a great potential for optimization in the quest for more effective treatments (Chang, 2011), as addressed in the following sections.

Current Anticancer Therapeutic Approaches

Besides surgery and radiotherapy, chemotherapy is, by far, the most implemented, classical and documented anticancer approach. The use of chemotherapy in cancer dates back to the 1940-decade when diethylstilbestrol (a synthetic nonsteroidal estrogen) and nitrogen mustard (an alkylating agent precursor) were used to cure prostate cancer and lymphoma, respectively (Begonha and Azevedo, 2006, ACS, 2012a). Not long after this, aminopterin (predecessor of methotrexate, a chemotherapeutic drug used commonly today) was found to produce remissions in children with acute leukemia (ACS, 2012a). Ever since, many chemotherapy drugs of natural and synthetic origins were successfully introduced in therapeutics for different neoplastic oncological diseases (Begonha and Azevedo, 2006, ACS, 2012a). The implementation of clinical trials has enabled comparative studies to be conducted, leading to significant advances in the development of new chemotherapeutic agents (ACS, 2012a).

However, along with the accumulation of knowledge about tumour cell biology, many other approaches have arisen in an attempt to complement chemotherapy and/or overcome its main limitations, such as chemoresistance and lack of selectivity (Abdullah and Chow, 2013, Suh et al., 2011, Chang, 2011).

New therapeutic approaches emerged in the search for more effective, specific and precise treatments that minimize side effects and increase survival and quality of life. As such, there are currently many approaches available for cancer management, the choice of which must take into account the location and grade of the tumour, the stage of the disease and the general state of the patient (ACS, 2012b). Although the most common and classical strategies consist of a combination of oncology surgery, chemotherapy, and radiotherapy, other therapies have arisen such as targeted therapy, immunotherapy, stem cell transplantation, gene therapy, and anti-angiogenic therapy (ACS, 2012b). These approaches aim to minimize side effects on healthy cells, and to optimize the elimination of residual and micrometastatic tumours that often lead to recurrence (Aly, 2012).

Some of the targeted therapy blocks cancer cell proliferation by interfering with molecules that are needed for carcinogenesis and tumour growth; the development of such approach was made possible by the growing knowledge about cancer

pathophysiology and biomarkers (Klener and Klener, 2012). Monoclonal antibodies (e.g., antiCD20 rituximab) and tyrosine-kinase inhibitors (e.g., imatinib) are two examples of molecularly targeted therapies. While the first targets a cancer cell surface biomarker (CD20 antigen), the latter aims to inhibit the molecule responsible for cancer pathophysiology (Klener and Klener, 2012, Kirkwood et al., 2012, Zhou and Levitsky, 2012).

Besides using therapeutic monoclonal antibodies, immunotherapy also comprises the transfer of tumour-reactive lymphocytes to tumour-bearing hosts, as well as the administration, through 'cancer vaccines', of tumour- specific or tumour-associated antigens that elicit an immunological response (Aly, 2012, Zhou and Levitsky, 2012). Due to its specificity, immunotherapy is expected to minimize damage to normal, proliferating cells (Aly, 2012). Tumour-specific antigens are molecules exclusive to tumours (e.g., products of mutated genes, viral antigens expressed on tumour cells, endogenous human retroviruses activated and expressed on tumour cells), whilst tumour-associated antigens may also be found in normal cells, but have an abnormal expression on the tumour ones (Aly, 2012). Cancer vaccines may be composed of whole cells or cell extracts (including protein or RNA), single proteins or peptides that work as tumour-associated or specific antigens, tumour cells that were genetically modified in order to express co-stimulatory molecules, dendritic cells presenting tumour-associated antigens, recombinant viruses or bacteria encoding tumour-associated antigens, and naked plasmid DNA encoding tumour-associated or specific antigens (DNA immunization), combined with appropriate immunomodulators (Aly, 2012, Kirkwood et al., 2012). Depending on their nature, these vaccines may be administered locally, systemically or even in the lymph nodes (Aly, 2012), either in a preventive or therapeutic perspective (Kirkwood et al., 2012). In fact, immune-based therapy has been successfully used in the treatment of melanoma and hepatocellular, prostate, ovarian and lung cancers (Kirkwood et al., 2012, Higano et al., 2008, Kantoff et al., 2010, Gao et al., 2007, Saga et al., 2013, Wilmott et al., 2012).

Anti-angiogenic therapy targets factors that mediate angiogenesis, since the budding of new capillary branches from pre-existent blood vessels occurs along with tumour development as a way to provide tumour cells oxygen and nutrients (Kubota, 2012). By targeting angiogenic factors such as vascular endothelial growth factor (VEGF), angiogenic inhibitors suppress tumour growth and metastasis (Kubota,

2012). Because, most of them, makes use of antibodies raised against specific angiogenic factors, anti-angiogenic therapy may also be considered a form of targeted, immune-based therapy (Klener and Klener, 2012). Besides bevacizumab, a humanized anti-VEGF monoclonal antibody, already used in clinical practice (Kirkwood et al., 2012, Kubota, 2012, Shojaei, 2012), other angiogenesis inhibitors have been approved or are at different stages of clinical trials (Shojaei, 2012).

Gene therapy is another valid approach for cancer treatment. Therapeutic genes may be delivered to tumour cells through viral or non-viral carriers (Jia et al., 2012). While the first mostly include adenoviruses that bind host cells through their envelopes, the latter comprise nanoparticles made of lipid-like materials (Jia et al., 2012).

Biological therapy, in turn, is a broad term that defines all therapeutic approaches that are based on a group of biomolecules (biological medicinal products, biologicals, biopharmaceuticals), most frequently protein macromolecules like cytokines, growth factors, antibodies, soluble receptors, which usually have high molecular weight, as opposite to what happens with classical low molecular weight chemotherapeutic agents (Klener and Klener, 2012). Their activity is not only dependent on their primary structure, but also on their secondary modification (for instance, glycosylation) (Klener and Klener, 2012). Because they are produced within living cells, these biomolecules may present a considerable degree of heterogeneity, which is a major drawback for their clinical use (Klener and Klener, 2012).

Lastly, in order to optimize chemotherapy and radiotherapy effectiveness, some approaches based on the properties of different components of the electromagnetic spectrum, as well as on thermotherapy, have arisen.

Because they have cytotoxic effects and induce microcirculatory damage, radiofrequency ablation, microwave ablation therapy, high-intensity focused ultrasound and laser-induced thermotherapy, with the use of high temperatures, as well as cryoablation, with induction of low temperatures, are used as adjuvants to chemotherapy and radiotherapy (Haen et al., 2011).

Despite all the advances in anticancer approaches, presented, chemotherapy remains as the most studied and the one with more background to prompt new discoveries and improvements of current solutions. In fact, over the years, many people have been successfully treated with chemotherapy thanks to ongoing research into the use of these drugs (ACS, 2012c).

Several exciting new uses of chemotherapy and other agents hold even more promise for treating or controlling cancer. New drugs, new combinations of drugs, and new delivery techniques will help curing or controlling cancer and improving patients' quality of life. There are many expected advances in coming years, such as new classes of chemotherapy medicines and their combinations and new forms of administration, such as using smaller amounts over longer periods of time or providing them continuously with special pumps (ACS, 2012c). In turn, liposomal therapy is expected to enhance the specific delivery of chemotherapeutic agents to cancer cells, thus minimizing side effects. Also, agents that reduce collateral damage, such as colony-stimulating factors and chemoprotective agents (e.g., dexrazoxane and amifostine), as well as agents that overcome multidrug resistance, may be co-administered with chemotherapeutics (ACS, 2012a).

Taken together, these factors emphasize the potential of chemotherapy as a field that presents many advantages over other approaches, thereby still offering many possibilities to explore.

Conventional Chemotherapy

Drugs that are currently used on a chemotherapeutic basis are categorized as cytotoxic agents, hormones and their antagonists, and others. Hormones are used in the treatment of hormone-sensitive cancers, such as breast, endometrial and prostate cancers. Cytotoxic drugs, in turn, comprise alkylating agents, antimetabolites, antimitotic agents, epipodophyllotoxins and cytotoxic antibiotics (Begonha and Azevedo, 2006).

Alkylating agents act by inducing the establishment of covalent bonds (alkylation) with several nucleophile substances. Bifunctional agents such as nitrogen mustards, bearing two alkylating chains, are able to crosslink both chains of nucleic acids or a nucleic acid chain with a protein, thus interfering with DNA, RNA and protein synthesis and, consequently, with mitosis, cell growth and differentiation (Bignold, 2006, Brulikova et al., 2012, Drablos et al., 2004). Their cytostatic activity is more pronounced in G_1 and S phases. Prototypical examples include cyclophosphamide, ifosfamide, mechloretamine, chlorambucil, busulfan, nitrosoureas, fotemustine, bendamustine, temozolomide and streptozotocin (Begonha and Azevedo, 2006).

Antimetabolites are structurally and chemically identical to compounds that are necessary for purine, pyrimidine and nucleic acid synthesis. They affect DNA synthesis by inhibiting folic acid regeneration or purine or pyrimidine activation, or by intercalating themselves, or their derivatives, into DNA molecules. Because of their detrimental influence on DNA synthesis, their cytostatic effect is specific for the S phase (Begonha and Azevedo, 2006).

Main antimetabolites are folic acid analogues (methotrexate, trimetrexate, pemetrexed) (Wright and Anderson, 2011), purine analogues (mercaptopurine, tioguanine, fludarabine, cladribine) and pyrimidine analogues (fluorouracil, cytarabine, gemcitabine) (Begonha and Azevedo, 2006, Peters et al., 2000).

Antimitotic agents comprise vinca alkaloids and taxanes. Vinca alkaloids are a group of chemically complex molecules derived from *Vinca rosea*. Their cytotoxic activity is cell cycle-dependent and is mainly due to their ability to bind microtubules and destroy mitotic spindles. Examples include vincristine and vinblastine, vindesine and vinorelbine. In turn, taxanes such as paclitaxel and docetaxel act by stabilizing

microtubules and inhibiting their dissociation (Begonha and Azevedo, 2006, Gascoigne and Taylor, 2009).

Epipodophyllotoxins, like etoposide and teniposide, are semi-synthetic derivatives of podophyllotoxin. Their mechanism of action is poorly understood; however, it is known that they act by arresting cells at the S/G_2 interphase of the cell cycle. Also, they appear to stimulate DNA topoisomerase II, preventing its dissociation from DNA and causing DNA fragmentation (Xu et al., 2009), and they are able to prevent nucleoside transport and incorporation into nucleic acids (Begonha and Azevedo, 2006).

Cytotoxic antibiotics are the result of a thorough screening of soil microorganism (*Streptomyces*) products. This category comprises anthracyclines, the cytostatics with the broadest spectrum of activity, which act by inhibiting DNA, RNA and protein synthesis, by affecting membrane sodium and calcium transport and by triggering the production of reactive oxygen species (Begonha and Azevedo, 2006, Khasraw et al., 2012). Anthracyclines include doxorubicin, daunorubicin, epirubicin, idarubicin, and their effects are maximal during the S phase. Other examples comprise actinomycin D, which inhibits DNA synthesis, and bleomycin, which fragments DNA through the generation of free oxygen radicals (Begonha and Azevedo, 2006).

Chemotherapeutic Targets

Cytotoxic drugs interfere with cell growth and proliferation through several ways. Nevertheless, from what has been exposed in the previous section, the most representative drugs currently used in conventional chemotherapy share mechanistic similarities and target mostly microtubules, cell cycle, apoptotic machinery, as well as autophagic and necrotic mechanisms (Oliveira, 2006). Some of these chemotherapeutic targets will be addressed below.

Cell cycle machinery

The cell cycle is an irreversible, continuous and highly coordinated process that underlies cell growth, proliferation and tissue regeneration. It comprises two main stages: interphase and mitosis. In mitosis, which encompasses prophase, prometaphase, metaphase, anaphase and telophase, cell division occurs (Schafer, 1998). Therefore, its proper accomplishment is the main responsible for the maintenance of genomic fidelity and stability (Silva et al., 2011). In interphase, the most time-consuming step, the cell prepares for mitotic division by synthetizing DNA, RNA and proteins. It comprises two gap phases (G_1 and G_2) separated by a synthesis step, the S phase (Schafer, 1998, Liu and Ander, 2012). Cell cycle progression is under tight supervision of G_1/S, S and G_2/M checkpoints, which are DNA-damage checkpoints, and the mitotic checkpoint (Schafer, 1998). While DNA-damage checkpoints check DNA integrity and replication status, the mitotic checkpoint verifies proper chromosome binding to the mitotic spindle and alignment at the metaphase plate, as well as the existence of bipolar tension across sister chromatids (Silva et al., 2011, Musacchio, 2011). These control loops make the entrance on a new stage dependent on the adequate execution of the previous one, providing the cell time enough to correct eventual errors. If their requirements are not fully met, they promote a cell cycle arrest that ultimately leads to cell death (Silva et al., 2011, Musacchio, 2011).

The orderly progress between all cell cycle stages is assured by the activity of cyclin-dependent kinases (CDKs), which, as their name suggests, act together with their corresponding cyclins (Hindley and Philpott, 2012). Different cyclin/CDK

complexes are intermittently active during the cell cycle, acting through substrate phosphorylation (Hindley and Philpott, 2012). Many compounds are being investigated for their ability to inhibit cyclins or CDKs themselves, as a way to deregulate tumour cell cycle progression, causing cell death (Holkova and Grant, 2011). CDK inhibitors (CDKIs) are particularly promising. Flavopiridol is a semi-synthetic flavone that was shown to inhibit CDKs 1, 2, 4/6, 7 and 9 and to downregulate cyclin D1 expression in certain cell types, as well as the expression of several anti-apoptotic proteins (Holkova and Grant, 2011). Besides flavopiridol, other CDKIs are in clinical trials, envisaging treatment for solid tumours and chronic lymphocytic leukaemia. Examples include indisulam, AZD5438, SNS-032, PD 0332991, SCH 727965, UCN-01, roscovitine, AT7519, bryostatin-1, seleciclib and P276-00 (Liu and Ander, 2012, Parekh et al., 2011). Some of these compounds exert their effects through mechanisms that go beyond cell cycle inhibition (Parekh et al., 2011).

In addition, several xanthones and natural compounds, which will be later addressed in this thesis, show the ability to cause a cell cycle arrest that ultimately leads to cell death. That is the case of gambogic acid (Pouli and Marakos, 2009), lucanthone and hycanthone (Naidu et al., 2011) and some prenylated xanthones (α-mangostin, β-mangostin, γ-mangostin and methoxy-β-mangostin) (Shan et al., 2011).

Some examples of marine origin include fucoxanthin (Kim et al., 2013) and elatol (Campos et al., 2012) as well as extracts from brown alga *Turbinaria ornata* (Shalaby, 2011).

Microtubules

Microtubules are non-covalent polymers composed of α- and β-tubulin units, forming a filamentous, tubular assembly (Perez, 2009, Kingston, 2009, Risinger et al., 2009). Together with actin microfilaments and intermediate filaments, they are main constituents of the cytoskeleton, playing important roles in intracellular transport, cell shape, motility and polarity, cell signalling and mitosis (Perez, 2009, Risinger et al., 2009). During mitosis, microtubules compose the mitotic spindle, along which chromosomes are pulled to opposite poles of the dividing cell (Perez, 2009). Mitotic microtubules are required to be highly dynamic in order to allow

chromosome alignment and separation; the success of mitosis is then dependent on the proper function of the mitotic spindle (Perez, 2009, Kingston, 2009). Given their relevant role in mitosis, microtubules appear as very attractive targets for new chemotherapeutic agents that aim to prevent tumour cell division (Perez, 2009). Indeed, compounds that interfere with microtubule dynamics compromise cell cycle progression and lead to an arrest at the G_2/M phase, eventually culminating in apoptotic cell death (Perez, 2009, Kingston, 2009, Yue et al., 2010).

Microtubule inhibitors are classified as stabilizing or destabilizing agents. Microtubule-stabilizing agents promote microtubule polymerisation and increase microtubule polymer mass, whilst microtubule-destabilizing agents depolymerize microtubules, inhibit their polymerisation and decrease their mass. However, at low concentrations, both stabilizers and destabilizers suppress microtubule dynamics without changing their mass (Perez, 2009, Gascoigne and Taylor, 2009, Kingston, 2009, Risinger et al., 2009).

Microtubule stabilizers include, as already mentioned, taxanes as paclitaxel and docetaxel, as well as epothilones (Perez, 2009). Taxanes bind β-tubulin only if it is incorporated into a microtubule; chromosomes may attach taxane-stabilized microtubules but, in the absence of microtubule dynamics, there is no tension across sister chromatids and no chromosome bi-orientation, thus leading to spindle assembly checkpoint activation and mitotic arrest (Gascoigne and Taylor, 2009). Taxanes have been used in the treatment of ovarian and breast cancers (Gascoigne and Taylor, 2009). Some taxanes, like larotaxel, paclitaxel poliglumex, cabazitaxel and taxoprexin, are in clinical trials (Kingston, 2009). Other microtubule stabilizers, such as laulimalide, peloruside A and taccalonolides, are also raising interest (Risinger et al., 2009, Yue et al., 2010). Taccalonolides, ferunerol, jatrophane esters, partenolide, costunolide, dicoumarol and evodiamine are examples of microtubule-stabilizing agents of plant origin. On the other hand, discodermolide, dictyostatin, eleutherobin, sarcodictyins, laulimalide, peloruside A and ceratamines are examples of microtubule-stabilizing agents of marine origin (Altmann and Gertsch, 2007, Risinger and Mooberry, 2010).

On the other hand, vinca alkaloids provide an example of microtubule destabilizers (Perez, 2009). They bind β-tubulin next to their GTP-binding site, known as the vinca domain (Gascoigne and Taylor, 2009). Vinca alkaloids are frequently used in combination therapies for haematological malignancies (Gascoigne and

Taylor, 2009). Although vinca alkaloids are the only inhibitors of microtubule polymerisation with clinical use, other compounds with this mechanism of action are at different stages of clinical trials. That is the case of combretastatins and analogues, dolastatins, noscapine, hemiasterlin and rhizoxin (Kingston, 2009, Risinger et al., 2009, Yue et al., 2010). Together, they illustrate how nature is a plentiful source of microtubule-targeting agents (Altmann and Gertsch, 2007, Risinger and Mooberry, 2010).

Other recent microtubule inhibitors comprise epothilone B (patupilone, EPO906) and its analogue ixabepilone, epothilone D and analogues (KOS-862 and KOS-1584), vinflunine (a vinca alkaloid), halichondrin D analogue erubilin mesylate and taxane analogue DJ-927 (Perez, 2009, Kingston, 2009).

Apoptosis and other types of cell death

Apoptosis is a form of programmed cell death that is essential for morphogenesis, tissue regeneration and elimination of abnormal or infected cells, thus being a prerequisite for homeostasis (Favaloro et al., 2012). It is, in most cases, a physiological form of cell death, being involved in ontogenesis, organ and tissue remodelling, immunity, prevention of abnormal cell proliferation and elimination of mutated cells (Boehm, 2006). It is an active process, tightly controlled at the gene level, triggered by internal and external stimuli such as reactive oxygen species, UV light, cytokines, infection, sub lethal heat, chemicals, drugs and physical damage (Boehm, 2006). Many cytotoxic agents used with chemotherapeutic purposes act by inducing apoptosis, making it an interesting target for cancer treatment (Lowe and Lin, 2000, Kasibhatla and Tseng, 2003, Cho et al., 2012). Apoptosis is characterized by a distinctive set of morphological features, including DNA and chromatin fragmentation, plasma membrane blebbing and cell shrinkage, cell fragmentation (with the formation of apoptotic bodies, which are later removed through phagocytosis), all of them directly related to the activity of aspartate-specific cysteine proteases, the caspases, which are responsible for substrate cleavage or activation within the cell (Kasibhatla and Tseng, 2003, Wong, 2011).

Apoptosis is primarily induced as a response to intracellular phenomena, occurring at the mitochondria or the nucleus – the intrinsic pathway – or to external

factors that bind specific receptors at the cell surface – the extrinsic pathway (Boehm, 2006). Members of the BCL-2 family are crucial for the apoptotic intrinsic pathway, either through its promotion or inhibition. The intrinsic pathway is activated as a consequence of different stressful conditions, such as DNA damage and oxidative stress (Favaloro et al., 2012). Anti-apoptotic members like BCL-2, BCL-XL, BCL-W, A1 and MCL-1 present two or more of four different types of BCL-2 homology (BH) domains. These domains mediate interactions with other BCL-2 family members, ultimately leading to the release of pro-apoptotic factors from the mitochondrion, such as SMAC/DIABLO (Cotter, 2009, Favaloro et al., 2012). Also, BCL-2 family members may bind pro-apoptotic molecules BIM, BID, PUMA, BAD and NOXA with different selectivity. These molecules contain a BH3 domain, for which they are called BH3-only proteins (Parekh et al., 2011). Their binding to BCL-2 family members leads to mitochondrial outer membrane permeabilization (MOMP). BAX and BAK are then released from mitochondria. Subsequently, cytochrome c is also released, activating caspases 9 and 3 and triggering apoptosis (Parekh et al., 2011, Cotter, 2009, Favaloro et al., 2012, Wong, 2011).

Targeting anti-apoptotic proteins by mimicking the activity of BH3-only proteins is an attractive therapeutic strategy for many tumours. For instance, obatoclax (GX15-070) and AT-101 have BAD- and NOXA-like activities, respectively (Parekh et al., 2011, Favaloro et al., 2012, Ocker and Hopfner, 2012, Wong, 2011). ABT-737 and its analogue ABT-263 show BAD-like specificity, with high affinity for BCL-2 and BCL-XL (Parekh et al., 2011, Favaloro et al., 2012, Ocker and Hopfner, 2012, Wong, 2011). Anti-sense oligonucleotides against BCL-2 have also been developed, having reached phase III clinical trials aiming the treatment of chronic lymphocytic leukaemia (Favaloro et al., 2012, Kasibhatla and Tseng, 2003). Oblimersen (G3139) is one such example (Ocker and Hopfner, 2012).

Apoptosis may also be elicited through binding of external factors to the FAS/CD95L death receptor, a member of the Tumour Necrosis Factor (TNF) receptor family (Cotter, 2009). Other components, including TRAIL (TNF-related apoptosis-induced ligand) and its receptors DR4 and DR5, also have a significant contribution (Cotter, 2009), and together mediate the apoptotic extrinsic pathway. Upon ligand binding, the death receptor recruits an adaptor, FAS-associated protein with a death domain (FADD), which also contains a death effector domain (DED) that binds an homologous domain in caspase 8, leading to its activation. This, in turn, causes the

activation of other caspase 8 molecules, as well as of other downstream caspases, including caspase 3 (Favaloro et al., 2012, Lowe and Lin, 2000, Wong, 2011).

In spite of the initial enthusiasm about exploiting the activation of death receptors as an anti-cancer approach, inherent toxicity curtailed investigation in this field (Favaloro et al., 2012). However, since recombinant TRAIL induces apoptosis on cancer cells, while sparing the healthy ones, it appears as a promising strategy (Favaloro et al., 2012). Also in this context, diverse DR5 agonists such as conatumab (AMG 655), tigatuzumab (CS-1008), lexatumumab and drozitumab (PRO95780) are in phase I and II trials, as single or combined agents, aiming to treat different solid tumours. In turn, mapatumumab is the only available fully human monoclonal antibody activating DR4 (Ocker and Hopfner, 2012). Dulanermin, which binds both DR4 and DR5, is also a promising ligand used in phase I and II trials for the treatment of solid tumours like colorectal and non-small cell lung cancers (NSCLC) (Ocker and Hopfner, 2012).

Other apoptosis-related proteins, such as p53, Survivin and caspases, may also be targeted for cancer treatment (Wong, 2011).

Several xanthones and natural compounds have been found to be able to induce apoptosis. Examples include α-mangostin, from the mangosteen extract (Shan et al., 2011, Kurose et al., 2012), γ-mangostin (Chang and Yang, 2012), gambogenic acid (Chen et al., 2012), pyranocycloartobiloxanthone A (Mohan et al., 2012) and DMXAA (Baguley and Siemann, 2010).

Some marine origin examples include sea cucumber extract (Wijesinghe et al., 2013) and extract from the microalga *Chaetoceros calcitrans* (Ebrahimi Nigjeh et al., 2013) as well as extracts from *Porphyra* sp. and *Corallina pilulifera* algae (Shalaby, 2011), all of them addressed in a later stage in this thesis.

Finally, it should be reminded that apoptosis is not the only mechanism of programmed cell death. In particular, autophagy is an alternative to apoptosis whose role, besides the participation in protein and cell component turnover, as well as in the physiological response to nutrient deprivation and other stresses, is still unclear (Ocker and Hopfner, 2012, Zhang et al., 2012). Although its contribution for cancer growth is poorly understood, many antitumour compounds have been found to induce autophagy (Zhang et al., 2012).

Biochemically, autophagy is also different from apoptosis in that there is no caspase involvement; instead, an autophagosome fuses with a lysosome forming an

autophagolysosome, within which organelles digestion takes place (Ocker and Hopfner, 2012, Kroemer et al., 2009). Autophagy has also been targeted as a therapeutic strategy; for instance, EZ235, AZD8055 or rapamycin analogues promote autophagosome formation, whilst chloroquine and hydroxychloroquine inhibit autophagolysosome maturation and degradation. While the first directly favour cancer cell death through autophagy, the latter overcome autophagy-mediated survival and resistance, which is observed in some tumour types (Ocker and Hopfner, 2012). In addition, several natural compounds have been shown to trigger autophagy, including curcumin, resveratrol, paclitaxel, oridonin, quercetin and plant lectins (Zhang et al., 2012).

Autophagy is an evolutionary conserved homeostatic and catabolic process that works by disposing damaged cellular organelles and proteins, as well as a physiologic response to nutrient deprivation, hypoxia, reactive oxygen species (ROS), anticancer treatments and other stress conditions (Yang et al., 2011, Zhang et al., 2012, Ocker and Hopfner, 2012).

It plays an important role in protein turnover and recycling of cellular components, by enabling the sequestration and lysosomal degradation of cytoplasmic organelles and proteins, which are important for the maintenance of genomic stability and cell survival (Yang et al., 2011, Ocker and Hopfner, 2012, Jo et al., 2012).

Autophagy involves the formation of double membrane vesicles (autophagosomes) that engulf intracellular contents and fuse with lysosomes for their degradation through their proteolytic enzymes (Barth et al., 2010, Kroemer et. al, 2009).

Morphologically, this process is characterized by the formation of cytoplasmic vacuoles containing cellular organelles or protein aggregates, as already mentioned, and by the absence of typical features of apoptotic cell death. Biochemically, cell death is also executed without activation of caspases (Ocker and Hopfner, 2012).

The assembly of autophagic vacuoles involves several members of the ATG family gene that can recruit the BH3-domain regulator beclin-1 and the phosphoinositide-3-kinase (PI3K) member VPS34. This complex activates other ATG members that recruit the protein LC3 and conjugate it with phosphatidylethanolamine, leading to the formation of the autophagosome vesicle that will eventually fuse with a lysosome. The newly formed autophagolysossome

would then lead to the digestion of its content (Ocker and Hopfner, 2012, Zhang et al., 2012, Huang and Klionsky, 2007).

Cytoprotective autophagy may facilitate tumour cell survival and progression, and promote resistance to anticancer treatments (Yang et al., 2011). On the other hand, depending on the context, autophagy induction may act as a pro-death factor, arising as an alternative approach to kill cells, especially when these are apoptosis-defective. It is still controversial whether autophagy is a defence tool against chemotherapy or if it contributes to cell death (Ocker and Hopfner, 2012).

Autophagy inhibition has been shown to trigger apoptosis (Yang et al., 2011). However, the crosstalk between autophagy and apoptosis is complex, and strategies exploiting it have a considerable therapeutic potential (Yang et al., 2011). The interplay between autophagy and apoptosis has been documented at many levels, where both mechanisms share common mediators, ranging from the core machinery (Atg5, Beclin 1-Bcl-2/Bcl-x_L interaction) to upstream regulating pathways (AKT, DAPK, ARF, E2F1, HMGB1 and p53). HMGB1 release has been shown to act both on autophagy and apoptosis, depending on its redox state. Cytoplasmic translocation of HMGB1 was shown to mediate stress-induced autophagy through the MEK-ERK pathway and limited apoptosis (Yang et al., 2011).

Prolonged autophagy may culminate in tumour cell death. In fact, several well-known chemotherapeutic agents interfere with autophagy programs in tumour cells (Ocker and Hopfner, 2012). Genetic or pharmacological inhibition of cytoprotective autophagy enhance drug-induced cell death, thus representing a valuable therapeutic approach (Yang et al., 2011). Indeed, many agents have been found to induce autophagy: kinase inhibitors, arsenic trioxide, tamoxifen, lucanthone, cyclooxygenase inhibitors and the HIV inhibitor, nelfinavir (Yang et al., 2011). In turn, rapamycin and its analogs (temsirolimus (CCI-779), everolimus (RAD-001) and deforolimus (AP-23573)) have been shown to activate the autophagosome formation (Ocker and Hopfner, 2012, Yang et al., 2011). When combined with cytotoxic chemotherapy, they were proven to enhance apoptosis *in vitro* and to enhance antitumour efficacy *in vivo* (Yang et al., 2011).

Preclinical studies have already shown that inhibition of cytoprotective autophagy by genetic or pharmacological means may overcome treatment resistance. Autophagy inhibitors such as chloroquine and hydroxychloroquine may prevent autophagolysosome maturation and degradation (Ocker and Hopfner, 2012,

Yang et al., 2011); Imatinib, a kinase inhibitor that promotes autophagy through the regulation of lysossomal components, may also have the same effects (Yang et al., 2011).

The elucidation of the mechanisms of autophagy activation, its interplay with apoptosis and the specific mechanisms by which autophagy confers resistance to treatments are a very important and promising area of investigation, since new antitumour therapy approaches are an urgent demand.

Chemical families with antitumour activity

In the last decades, a lot of research for new natural and synthetic antitumour compounds has been made in order to define the therapeutic potential of different chemical families and to characterize structure-activity relationships.

In this work, some xanthones and natural compounds, including some of plant and marine origin, were studied.

Natural compounds

Nature has always been a very rich source of compounds with the most diverse pharmacological activities, and a considerable amount of natural compounds or natural product-derived drugs is currently being used for several therapeutic applications (Yue et al., 2010). Although they have been in disuse for a while because of their lack of selectivity against cancer cells, they have regained attention due to the inability of targeted therapies to face the urgent demands of the fight against cancer (Yue et al., 2010). Indeed, it is estimated that more than 79% of all the commercially available drugs on the United States from 1985 to 2002 are of natural origin or derived from natural compounds (Javed et al., 2011).

In fact, compounds from microorganisms, plants and marine organisms have added valuable information about mechanisms of action. Two main reasons underlie the effectiveness of natural compounds: a very high correlation between drug properties and those of the natural products from which they derive, and their built-in chirality, which makes them the best suited to fit cell receptors and proteins (Kingston, 2009).

Natural products, semi-synthetic natural products or natural product-derived compounds have been used in a wide range of medical fields, including diseases of infectious, neurological, cardiovascular, metabolic, immunologic, inflammatory and oncological nature (Butler, 2008).

Plants are a remarkable source of chemotherapeutic agents. Examples include camptothecins, combretastatins, noscapine, vinblastine, paclitaxel, epipodophyllotoxins, genistein, isoflavones, gossypol, β-lapachone, rohitukine,

resveratrol, curcumin, oleanolic and betulinic acids, and silybin (Butler, 2008, Mishra and Tiwari, 2011).

In the present work, extracts from *Cecropia catarinensis* were tested for their chemotherapeutic potential. Plants from the *Cecropia* genus are common in Latin America, in which they have been used for their antidiuretic, hypoglycemiant, anti-asthmatic, anti-depressant and anti-malarial properties (Durães, 2013). *C*-glycosylflavones and terpenes have been found to occur in *Cecropia* plants (Durães, 2013). Extracts from *Cecropia catarinensis* have shown variable degrees of activity against breast cancer (MCF-7) and melanoma (A375-C5) cell lines (Durães, 2013).

Marine organisms are also abundant sources of anticancer compounds. Algae, bacteria and cyanobacteria, sponges, nematodes and fungi are a great source of highly bioactive secondary metabolites that may be useful as lead compounds for the development of new chemotherapeutic agents (Gamal, 2010, Javed et al., 2011). Ascidians have provided plitidepsin and marine sponges have been the source of halichondrin B, hemiasterlin and psammaplin A (Butler, 2008, Mishra and Tiwari, 2011). Dolastatins were isolated from cyanobacteria, while kahalalide F originated from green algae (Butler, 2008, Mishra and Tiwari, 2011, Javed et al., 2011). Several studies with anti-tumour compounds from algae, such as Halomon, have been conducted to the clinical phase (Nianjun Xu, 2004). Jorumycin and bryostatin 1 are other examples of marine compounds with anticancer activity (Butler, 2008, Mishra and Tiwari, 2011). Compounds as diverse as bifurcadiol 76 (from brown alga *Bifurcaria bifurcata*) and diterpenes (Gamal, 2010), palmitic acid (from red alga *Amphiroa zonata*), porphyrans and polysaccharides (Shalaby, 2011) have been shown to induce cytotoxicity through different mechanisms, including cell cycle arrest and apoptosis (Shalaby, 2011).

In this work, extracts from *Ulva rigida* (a green alga) and *Gelidium microdon* (a red alga) were analysed for their bioactivity against cancer cells. Previous works demonstrated that *Ulva rigida* and *Gelidium microdon* revealed cytotoxicity against the A375-C5 human tumour cell line (Medeiros, 1999), as well as antioxidant properties (Khanavi et al., 2012, Paiva, 2011). *Ulva rigida* has also been found to be anti-mutagenic (Khanavi et al., 2012).

Xanthone derivatives and their biological activities

Xanthones (9*H*-xanthen-9-ones) are a class of compounds consisting of an oxygenated heterocyclic scaffold (dibenzo-γ-pirone; Figure 1) (Pedro et al., 2002, Pinto et al., 2005) that can be found in nature as secondary metabolites of higher plants and microorganisms such as fungi and lichens (Na, 2009, El-Seedi et al., 2009).

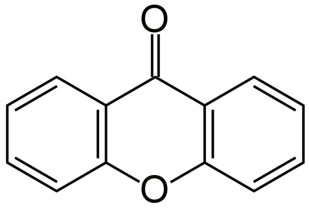

Figure 1: Xanthone (dibenzo-γ-pirone) scaffold.
Picture adapted from Pinto et al., 2005.

These compounds have drawn the attention for the numerous biological activities that are strictly connected to the dibenzo-γ-pirone structure and to the different substituents on the ring system (Pedro et al., 2002, Na, 2009). Since natural occurring xanthones are limited in type and position of the substituents by the biosynthetic pathways, the chemical synthesis of new compounds can provide new molecules containing the xanthone scaffold with substituents of distinct natures at different positions (Pedro et al., 2002, Sousa and Pinto, 2005).

In the past few years, a large number of natural and synthetic xanthones with interesting biological and pharmacological activities has been reported (Palmeira et al., 2010).

Currently, many studies and research of clinical relevance have been made around this class of compounds and some remarkable examples such as DMXAA will be approached later in this section.

Biological activities

Many structure-activity relation studies have been conducted in order to establish a connection between different xanthonic compounds, their substituents

and different biological activities. These facts raised increasing interest in the search and optimization of anticancer compounds, from whose structures several others may be developed. In fact, several natural and synthetic xanthones have been described for their effects on crucial enzyme and functional systems, leading to many biological activities (Pinto et al., 2005).

Various xanthonic derivatives revealed to be able to modulate many important enzyme systems such as acetylcholinesterase, aldose reductase, aromatase, cyclic AMP-phosphodiesterase, human cytochrome P450 17 α-hydrolase-17, 20-lyase, monoamine oxidases A and B, proteases (such as caspase-3 and caspase-9), sphingomielinases, topoisomerases I and II, and protein kinases (Pinto et al., 2005, Urbain et al., 2008, Jun et al., 2011).

Among diverse biological activities, xanthones have shown the following effects: antimicrobial (antibacterial, antimycotic, antimalarial, antileishmanial, antiviral), central nervous system stimulation, anticonvulsant, analgesic, antiarrhythmic and antihypertensive, diuretic, antidiabetic, antiplatelet/anticoagulant, antiallergic, antioxidants, hepatoprotective, anti-inflammatory, antimutagenic, antitumour, and immunomodulator (Pinto et al., 2005, Pouli and Marakos, 2009, El-Seedi et al., 2010, Szkaradek et al., 2013, Batova et al., 2010, Ryu et al., 2011, van der Merwe et al., 2012, Gong et al., 2012).

Antitumour activity

The *in vitro* growth inhibitory activity on tumour cell lines appeared to be a quite remarkable biological effect of numerous xanthones, since they exert their effect on a wide range of more than 20 different tumour cell lines of different origins and characteristics (Pinto et al., 2005, Leao et al., 2013, Azevedo, 2013). In fact, cytotoxicity assays have been performed for xanthones and their derivatives with a wide diversity of human cell lines, including those from colorectal, lung, breast, cervical, pancreatic, oral squamous cell, nasopharyngeal and liver carcinomas, fibrosarcomas, adenocarcinomas, leukaemias and melanomas (Su et al., 2011, Garcia-Rivera et al., 2011, Aisha et al., 2012, Deng et al., 2012, Jia et al., 2011, Pinto et al., 2005, Zou et al., 2012). Many of these studies have also evaluated additional

parameters, such as viability, apoptosis, mechanisms of action, anti-tumourigenicity and effects on cell signalling pathways (Aisha et al., 2012, Garcia-Rivera et al., 2011, Su et al., 2011). In addition, some of these studies are being complemented with *in vivo* assays with tumour-bearing animal models, thus reinforcing *in vitro* results (Aisha et al., 2012).

The chemopreventive and chemotherapeutic potential of xanthones and their derivatives are mainly due to their ability to suppress tumour initiation and growth, to induce cell cycle arrest and apoptosis, as well as their anti-inflammatory, anti-invasive and anti-metastatic effects (Shan et al., 2011, Azevedo, 2013, Leao et al., 2013).

Xanthones may prevent the production of carcinogenic compounds/metabolites and the formation of DNA adducts that are implied in carcinogenesis. In fact, xanthones can modulate the activity of phase I and phase II enzymes, influencing carcinogen metabolism. Furthermore, mangosteen is a great natural source of antioxidant xanthones that can inhibit or delay oxidative damage through mechanisms such as free radical scavenging (Shan et al., 2011).

The release of many inflammatory mediators that might be associated with carcinogenesis can be effectively inhibited by xanthones in non-malignant cells. However, further studies are needed to determine this effect in cancer cells. γ-mangostin exhibited a potent concentration-dependent inhibitory activity of prostaglandin E2 (PGE2) release, mediated through the inhibition of both cyclooxigenase-1 and -2 (COX-1 and COX-2) activities. α- and β-mangostin decrease the expression of pro-inflammatory molecules by interfering with several intracellular signaling pathways (Shan et al., 2011).

In turn, xanthones are able to induce apoptosis in cancer cells by interfering with pathways that are crucial for cell death and survival. It has been reported that mangosteen extract induces apoptosis in various cell types by activating pro-apoptotic signalling molecules and by inhibiting anti-apoptotic molecules within intracellular signal transduction pathways. α-mangostin induced apoptosis in human promyelocytic leukaemia cells through the activation of caspase-9 and caspase-3, but not caspase-8, and also inhibited Ca^{2+}-ATPase, showing that α-mangostin may be involved in the intrinsic apoptotic pathway (Shan et al., 2011, Pinto et al., 2005, Kurose et al., 2012). α-mangostin was also able to cause apoptosis either by activating caspases or by changing BAX, BCL-2 and apoptosis-inducing factor (AIF)

levels. Also, combined treatment with gartanin and TRAIL led to a potential sensitization of TRAIL-resistant human tumours (Shan et al., 2011).

In addition, xanthones can directly inhibit the proliferation and decrease the viability of various cancer cell types *in vitro*, as manifested by the significant arrest of cells at various phases of the cell cycle. Four structurally similar prenylated xanthones (α-mangostin, β-mangostin, γ-mangostin, and methoxy-β-mangostin) were associated with cell cycle arrest through modulation of the expression of several cyclins and other cell cycle-related proteins in human colon cancer cells. While the exposure to α- and β-mangostin resulted in a G1 arrest, treatment with γ-mangostin led to a S arrest (Shan et al., 2011).

Although the exact underlying mechanism is unknown, xanthones have shown anti-invasive and anti-metastatic activity. α-mangostin is a novel anti-metastatic agent that exhibited an inhibitory effect on the adhesion, migration, and invasion of human cancer cells, which is associated with decreased expression of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9). *In vivo* studies in a mice model with breast cancer also revealed that metastasis tended to decrease using panaxanthone in their diet (Shan et al., 2011).

Remarkable examples

DMXAA

DMXAA (5,6-dimethylxanthenone-4-acetic acid; ASA404; Vadimezan; Figure 2) (Jameson and Head, 2011) is a simple carboxylated xanthone (Pinto et al., 2005) that has shown very promising therapeutic features, with both immune and non-immune-mediated mechanisms of action (Buchanan et al., 2012). This compound is a vascular-disruptor agent that activates macrophages and dendritic cells, and is the most potent and most advanced of its class in clinical development (Jameson and Head, 2011, Kim et al., 2012a, Sun et al., 2011). Although its action on endothelial hyperpermeability is thought to be mostly indirect (through leukocyte-mediated signalling), DMXAA has also been shown to directly trigger signalling and apoptotic events in endothelial cells (Kim et al., 2012a). Unlike other vascular-disrupting

agents, DMXAA does not seem to target microtubules (Kim et al., 2012a, Buchanan et al., 2012).

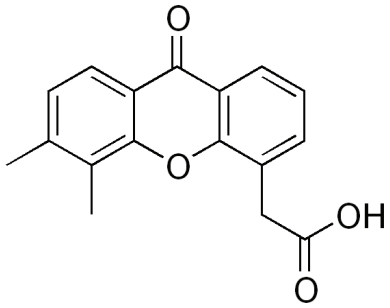

Figure 2: DMXAA chemical structure.
Picture adapted from Pinto et al., 2005.

Studies revealed that DMXAA is capable of causing fast vascular collapse and tumour haemorrhagic necrosis by immunomodulation and pro-inflammatory cytokine induction (Sun et al., 2011, Kim et al., 2012a, Ellis et al., 2012). The production of cytokines (such as TNF-α, granulocyte-colony stimulating factor G-CSF and IL-6) by white blood cells seems to be induced by DMXAA in a TANK binding kinase 1 (TBK1)-dependent manner (Kim et al., 2012a). Besides that, it can also induce the expression of 5-hydroxytryptamine (5-HT), nitric oxide (NO), and transcription factors STAT (signal transducers and activators of transcription) and nuclear factor κB (NFκB) (Pinto et al., 2005, Gridelli et al., 2009, Lara et al., 2011). DMXAA ability to modulate mitogen-activated protein kinases (MAPK) is also documented (Sun et al., 2011). In particular, p38MAPK and extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) have been found to mediate DMXAA regulation of both TNF-α and IL-6 production (Sun et al., 2011). Also, DMXAA was found to strongly inhibit members of the VEGFR (vascular endothelial growth factor receptor) tyrosine kinase family. This mechanism may be of special importance for its non-immune-mediated effects on tumour vasculature (Buchanan et al., 2012).

Besides its antivascular effects, DMXAA has an anti-angiogenic action, mediated by the anti-angiogenic chemokine interferon-inducible protein 10 (IP-10), which inhibits neovascularization in several *in vitro* and *in vivo* models (Milanovic et al., 2012).

DMXAA may be applied in synergy with conventional cytotoxic agents (such as paclitaxel, docetaxel or carboplatin) (Jameson and Head, 2011, Milanovic et al., 2012) and other antivascular agents (Silva and Pinto, 2005, Ellis et al., 2012).

Immunomodulatory agents that increase host-mediated responses, such as cytokine and NO production, may be concomitantly administered. A synergic effect was observed when combined with chemotherapeutic agents and thermal radiation therapy (Pinto et al., 2005).

DMXAA pharmacokinetics has been well characterized and, in spite of its complexity, its behaviour is relatively predictable (Jameson and Head, 2011).

Phase I studies concluded that DMXAA has anti-tumour activity at well-tolerated doses (Daga et al., 2011, Kelland, 2005). In spite of the encouraging results of phase II trials, in phase III DMXAA failed to demonstrate any advantage to its addition to standard platinum-based chemotherapy for the first line treatment of advanced non-small lung cancer cells (Lara et al., 2011, Buchanan et al., 2012).

Even so, DMXAA remains a conceptually exciting drug that can bring new future perspectives in cancer treatment (Kim et al., 2012a).

Psorospermin

Psorospermin is a natural dihydrofuranoxanthone (Figure 3) isolated from the roots and bark of a tropical African plant, *Psorospermum febrifugum*, which has been studied for over thirty years (Carey et al., 2008, Pinto et al., 2005).

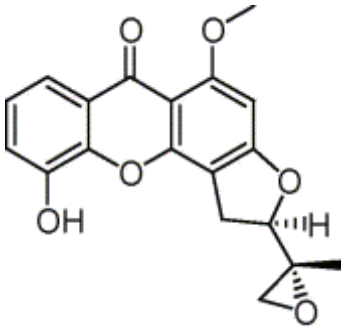

Figure 3: Psorospermin chemical structure.
Picture adapted from Palmeira et al., 2010.

This compound has demonstrated significant cytotoxic activity against several human tumour cell lines such as breast, colon, lymphocytic leukemia, drug-resistant leukaemias, and AIDS-related lymphomas, both *in vitro* and *in vivo* (Pinto et al., 2005, Carey et al., 2008).

Psorospermin intercalates the DNA molecule and is able to promote its alkylation. This ability is significantly enhanced by topoisomerase II, an enzyme that is involved in DNA breakage and re-ligation. Psorospermin antitumour activity may then be associated with its interaction with the topoisomerase II-DNA complex. By entrapping this complex, psorospermin leads to DNA cleavage and, thus, to cancer cell death (Carey et al., 2008, Pinto et al., 2005).

Therefore, this drug exhibits a novel mechanism of action, being an irreversible topoisomerase II poison. Rapidly proliferating and neoplastic cells usually contain higher levels of topoisomerase II than normal cells. This selectivity is considered a therapeutic advantage over other clinically used topoisomerase II poisons (Pouli and Marakos, 2009).

Evaluation of psorospermin/quinobenzoxazine hybrids already gave rise to structurally novel and even more potent antitumour agents (Pinto et al., 2005).

Prenylated xanthones and pyranoxanthones

Mangosteen (*Garcinia mangostana* L.) is a tropical tree whose fruits, bark, and roots have been used for thousands of years in traditional medicine (Akao et al., 2008, Pinto et al., 2005, Obolskiy et al., 2009) to treat a great variety of medical conditions. It has been used mainly for its anti-microbial and anti-inflammatory activities (Obolskiy et al., 2009).

Prenylated xanthones are the main components of the pericarp of the mangosteen fruit. In fact, they constitute the most abundant family of naturally-occurring xanthones (Chin and Kinghorn, 2008, Pinto and Castanheiro, 2009). They are responsible for most of mangosteen fruit properties (Zhang et al., 2010). Prenylated xanthones extracted from the fruit have already found many applications such as antibacterial, antifungal, antiretroviral, antiallergic, antioxidant and anti-inflammatory purposes, and also for the treatment of rosacea, telangiectasia and skin aging (Obolskiy et al., 2009, Pinto et al., 2005, Zhang et al., 2010). Moreover, prenyl substituents are thought to enhance xanthone potency and selectivity for some of these properties (Castanheiro et al., 2009, Azevedo, 2013). Indeed, xanthones bearing prenyl side chains have enhanced activity when compared with their non-prenylated analogs (Palmeira et al., 2010).

Concerning their antitumour effects, xanthone extracts from the fruit pulp and pericarp have become very attractive as useful sources of cancer chemopreventive and chemotherapeutic agents (Pinto et al., 2005).

Extracts from *Garcinia mangostana* were shown to have anti-proliferative effects on leukemia and breast tumour cell lines, as well as a potent cytotoxic effect against hepatocellular carcinoma, gastric and lung cancer cell lines. Moreover, they have been found to prevent initial carcinogenesis events and to inhibit the production of reactive oxygen species, thereby showing important apoptotic and antioxidant properties (Pinto et al., 2005).

α-Mangostin has significant antiulcer activity *in vivo* and is a potent inhibitor of acidic sphingomyelinase, which is involved in cancer pathogenesis. In turn, γ-mangostin shows important anti-inflammatory properties, since it can inhibit COX and PGE2 synthesis. α-, β- and γ-mangostins also induce apoptosis through differential activation of caspases. Finally, both α- and γ-mangostins seem to inhibit protein kinases such as those from the PKC family (Pinto et al., 2005, Matsumoto et al., 2004).

Besides naturally occurring xanthones, several synthetic prenylated xanthones have been being developed, leading to promising biological activity results (Castanheiro et al., 2007, Palmeira et al., 2010, Paiva et al., 2012, Azevedo, 2013). For instance, 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one, a synthetic pyranoxanthone, has been shown to have antiproliferative and pro-apoptotic activities (Paiva et al., 2012, Palmeira et al., 2010). In particular, it also has the ability to enhance the anti-estrogenic effect of 4-hydroxytamoxifen in an estrogen-dependent tumour cell line (MCF-7) (Paiva et al., 2012).

In fact, pyranoxanthones are another promising group of xanthones that can be obtained both from natural sources and through synthesis. A significant number has been synthetized using acronycine as lead compound. This, in turn, has been isolated from stem bark of *Acronychia baueri* (Rutaceae), a small Australian tree, showing antileukaemic activity, but also large spectrum toxicity (Pouli and Marakos, 2009). Depending on their structure, pyran substituents were found to act on specific cell cycle stages, being more potent and selective than acronycine itself (Pouli and Marakos, 2009). Their cytotoxicity has been demonstrated against several tumour cell lines, including breast adenocarcinoma MCF-7, non-small cell lung NCI-H460, central nervous system SF-268, and melanoma UACC-62 cell lines (Pouli and

Marakos, 2009). In turn, most naturally-occurring pyranoxanthones bear prenyl groups on key positions of their xanthone nuclei (Castanheiro et al., 2009, Pinto et al., 2005, Paiva et al., 2012), which are associated with inhibition of human lymphocyte proliferation, protein kinase C (PKC) modulation, antitumour and anti-inflammatory activities. Extracts from *Cratoxylum formosum*, *Cudrania tricuspidata, Garcinia mangostana*, *Ovomita brevistaminea*, *Cratoxylum sumatranum*, for instance, have been found to contain pyranoxanthones and to exhibit antiproliferative and cytotoxic activities (Pouli and Marakos, 2009, Castanheiro et al., 2009, Pinto et al., 2005, Paiva et al., 2012).

These findings emphasize the potential of synthetic and natural prenylated xanthones as pyranoxanthones as scaffolds for the design of new, more potent and specific antitumour compounds.

Thioxanthones

Thioxanthones are isosteric analogues of xanthones, consisting of S-heterocycles with a dibenzo-γ-thiopyrone scaffold (Figure 4) (Palmeira et al., 2012b).

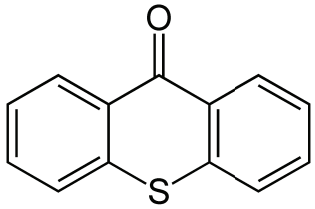

Figure 4: The thioxanthone (dibenzo-γ-thiopyrone) scaffold.

Picture adapted from Paiva et al., 2013.

The first thioxanthone with promising therapeutic value, lucanthone (Miracil D) (Figure 5A), appeared in the 1940's decade as an antischistossomal agent (Hawking and Ross, 1948, de Paiva et al., 2013). Together with hycanthone (Figure 5B) (its derivative), it has shown the ability of this class of molecules to intercalate into DNA, and to inhibit RNA synthesis, as well as the DNA processing enzymes topoisomerases I and II (Naidu et al., 2011). These two compounds presented similarity with other intercalating agents on the tricyclic moiety and aminated side chain. Nevertheless, their mutagenicity, due to their methylene moiety directly bound

to C-4, disencouraged their use in cancer chemotherapy (Palmeira et al., 2012a). Recently, lucanthone proved to be a novel inhibitor of autophagy, being even more potent against a panel of breast cancer cell lines than chloroquine (chloroquine and its analog hydroxychloroquine are the only clinically relevant autophagic inhibitors used in cancer therapy) (Carew et al., 2011).

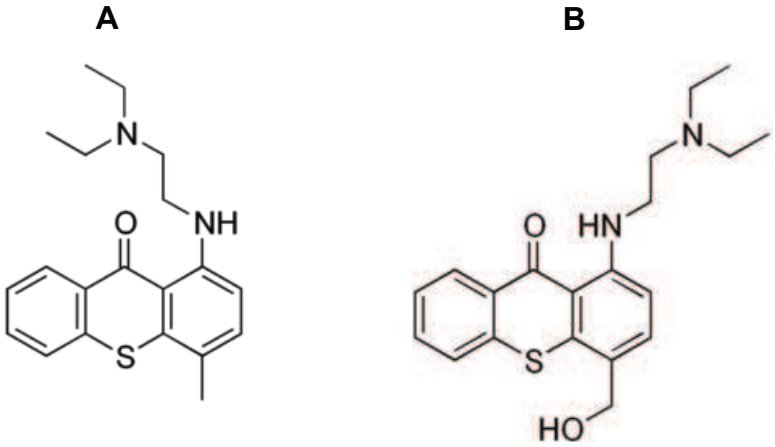

Figure 5: The lucanthone (A) and hycanthone (B) chemical structures.
Pictures adapted from Paiva et al., 2013.

SR233377, a hycanthone derivative, is a second-generation aminated thioxanthone. It has shown solid tumour selectivity *in vitro*, and this activity was confirmed *in vivo* against several murine tumours (LoRusso et al., 2000, Goncalves et al., 2008). However, it was found to be hepatotoxic. This issue was overcome by SR271425, a third-generation thioxanthone, which showed broad spectrum solid tumour activity both *in vitro* and *in vivo* in murine as well as in human xenograft tumour models (Goncalves et al., 2008). Both hycanthone analogs were driven until phase I trials, but were abandoned because of their cardiotoxicity (Stevenson et al., 1999, LoRusso et al., 2000, Goncalves et al., 2008). The continuous search for new thioxanthones and their derivatives is yielding increasingly better results, thus encouraging efforts in this research field.

Some thioxanthone derivatives, such as 2,7-disubstituted xanthones and thioxanthone derivatives (in particular, one with an aminoalkoxy substituent) have also been described as potential inhibitors of miRNA pathways in order to modulate gene expression (Murata et al., 2013), which have been studied for their relevant role in cancer diseases (Sassen et al., 2008).

Palmeira et al. have evaluated the biological effects of a series of

thioxanthones. Two aminoalkylthioxanthones have been described as potential dual non-competitive inhibitors of P-glycoprotein (P-gp) and cell growth (Palmeira et al., 2012b). In a similar study, the same authors have found that one of those thioxanthones, as well as a similar one, are multi-target inhibitors of P-gp, BRCP (breast cancer resistance protein), and multidrug resistant proteins (MRP) 1 and 3. These are important findings that emphasize the relevance of a deeper knowledge of the interplay between these compounds and typical resistance mechanisms, as a requirement for enhanced therapeutic approaches (Palmeira et al., 2012a).

TXA1 (Figure 6A) is a new aminated thioxanthone synthesized by the CEQUIMED-UP research group. This small molecule has revealed to have a potent antitumour activity in *in vitro* assays using several tumour cell lines such as MCF-7, NCI-H460, A375-C5, and K562 (GI_{50}<10μM) (data obtained by CEQUIMED-UP group, not published). Furthermore, there is preliminary evidence that this compound affects the cell cycle. Flow cytometry results showed that TXA1 significantly increases the percentage of leukaemic cells in the G1 phase and decreases the amount of cells in the G2 phase, which means a G1 arrest in cell cycle (data obtained by CEQUIMED-UP group, not published).

The TXA1.HCl salt (Figure 6B) was originally synthesized from TXA1 in order to improve its solubility and bioavailability characteristics. A rodent erythrocyte micronucleus assay suggested a direct DNA reactivity of TXA1.HCl for concentrations above 5,75 mM (Gomes, 2011, Vieira, 2010).

There is little information available about the antitumour mechanism of action of these compounds, which substantiates the need to elucidate it.

From what has been exposed so far, the need to pursue studies that correlate compound chemical structures and modifications with their anticancer effects becomes clear.

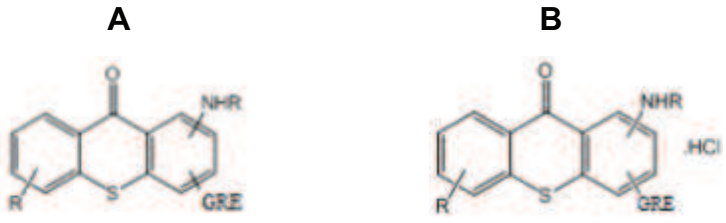

Figure 6: The thioxanthonic scaffold of TXA1 (A) and TXA1.HCl (B).

Pictures taken from Vieira, 2010.

Chapter II.

Aims

This work aimed at the screening of antitumour activity on both natural and synthetic compounds. Nine natural product-derived samples and 12 synthetic compounds were screened on different cell lines from different origins.

One thioxanthone revealed an important cell growth inhibitory effect during the previous screening work (as previously demonstrated by CEQUIMED-UP group) and prompted the search for insights of its molecular mechanism of action. The work reported in the present thesis aimed at characterizing the compound's activity profile through proliferation and viability assays and at clarifying how the cytotoxicity is performed.

Chapter III. Materials and Methods

Cell Culture

Cell lines

In the present study, four different human tumour cell lines were used:

- **A375-C5** – malignant melanoma (European Collection of Cell Cultures, UK);
- **MCF-7** – breast adenocarcinoma (Eutopean Collection of Cell Cultures, UK);
- **NCI-H460** – non-small cell lung cancer (National Cancer Institute, USA);
- **HeLa** – cervical adenocarcinoma (Molecular Medicine Institute – University of Lisbon).

Cell culture conditions

A375-C5, MCF-7, and NCI-H460 cell lines were cultured in complete growth medium consisting of Roswell Park Memorial Institute-1640 medium (RPMI-1640, BioWhitakker®, Lonza) supplemented with 5% (v/v) Foetal Bovine Serum (FBS, Gibco). HeLa cells, in turn, were cultured in Dulbecco's Modified Eagle's Medium (D-MEM, PAA – The Cell Culture Company) supplemented with 10% (v/v) FBS.

All cell lines were grown in monolayer cultures in adequate flasks and kept permanently in exponential growth in a humidified incubator (Heraeus Hera Cell), at 37ºC and a 5% CO_2 atmosphere. Cells were trypsinized and subcultured twice a week in order to avoid confluence and ensure their healthy exponential growth.

All procedures involved in cell culture were performed in a Telstar Bio-II-A/P laminar flux biological safety cabinet, biosafety level II, and aseptic techniques were always regarded.

Cell subculture

Cells were maintained in 25 cm^3 flasks and regularly observed under microscopy (Zeiss Primo Vert) to check for the confluence and to assure they looked healthy and free of contamination to use in the experiments. At approximately 70-80% confluence, they were subcultured to new flasks, according to the following procedure.

Growth medium was discarded and the cells were washed with 2ml of sterile Phosphate-Buffered Saline (PBS, pH 7.2: 137 mM NaCl; 2.7 mM KCl; 6.4 mM K_2HPO_4; 1 mM $NaHPO_4$) to remove trypsin inhibitors. In order to promote cell detachment, 500µL of Trypsin EDTA 1× (ScienCell) were added and allowed to act for 5 minutes at 37.0 °C. Cell detachment was then confirmed through microscopic observation. 4.5ml of complete growth medium were added to stop the reaction. At this point, two different approaches were performed, in accordance to the final purpose of the cell suspension (experimental assays or cell culture maintenance).

When cells were intended to be seeded at a definite density for experimental assays, they were counted through the trypan blue exclusion method. In such cases, a 30µL aliquot of the cell suspension was collected. 30 µl of trypan blue were then added to this aliquot and the suspension was homogenized. Aliquots of 15 µl were then transferred to a Neubauer counting chamber. Cells were then counted using the trypan blue exclusion method and cell density (number of cells per ml) was determined[1]. Finally, an equivalent volume to the intended density of cells (according to each experiment) was transferred to an adequate final volume of fresh growth medium.

Whenever the purpose was to maintain cell culture stocks, cells were split and diluted. Cell splits were based on the percentage of cells taken from the trypsinized flask and put into a new flask.

In general, according to the amount of cells recovered from the trypsinized flask, 10% or 20% splits were performed. To perform a 10% split (1:10), 500 µL of cell suspension were transferred into a new flask containing 4.5 ml of growth medium and, to do a 20% split (1:5), 1ml of cell suspension were pipetted into the new flask.

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[1] $$\text{No. of cells/ml} = \frac{\text{total cell number}}{\text{number of squares}} \times \text{dilution factor} \times 10^4$$

Cryopreservation of cell lines

Cells were stored when growing exponentially in order to maintain a stock for posterior thawing and preventing phenotypic degeneration / dedifferentiation.

Cells were harvested when they were approximately 80% confluent.

Cell suspensions were obtained as mentioned in the “cell subculture” section by trypsinization and centrifuged at 1,000 rpm for 5 minutes; the supernatant was discarded. Each pellet was then gently resuspended in 1 ml of freezing medium (RPMI complete medium containing 10% (v/v) dimethyl sulfoxide, DMSO, as cryoprotective agent) and transferred to adequate cryovials. Upon remaining 15 minutes at room temperature, the cryovials were transferred to a -80°C freezer, in an isopropanol container, in order to ensure a steady cooling rate (-1ºC/minute).

Cell defrosting

Cryovials containing cells were removed from the freezer and rapidly thawed in a 37°C water bath. The cells were then transferred to pre-warmed growth medium and centrifuged at 1,000 rpm for 5 minutes; the supernatant was discarded due to DMSO toxicity and cells were transferred to growth medium supplemented with 10% (v/v) FBS for all cell lines except for HeLa (20%).

Compound Origin

Natural product-derived samples and synthetic origin compounds were obtained or synthesized by the CEQUIMED-UP research group.

The extracts and compounds obtained from *Ulva rigida* and *Gelidium microdon* were isolated by Prof. Anake Kijjoa at Instituto de Ciências Biomédicas Abel-Salazar (ICBAS).

The extracts obtained from *Cecropia catarinensis* were isolated by Prof. Ana Paula Almeida at Faculdade de Farmácia da Universidade do Porto – Laboratório de Química Orgânica.

Pyranoxanthones and thioxanthones were supplied by Prof. Madalena Pinto, Prof. Emília Sousa and Prof. Carlos Afonso at Faculdade de Farmácia da Universidade do Porto – Laboratório de Química Orgânica.

Drug Screening

Compound dilutions

All the compounds were aseptically dissolved in DMSO (except TXA1.HCl which was dissolved in water) to a final concentration of 60 mM and stored at -20ºC. For subsequent assays, they were diluted in growth medium.

SRB assay

The sulforhodamine B (SRB) method was performed according to the procedure adopted by the National Cancer Institute's Developmental Therapeutics Program (NCI, 2012), which was originally published by Skehan and co-workers (Skehan et al., 1990).

Briefly, cells were trypsinized and counted according to the procedure described in the "Cell culture" section. Ideal cell densities were previously determined by plotting calibration curves for each cell line. Cell suspensions with a final density of 7.5 x 10^4, 5 x 10^4, and 5 x 10^4 cells/ml were prepared, for A375-C5, MCF-7, and NCI-H460, respectively, in appropriate growth medium containing 5% FBS and lacking antibiotic/antimycotic mixture (which could influence the results). HeLa cell suspension was prepared with a final density of 2 x 10^4 cells/ml in appropriate growth medium containing 5% FBS (a lower FBS concentration is required because of its interference with the results) and lacking antibiotic/antimycotic mixture. 100 µl cells were plated per well in two 96 well-plates, with the exception of the wells in lines A and H (see Figure 7). These were inoculated with growth medium only and were used as a negative control. Three cell lines were inoculated in each plate.

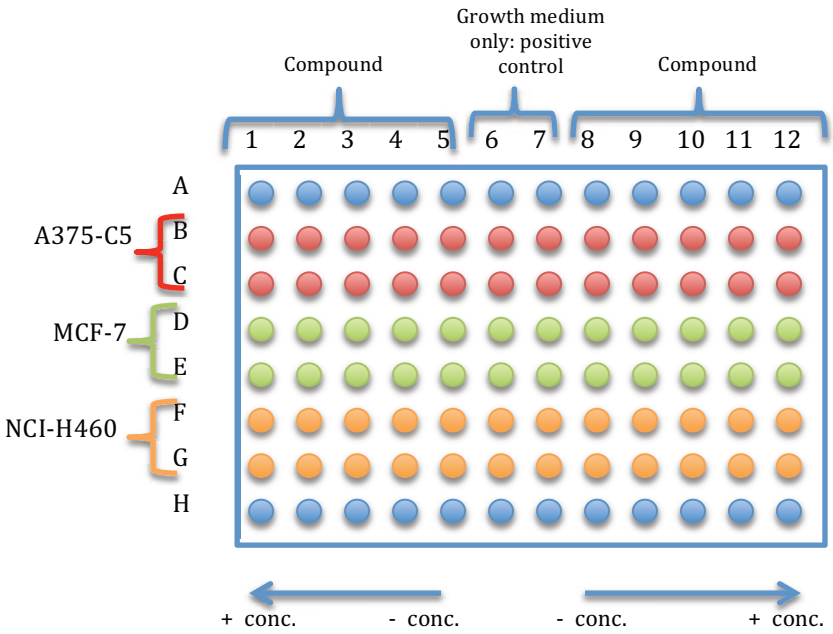


Figure 7: Schematic representation of the experimental design of a 96-well plate for drug screening through the SRB assay.

Duplicates were run with three different cancer cell lines – A375-C5, MCF-7 and NCI-H460. Lines A and H, containing growth medium but no cells, were used as negative controls; rows 6 and 7, containing cells and growth medium, but lacking compound, were used as positive controls. Doxorubicin was used as a reference drug. The compounds under study were serially diluted in growth medium and added to each cell line, plated in the appropriate density.

Cells were incubated for 24 hours in the humidified incubator, allowing them to stabilize and adhere. Then, in one of the two 96-well plates, cells were fixed by adding 50 µl of 50% (w/v) trichloroacetic acid (TCA) to each well and by incubating at 4ºC for 1 hour. The plate was then washed 5 times with distilled water and was allowed to dry at room temperature. This plate was named T_0 and was later used to determine the amount of cells present by the time that the compound was added.

In a second 96-well plate, the compound under study was added to different final concentrations. The most concentrated solution was added to columns 1 and 12; the other solutions were then serially added to the remaining columns in descending order of their concentration and in a symmetrical way. In columns 6 and 7, only 100 µl of growth medium were added (according to Figure 7). The compound was allowed to act for 48 hours at 37ºC and 5% CO_2. Following this incubation, the cells were fixed and washed as described for the T_0 plate.

Once both plates were completely dry, cell proteins were stained by adding 50 µl of 0,4% (w/v) SRB in 1% (v/v) acetic acid and incubating for 30 minutes at room temperature. SRB was then removed through five successive washings with 1% (v/v) acetic acid. The plates were allowed to dry again at room temperature.

In order to solubilize protein/SRB complexes, 100 µl of 10 mM Tris buffer were added to each well and, then, plates were shaken in an agitator to ensure homogenization and allow absorbance (Abs) determination in a plate reader (Biotek Synergy 2), at 515 nm. Abs values were retrieved using Gene5 software (Biotek).

Growth inhibition (GI) was determined in accordance to the formula $GI = [(T - T_0) / (C - T_0)] \times 100$, in which T represents the mean Abs value of the wells containing the compound concentration under analysis, T_0 represents the mean Abs value of the T_0 plate and C stands for the mean Abs value of control wells, to which no compound was added. Once this formula is applied to the several concentrations under study, it is possible to plot a graphic in which GI is a function of the compound concentration. Through graphic interpolation and using a NCI datasheet, the concentration that inhibits 50% of cell growth (GI_{50}) was determined.

Analysis of the effect of the compounds under study

MTT assay

Cells were trypsinized and counted according to the procedure described in the "Cell culture" section. Cells were then plated in ideal densities (previously determined by plotting calibration curves for each cell line), in 96-well plates, and allowed to stabilize and adhere for 24 hours. Cell suspensions with a final density of 2×10^4, 5×10^4, and 5×10^4 cells/ml were prepared, for A375-C5, MCF-7, and NCI-H460, respectively, in appropriate growth medium containing 5% FBS and lacking antibiotic/antimycotic mixture (which could influence the results). HeLa cell suspension was prepared with a final density of 5×10^4 cells/ml in appropriate growth medium containing 5% FBS (a lower FBS concentration is required because of its interference with the results) and lacking antibiotic/antimycotic mixture. 100 µl cells were plated per well. Following dilution, the compounds under analysis were then added, according to Figure 8.

Following 48 hours of exposure to the compound at 37ºC and 5% CO_2, 20 µl of a 5 mg/ml MTT ((3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)) solution in PBS were added to each well and incubated for 4 additional hours in the same conditions. Formazan crystals were then solubilized by adding 100 µl of solubilization solution (89% (v/v) isopropanol, 10% (v/v) triton X-100, 0,37% (w/v) HCl). Upon homogenization, absorbance values were read at 550 nm in a plate reader (Biotek Synergy 2) and retrieved using Gene5 software (Biotek). Dose-response curves were plotted using GraphPad Prism® version 6.0c.

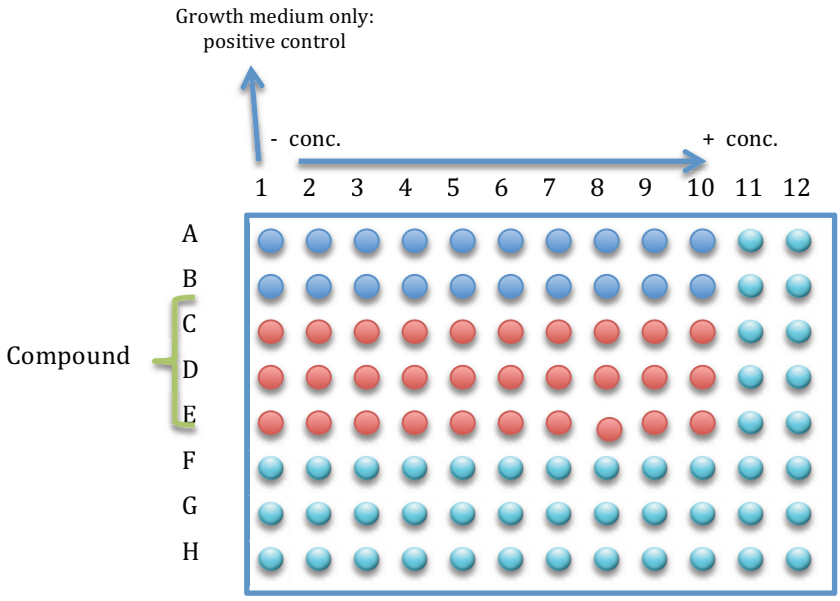


Figure 8: Schematic representation of the experimental design of a 96-well plate for drug screening through the MTT method.

Lines A and B, containing growth medium but no cells, were used as negative controls; row 1, containing cells and growth medium, but lacking compound, was used as positive control. Doxorubicin was used as a reference drug. The compounds under study were previously diluted in growth medium at specific concentrations and added to each cell line, plated in the appropriate density. Blue wells were filled with PBS.

Trypan blue assay

Cells were trypsinized, counted through the trypan blue exclusion method (as detailed in the "Cell culture" section) and plated in 6-well plates, in appropriate growth medium. 1.5 ml of 7 x 10^4 cells/ml (for MCF-7 cells) or 2.5 x 10^4 cells/ml (for HeLa cells) were plated in each well. In the following day, the compound under study (TXA1.HCl) was added to a final concentration of 5, 10 and 20 μM. Additional controls, containing or lacking water (the compound solvent), were included. The compound was then allowed to act for 24, 48 and 72 hours. In the end of each time point cells were trypsinized, cell viability and cell number were determined through the trypan blue exclusion method. Again, both cell trypsinization and the trypan blue method were performed according to the procedures described in the "Cell cultures" section. The assays were run in triplicate and the results were plotted and statistically analyzed using GraphPad Prism® version 6.0c.

Morphology assay

In order to evaluate the effect of the compound on cell morphology along with time and with different concentrations, cells were plated onto slides in 6-well plates. Cell densities and compound inoculation were performed as described in the "Trypan blue assay" section. In the end of each time point, cells were observed under phase contrast microscopy, in a Nikon Eclipse TE2000-U microscope equipped with a DXM1200F digital camera and controlled by Nikon ACT-1 software, and images were acquired with a 40x magnification. Slides were then mounted in 8 µl Vectashield containing 0,5 µg/ml DAPI and secured with nail polish. They were observed under fluorescence microscopy in a Zeiss Spinning Disc AxioObserver Z.1 SD microscope, coupled to an AxioCam MR3 camera. Representative focus plans were chosen for image acquisition, which was performed using the AxioVision 4.8.2 software.

Reversibility studies

MTT assay

MCF-7 cells were plated per well in five 96-well plates as described in the "MTT assay" section, and allowed to stabilize and adhere for 24 hours. Following dilution at specific concentrations, the compounds under analysis were added according to Figure 9.

Upon 6, 12, 24, or 48 hours of exposure to the compound, an MTT assay was performed in one of the plates, according to the described in the corresponding section. As for the other three plates, the compound-containing medium was discarded and cells were washed twice with PBS. Then, they were incubated with 200 µl of fresh growth medium (without compound), at 37ºC and 5% CO_2, for additional 24 and 48 hours. In the end of each time point, an MTT assay was performed in the corresponding plate, again in accordance to the "MTT assay" section. Data was analyzed using GraphPad Prism® version 6.0c.

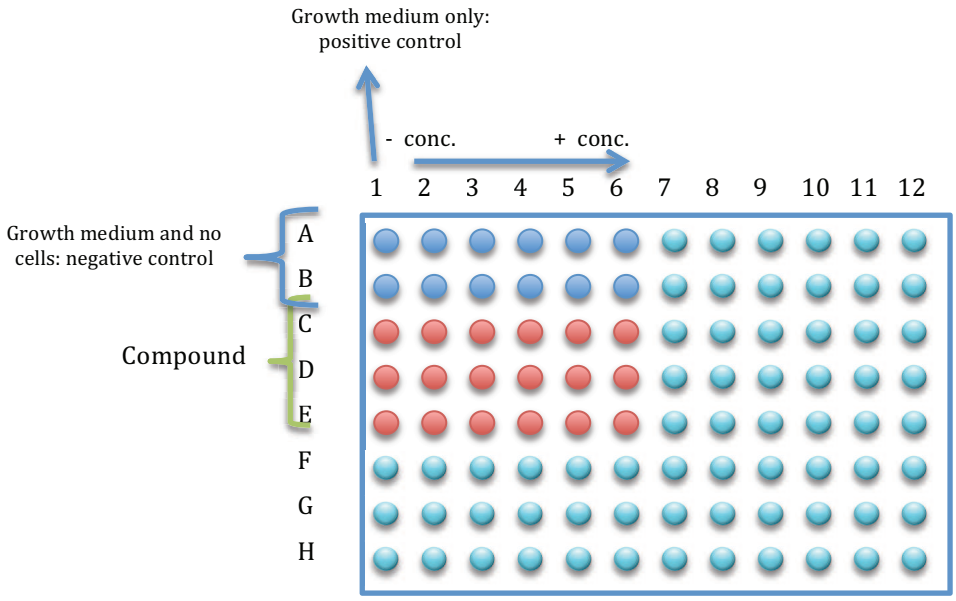


Figure 9: Schematic representation of the experimental design of a 96-well plate for the reversibility assay.

Lines A and B, containing growth medium but no cells, were used as negative controls; row 1, containing cells and growth medium, but lacking compound, was used as positive control. The compounds under study were previously diluted in growth medium at specific concentrations and added to 5 x 10^3 cells/well. Blue wells were filled with PBS.

Colony forming assay

7.5 x 10^2 MCF-7 cells were plated per well in 6-well plates and allowed to adhere and stabilize for 24 hours.

Cells were then exposed to 5, 10 and 20 μM TXA1.HCl and incubated for 6 and 24 hours. Additional controls, containing or lacking water (the compound solvent), were included in the assay. Thereafter, the compound-containing medium was discarded and cells were washed twice with PBS. Then, they were incubated with fresh growth medium, at 37ºC and 5% CO_2, for additional 14 days.

The resulting colonies were fixed with 3.7% (v/v) paraformaldehyde for 5 minutes and stained with crystal violet (0.05% (w/v) in distilled water) for 20 minutes. The plates were then washed twice and were allowed to dry at room temperature.

All plates were photographed in a Gel Doc™ XR system (Bio-Rad) and colonies were manually counted.

TUNEL assay

Cell death by apoptosis was assessed with TUNEL assays. The effect of the compound on cell death was evaluated after different time expositions to different compound concentrations. Cells were plated in 6-well plates. Cell densities, compound inoculation, and exposure times (with the exception for a 14 hours exposure) were performed as described in the “Trypan blue assay” section. After the compound exposition, cells were trypsinized and counted by the trypan blue exclusion assay. Cells were then resuspended in PBS at a $1x10^5$ cells/ml density, and submitted to a cytospin (Thermo Scientific Cytospin 4).

The TUNEL assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega), according to the manufacturer’s instructions. In brief, cells were fixed by immersing the slides in a Coplin jar in a fresh 4% (v/v) paraformaldehyde solution in PBS, for 25 minutes, at 4ºC. Then, cells were washed twice with PBS and permeabilized with a 0,2% (v/v) Triton X-100 solution in PBS. Slides were then rinsed twice with PBS. Following excess liquid removal, cells were incubated with 100 µl of Equilibration Buffer for 10 minutes. The equilibrated areas were blotted around with paper and incubated with rTdT Incubation Buffer (composition per standard 50 µl reaction: 45 µl Equilibration Buffer, 5 µl Nucleotide Mix, 1 µl rTdT Enzyme). Negative controls, in which rTdT Incubation Buffer had 1 µl sterile deionized water instead of rTdT Enzyme, were included, as well as positive controls, treated with DNaseI (Fermentas) or H_2O_2. Care was taken in order to prevent cells from drying out. Cells were incubated for 1hour, at 37ºC, in a humidified chamber and protected from direct light, to allow the tailing reaction to occur. The reaction was stopped by immersing the slides in 2x SSC for 15 minutes, at room temperature. Unincorporated fluorescein-12-dUTP was then washed through three washing steps with PBS. Unless otherwise stated, each washing or incubation step proceeded for 5 minutes, at room temperature.

Slides were then mounted in DAPI and analyzed by fluorescence microscopy as described in the “Morphology assay” section.

Analysis of genomic DNA fragmentation by electrophoresis

In order to further assess TXA1.HCl effects on cells, fragmentation of genomic DNA, a hallmark of apoptosis, was evaluated through electrophoresis.

MCF-7 cells (1.5 ml of a 5 x 10^4 cell/ml suspension) were seeded onto 6-well plates, and exposed to TXA1.HCl for 48 hours, at a final concentration of 0 (negative control), 5, 10 and 20 µM. H_2O_2 was also added, at a final concentration of 0,2 mM, as an inducer of apoptosis (positive control).

Genomic DNA extraction was then performed used the QIAamp® DNA Mini and Blood Mini kit (Qiagen), according to the "Protocol for Cultured Cells". In the final step, genomic DNA was eluted in 100 µl deionized water, instead of 200 µl Buffer AE.

Genomic DNA concentration was determined using a Nanodrop 2000 spectrophotometer and its software (Thermo Scientific), and considering that one absorbance unit at 260 nm is equivalent to 50 µg/ml double-stranded DNA. 93 ng of each genomic DNA sample were loaded with appropriate loading dye, along with 5 µl Hind*III* lambda digest molecular weight standards (Bio-Rad), in a 0.8% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis was run in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), at 80 V, for 1 hour.

Electrophoretic profiles were then analysed in a Gel Doc™ XR densitometer, using The Discovery Series™ Quantity One 1-D analysis software, version 4.6.1, both from Bio-Rad.

Autophagy

Monodansylcadaverine incorporation

7.5 x 10^4 cells were plated on each well of a 6-well plate and were allowed to adhere and stabilize overnight. In the following day, TXA1.HCl was added to a final concentration of 5, 10 and 20 µM. Additional controls, containing or lacking water (the compound solvent), were included. The compound was then allowed to act for 48 hours. After the compound exposition, cells were incubated with 20 µl of

monodansylcadaverine (MDC) 35.5 µg/µl (MDC in methanol) for 60 minutes at 37ºC. Cells were trypsinized and counted by the trypan blue exclusion assay and were then ressuspended in PBS, and submitted to a cytospin (Thermo Scientific Cytospin 4).

Cells were washed once with PBS, for 5 minutes. They were then fixed for 30 minutes with a 4% (v/v) paraformaldehyde solution, prepared in PBS. They were finally washed twice in PBS, for 5 minutes, and analysed under fluorescence microscopy, with the help of IPATIMUP staff (Diana Sousa) and equipment.

Transfection with LC3-mCherry expression vector

2.5 x 10^4 MCF-7 cells were plated on each well of a 24-well plate, onto glass coverslips. Cells were allowed to adhere and stabilize overnight.

On the following day, culture medium was removed and replaced by new medium (RPMI containing 5% (v/v) FBS). In the meanwhile, two separate tubes were prepared: tube A, containing 0.635 µl LC3-mCherry expression vector and 25 µl Opti-MEM I Reduced Serum Media, and tube B, containing 1.5 µl Lipofectamine and 25 µl Opti-MEM I Reduced Serum Media. Both Opti-MEM and Lipofectamine were purchased from Invitrogen. Total volumes were determined according to the number of wells to transfect. These solutions were gently homogenized and allowed to rest for 10 minutes at room temperature, upon which they were combined, homogenized and allowed to rest for 30 additional minutes at room temperature. Then, 52 µl of this mixture were added to each well of the plate and carefully homogenized. Cells were incubated for 4 hours at 37ºC, upon which the transfection medium was removed and replaced by new medium (RPMI containing 5% (v/v) FBS). Cells were then incubated overnight, at 37ºC.

On the following day, cells were observed under fluorescence microscopy. Once the transfection was proven to be successful, TXA1.HCl was added at a final concentration of 5, 10 and 20 µM. Appropriate controls, containing or lacking the solvent (water), were also prepared.

48 hours later, culture medium was discarded and cells were washed once with PBS, for 5 minutes. They were then fixed for 30 minutes with a 4% (v/v) paraformaldehyde solution, prepared in PBS. They were finally washed twice in PBS,

for 5 minutes, and analysed under fluorescence microscopy, with the help of IPATIMUP staff (Diana Sousa) and equipment.

Statistical analysis

Data was expressed as the mean ± SD and analysed by the paired Student's T test. P-values below 0.05 were considered statistically significant.

Chapter IV.
Results and Discussion

Part I - Screening of Antitumour Activity on Natural and Synthetic Compounds

Chemopreventive and chemotherapeutic agents compose a remarkable fraction of the drugs discovered in the last decades (Houghton et al., 2007). Although there have been many progresses in this research area, many efforts are still being directed towards the screening of new compounds (Houghton et al., 2007). Large throughput, and often automated, screening methods have been developed in order to test a considerable number of compounds in a large range of concentrations, within a short period of time (Houghton et al., 2007). Many are used by industry and research institutions such as the NCI (Houghton et al., 2007). Examples include the SRB and the tetrazolium salt-based methods, MTT and XTT (Houghton et al., 2007).

SRB is a widely used method to evaluate drug-induced cytotoxicity and/or cytostatic activity, including those induced by xanthones and their derivatives (Pedro et al., 2002, Batova et al., 2010, Ding et al., 2011) and by natural extracts (Houghton et al., 2007, Ding et al., 2011, Aranjani et al., 2013).

Four different cell lines were used in the present work: A375-C5 (melanoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non small cell lung cancer) and HeLa (cervical adenocarcinoma). The choice of these cell lines was based on the previous experience of the group, their well-known morphology and behavior, as well as on the fact that they are commonly used in drug screening assays.

Upon a 48-hour exposure period to the compounds, cells were fixed by adding TCA. In acidic conditions, SRB binds cell proteins through electrostatic interactions. Upon an increase in pH, which is accomplished through the addition of Tris buffer, SRB is solubilized. The amount of SRB-protein complexes is then proportional to the amount of cells in each experimental condition (Houghton et al., 2007).

When compared with other colorimetric methods used, such as MTT, SRB provides a better linearity with cell number, higher sensitivity, lower variation between cell lines and good signal-to-noise ratios (Houghton et al., 2007, Keepers et al., 1991, Pauwels et al., 2003). Besides, it presents some practical advantages over other methods, such as the high durability of staining (due to the more stable end-point)

and the possibility of interrupting the staining procedure at several steps (Houghton et al., 2007, Pauwels et al., 2003).

In the present work the SRB method was performed with 9 natural product-derived samples (2 methanol crude extracts from *Gelidium microdon*, and 2 methanol crude extracts plus 2 compounds from *Ulva rigida*; and 3 extracts from *Cecropia catarinensis*) and 12 synthetic compounds (11 pyranoxanthones and 1 thioxanthones).

Concentrations ranging from 2.34 μM to 37.50 μM (for the thioxanthone), or 4.68 μM to 150 μM (for the pyranoxanthones) were tested at least in three independent assays. Natural extracts were tested from 6.25 μg/ml to 200 μg/ml and isolated compounds were tested from 6.25 μM to 200 μM.

The action profile of a given compound can be characterized by its GI_{50} value, which is provided by the SRB method. GI_{50} is the compound concentration that leads to a 50% reduction in the amount of untreated cells (NCI, 2012). GI_{50} values presented in the present work were determined using a NCI datasheet. GI_{50} values are inversely proportional to the compound's efficacy, since a low GI_{50} means that a lower compound concentration is required to have an inhibitory effect on cell proliferation.

Considering natural extracts, it should be mentioned that they are more complex matrices due to their fairly unknown composition. As such, it is more difficult to ascertain which compound(s) and concentrations are responsible for their cytostatic/cytotoxic activity.

Results obtained for the different cell lines with the different compounds/extracts under study are shown in the correspondent further subsections.

Evaluation of the antitumour effect of extracts obtained from two Azorean macroalgae: *Ulva rigida* and *Gelidium microdon*

All the extracts obtained from *Ulva rigida* and *Gelidium microdon*, collected from S. Miguel Island, were gently provided by Prof. Anake Kijjoa. Crude methanolic extracts were obtained and purified in order to get new chlorophyll-free isolates. The chemical purification of the methanolic extract without chlorophyll from *U. rigida* led to the isolation of two compounds: isofucosterol and 7(E)-3β-hidroxy-5α,6α-epoxymegastigmane.

SRB assays were carried out with A375-C5, MCF-7, and NCI-H460 cell lines. Results are depicted in Table 1 for the obtained extracts, and Table 2 for the isolated compounds.

All the extracts moderately inhibited cell growth of the three cell lines tested. The extracts without chlorophyll from both *U. rigida* and *G. microdon* were slightly more active for MCF-7 and NCI-H460. This trend seemed to invert for A375-C5 so that extracts with chlorophyll from both algae were somewhat more active.

From both compounds isolated from *U. rigida*, only isofucosterol poorly inhibited cell growth of the three cell lines. 7(E)-3β-hidroxy-5α,6α-epoxymegastigmane was found to be inactive at the highest concentration tested (GI_{50} >200 μM).

Table 1: Growth inhibitory effect of the extracts obtained from *Ulva rigida* and *Gelidium microdon* on human cancer cell lines.

Extracts	GI_{50} (µg/ml)		
	A375-C5	MCF-7	NCI-H460
***U. rigida* (before removal of chlorophyll)**	40.8 ± 10.2	44.5 ± 18.4	49.1 ± 14.0
***U. rigida* (after removal of chlorophyll)**	44.5 ± 7.6	43.0 ± 10.3	41.9 ± 12.1
***G. microdon* (before removal of chlorophyll)**	36.3 ± 8.0	75.9 ± 16.1	70.6 ± 20.1
***G. microdon* (after removal of chlorophyll)**	62.6 ± 15.9	63.1 ± 14.1	64.9 ± 16.6

Results are expressed as GI_{50} (µg/ml) values of the extracts tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in duplicate. Doxorrubycin was used as a positive control (data not shown).

Table 2: Growth inhibitory effect of the compounds isolated from *Ulva rigida* on human cancer cell lines.

Compounds	GI_{50} (µM)		
	A375-C5	MCF-7	NCI-H460
Isofucosterol	119.2 ± 28.9	122.2 ± 17.9	128.4 ± 32.4
7(*E*)-3β-hydroxy-5α,6α-epoxymegastigmane	≥ 200	≥ 200	≥ 200

Results are expressed as GI_{50} (µM) values of the compounds tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in duplicate. Doxorrubycin was used as a positive control (data not shown).

Evaluation of the antitumour effect of the crude extract obtained from *Cecropia catarinensis*

Extracts from *Cecropia catarinensis*, collected in Brazil, were gently provided by Prof. Ana Paula de Almeida. A crude methanolic extract (CME) was obtained from the *Cecropia catarinensis* leaves and was purified in order to obtain two different fractions: Fraction 1 (CH_2Cl_2 and AcOEt) and Fraction 2 (MeOH).

SRB assays were carried out with A375-C5, MCF-7, and NCI-H460 cell lines. Results are depicted in Table 3.

All the extracts inhibited cell growth of the three cell lines tested, except Fraction 2 that was inactive for the melanoma cell line (A375-C5). Regarding the results obtained for both fractions, we can conclude that Fraction 1 present a better activity for all cell lines tested. More studies will be performed with this Fraction.

Table 3: Growth inhibitory effect of the extracts obtained from *Cecropia catarinensis* on human cancer cell lines.

Extracts	GI_{50} (µg/ml)		
	A375-C5	MCF-7	NCI-H460
CME	80.2 ± 4.9	58.8 ± 9.1	68.3 ± 7.2
Fraction 1	42.1 ± 8.8	31.8 ± 6.7	36.1 ± 9.8
Fraction 2	≥ 200	80.9 ± 19.2	85.4 ± 27.2

Results are expressed as GI_{50} (µg/ml) values of the extracts tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in duplicate. Doxorrubycin was used as a positive control (data not shown).

These findings contributed to two poster communications, and one publication that is being prepared:

- Durães, F.; Cardoso É.; Cravo, S.; **Barbosa, J. F.**; Pedro, M.; Pinto, M.; Almeida, A. P. "*Cecropia catarinensis*: A plant with antitumoral potencial." IJUP'13 – 6º Encontro de Jovens Investigadores da Universidade do Porto. Porto. February, 2013.

- Cardoso, É. C.; Serdeiro, M. T.; Durães, F.; **Barbosa, J. F.**; Pedro, M.; Pinto, M.; Almeida, A. P. "Antitumoral potencial of crude extract of *Cecropia catarinensis* for breast cancer and melanoma". 1st Luso-Brazilian Symposium on Science. Vassouras, Brazil. October, 2012.

- Almeida, A. P.; Cardoso, É.; Faria, A.; Serdeiro, M.; **Barbosa, J. F.**; Pinto, M.; Pedro, M. "The *in vitro* anticancer activity of the crude extract of the *Cecropia catarinensis*." (in preparation).

Evaluation of the antitumour effect of pyranoxanthones on cell growth

Eleven pyranoxanthones were synthesized and gently provided by Prof. Madalena Pinto and Prof. Carlos Afonso.

The evaluation of the growth inhibitory activity on the three tumour cell lines demonstrates that from all the set of the assayed pyranoxanthones only three did inhibit cancer cell growth (see Table 4): XC20, XC21, and XC27.

From all the set of the assayed pyranoxanthones only three did inhibit cancer cell growth: XC20, XC21, and XC27.

Table 4: Growth inhibitory effect of the pyranoxanthones on human cancer cell lines.

	GI_{50} (μM)		
Compound	**A375-C5**	**MCF-7**	**NCI-H460**
XC14	≥150	≥150	≥150
XC15	≥150	≥150	≥150
XC17	≥150	≥150	≥150
XC18	≥150	≥150	≥150
XC19	≥150	≥150	≥150
XC20	-	<4,688	-
XC21	-	<4,688	-
XC22	≥150	≥150	≥150
XC25	≥150	≥150	≥150
XC26	≥150	≥150	≥150
XC27	-	43,9 ± 2,08	-

Results are expressed as GI_{50} (μM) values of the pyranoxanthones tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in duplicate. Doxorrubycin was used as a positive control (data not shown).

Since compounds XC20 and XC21 revealed to be potent inhibitors of cell growth (GI_{50} < 4,688, for both compounds) they were further studied by Prof. Helena Vasconcelos research group. Results obtained from these studies, together with the inhibitory effect of other xanthone derivatives, their synthesis and biological activities generated two publications:

- Azevedo, C. M. G.; Afonso, C. M. M.; Sousa, D.; Lima, R. T.; Vasconcelos, M. H.; Pedro, M.; **Barbosa, J.**; Corrêa, A. G.; Reis, S.; Pinto, M. M. "Multidimensional Optimization of promising antitumor xanthone derivatives." Accepted for publication by BMC in March, 2013. Online publication complete: 25-APR-2013, DOI: http://dx.doi.org/10.1016/j.bmc.2013.03.079

- Azevedo, C. M. G.; Afonso, C. M. M; Soares, J. X.; Reis, S.; Sousa, D.; Lima, R. T.; Vasconcelos, M. H.; Pedro, M.; **Barbosa, J.**; Gales, L.; Pinto, M. M. "Pyranoxanthones: synthesis, growth inhibitory activity on human tumor cell lines and determination of their lipophilicity in two membrane models." Submitted to Eur J Med Chem in April, 2013.

Evaluation of the antitumour effect of Thioxanthones on cell growth

TXA1 is an aminated thioxanthone that was synthesized and previously identified as a potent tumour cell growth inhibitor by CEQUIMED-UP. In order to improve its solubility and bioavailability characteristics, the hydrochloride salt (TXA1.HCl) was also prepared. TXA1 and TXA1.HCl were gently provided by Prof. Madalena Pinto and Prof. Emília Sousa.

SRB assays were carried out with TXA1 and its salt on A375-C5, MCF-7, and NCI-H460 cell lines. Results are depicted in Figure 10 and Table 5.

Figure 10A and Figure 10B show the dose-response curves obtained from the SRB assay with TXA1 and TXA1.HCl, respectively. Typically, compounds with good antitumoural activities follow a sigmoidal pattern: an initial concentration range that gives rise to no significant response, followed by a range in which cell death is evident; this, in turn, is followed by a final concentration range in which no further changes are observed in the number of viable cells.

As expected, different cell lines show distinct GI_{50} values (see Table 5), which can be explained by their different patterns of sensitivity to the compounds under study.

However, as seen on Table 5, TXA1 presents lower GI_{50} values than its hydrochloride salt, TXA1.HCl, unlike what was expected, considering its higher solubility.

Among all the GI_{50} values from the extracts and compounds that were tested, besides xanthones XC20 and XC21 (which were further tested by other members of CEQUIMED-UP) the thioxanthone is the lowest, thus showing that it is the most promising antitumoural compound under study. Their dose-response curve crosses the *x*-axis, reflecting that they effectively reduce the amount of viable cells, even if in low concentrations.

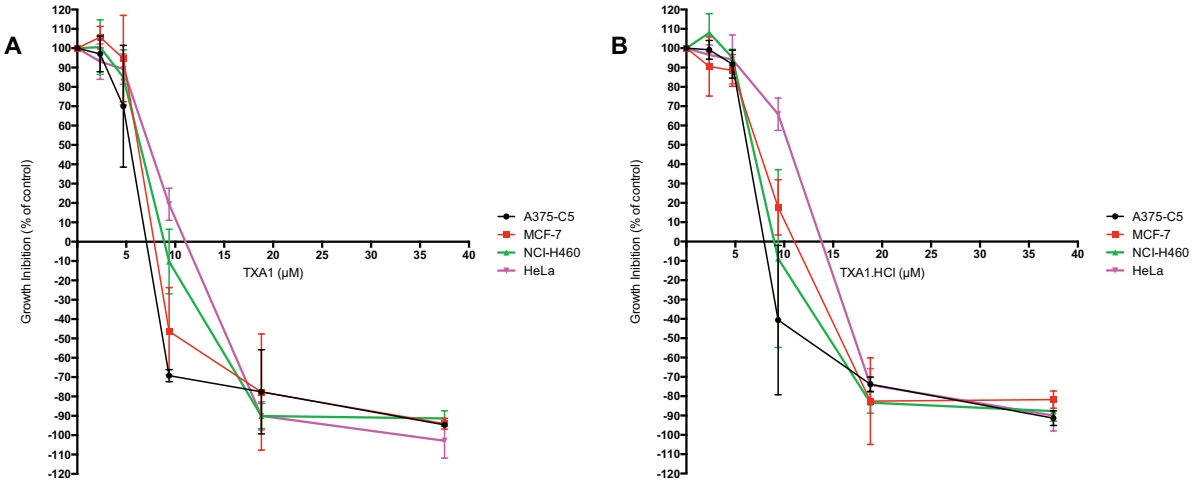


Figure 10: TXA1 and TXA1.HCl dose-response curves for human cancer cell lines, obtained through SRB assays.

TXA1 (A) and TXA1.HCl (B) dose-response curves for A375-C5, MCF-7 and NCI-H460 cell lines, obtained through SRB assays. Growth inhibition was expressed in percentual terms, as a fraction of the initial amount of cells (set as 100%). Results are expressed by means ± SD, from at least 3 independent assays, run in duplicate. Doxorrubycin was used as a positive control (data not shown).

Table 5: Growth inhibitory effect of TXA1 and TXA1.HCl on human cancer cell lines.

	GI_{50} (µM)			
Compound	**A375-C5**	**MCF-7**	**NCI-H460**	**HeLa**
TXA1	4.92 ± 0.77	5.91 ± 0.37	6.04 ± 0.48	6,77 ± 0.49
TXA1.HCl	5.92 ± 0.39	6.90 ± 0.59	7.26 ± 0.61	10.12 ± 0.34

Results are expressed as GI_{50} (µM) values of TXA1 and TXA1.HCl tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in duplicate. Doxorrubycin was used as a positive control (data not shown).

Part II - Insights Into a Thioxanthone Mechanism of Action

Effect of TXA1 and TXA1.HCl on metabolic activity

The MTT assay is a colorimetric assay, which was first described by Cole in 1986, that reflects cell metabolic activity (Cole, 1986, Houghton et al., 2007). MTT is a tetrazolium salt that is reduced by mitochondrial enzymes, in a NAD(P)H-dependent way, to formazan, a purple, insoluble crystal. Upon solubilization, the amount of formazan is determined through spectrophotometry. MTT reduction increases with metabolic activity; the amount of formazan is thus an indicator of cell viability (Houghton et al., 2007). In this sense, MTT assays provide additional information, besides that provided by the SRB method. If a compound is proven to have cytostatic effect, MTT assays may inform if the cells are still viable, that is, if they are still able to proliferate when in non-adverse conditions. However, it should be mentioned that these assays present some disadvantages when compared with SRB. For instance, they show poor linearity at high cell densities and their end-point requires time-sensitive measurements. Besides, since they are dependent on mitochondrial activity, their results are more prone to variability between different cell lines, thus requiring cell line-specific optimization (Houghton et al., 2007, Keepers et al., 1991, Pauwels et al., 2003).

In order to better characterize TXA1 and TXA1.HCl mechanism of action, MTT assays were performed to complement the results obtained through the SRB assay.

All the MTT assays were performed on A375-C5, MCF-7 and NCI-H460 cell lines. The compounds were also allowed to act for 48 hours and concentrations were in the same ranged from 2.3 μM to 21.1 μM. IC_{50} values were determined using GraphPad Prism®. Results are depicted in Figure 11 and Table 6.

Figure 11A and Figure 11B show the dose-response curves obtained from the MTT assay with TXA1 and TXA1.HCl, respectively. Both curves describe a dose-dependent decrease in metabolic activity, and thus in cell viability.

As seen on Table 6, IC_{50} values for TXA1 and TXA1.HCl describe different activity profiles. TXA1.HCl was more active in decreasing metabolic activity of A375-C5 and HeLa than TXA1. On the other hand, TXA1 was more active on MCF-7 and NCI-H460. These profiles are different from those obtained with the SRB assay (see

Table 5), which demonstrated that TXA1 was more active than TXA1.HCl on all cell lines assayed.

However, results provided from the MTT assay (Table 6) are in the same order of magnitude than those obtained through the SRB method (Table 5). The results are different, given the fact that both methods provide distinct, but complementary, information about cell growth. Similarly to what happened in the SRB assays, different cell lines led to different IC_{50} values, which was expectable due to their distinct origins and sensitivities to the compounds.

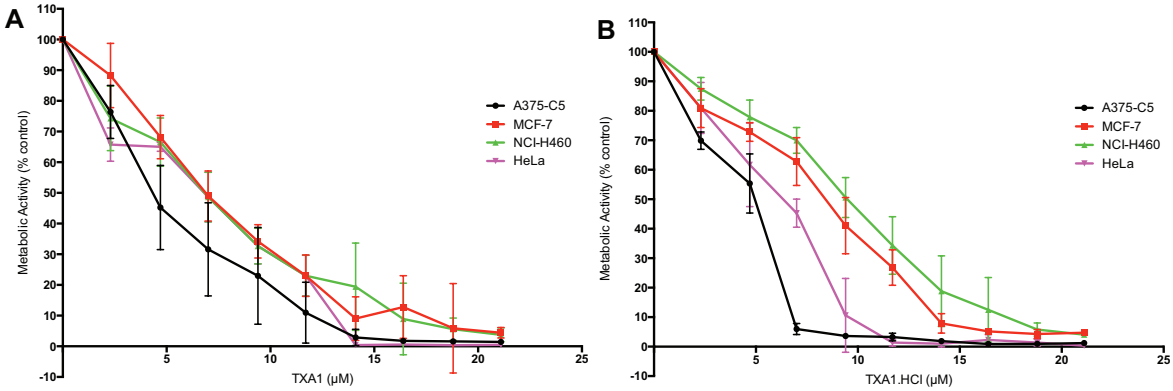


Figure 11: TXA1 and TXA1.HCl dose-response curves for human cancer cell lines, obtained through MTT assays.

TXA1 (A) and TXA1.HCl (B) dose-response curves and respective IC_{50} values for A375-C5, MCF-7 and NCI-H460 cell lines, obtained through MTT assays. Metabolic activity was expressed in percentual terms, as a fraction of the initial amount of cells (set as 100%). Results are expressed by means ± SD, from at least 3 independent assays, run in triplicate. Preliminary assays included a positive control with doxorubicin (data not shown).

Table 6: TXA1 and TXA1.HCl effect on metabolic activity of human cancer cell lines.

	IC_{50} (μM)			
Compound	**A375-C5**	**MCF-7**	**NCI-H460**	**HeLa**
TXA1	5.92 ± 0.89	8.24 ± 0.27	7.88 ± 0.47	7.10 ± 0.10
TXA1.HCl	4.53 ± 0.42	8.64 ± 0.72	9.79 ± 0.87	6.42 ± 0.57

Results are expressed as IC_{50} (μM) values of TXA1 and TXA1.HCl tested using A375-C5, MCF-7, and NCI-H460 cell lines. Results are expressed by means ± SD of at least three independent experiments performed in triplicate. Doxorrubycin was used as a positive control (data not shown).

Effect of TXA1.HCl on cell viability

TXA1.HCl reflected better solubility characteristics, which encouraged further elucidation of its mechanism of action.

As a complement to SRB and MTT methods, trypan blue assay was performed as a way to evaluate cell viability upon exposure to different TXA1.HCl concentrations, at different time points.

Trypan blue stains dead cells because their membranes have been damaged; in contrast, healthy cells actively exclude the dye (for which the assay is also known as a dye exclusion method). It cannot distinguish apoptotic from necrotic cells; all dead cells are colored blue, irrespectively of the mechanism responsible for their death.

Trypan blue assays were performed for TXA1.HCl at a final concentration of 5, 10 and 20 μM and upon 24, 48 and 72 hours of exposure. MCF-7 and HeLa cell lines were used. Concentration points were selected so as to include TXA1.HCl GI_{50}. In turn, time points were chosen in order to include the ones tested in the previously described methods. Negative controls, containing or lacking water (the solvent for TXA1.HCl), were also included, and, as expected, led to no changes in cell viability.

Cell viability was expressed in terms of the ratio between the number of viable cells and the total amount of cells. Figure 12 depicts the data resulting from, at least, three independent assays.

As it can be deduced from Figure 12, cell death increases along with the exposure to the compound and with its concentration. As seen in Figure 12A and Figure 12B, there is a time and dose-dependent effect of TXA1.HCl both in MCF-7 and HeLa cell lines. For a 24h exposure, 20 μM TXA1.HCl significantly decreased cell viability in both MCF-7 (40.1 ± 8.0%, $p \leq 0.001$) and HeLa (73.0 ± 5.4%, $p \leq 0.01$). For a 48h exposure, 10 μM significantly decreased cell viability to approximately 80% (77.3 ± 9.1%, $p \leq 0.05$ for MCF-7; 84.0 ± 5.2%, $p \leq 0.01$ for HeLa) and 20 μM significantly decreased cell viability to approximately 5% (4.7 ± 3.0%, $p \leq 0.0001$ for MCF-7; 2.2 ± 2.9%, $p \leq 0.0001$ for HeLa). This trend continued in a 72h exposure period, in which cell viability continued to decrease to approximately 70% (71.7 ± 8.0%, $p \leq 0.01$ for MCF-7; 72.5 ± 6.3%, $p \leq 0.001$ for

HeLa) with 10 µM TXA1.HCl. Cell viability drastically decreased to 0.0% ($p \leq 0.0001$) for both cell lines with a 72h exposure to 20 µM TXA1.HCl.

These results are in accordance with the dose-response curves obtained from the SRB and MTT assays that show the TXA1.HCl dose-dependent activity profile.

These assays have also shown that TXA1.HCl GI_{50} is around the 10 µM range, since pronounced cell death (and a significant decrease in cell viability) was observed for this upper limit after a 48h exposure. These observations are in harmony with the results from SRB and MTT methods, which led to the determination of a GI_{50} value of 6.90 ± 0.59 µM and an IC_{50} value of 8.64 ± 0.72 µM for MCF-7, while for HeLa cells the GI_{50} and IC_{50} values were 10.12 ± 0.34 µM and 6.42 ± 0.57 µM, respectively.

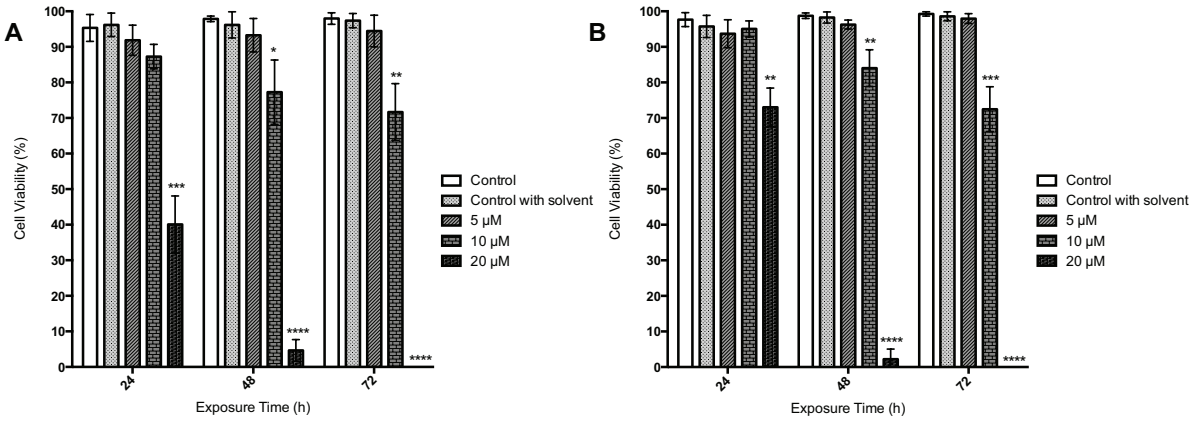


Figure 12: MCF-7 (A) and Hela (B) cell viability results obtained through the trypan blue assay. Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means ± SD from at least three independent assays. Statistical significance was tested by paired t-test, comparing results to control with solvent. *Indicates $p \leq 0.05$; **Indicates $p \leq 0.01$; *** indicates $p \leq 0.001$; ****Indicates $p \leq 0.0001$.

Effect of TXA1.HCl on cell morphology

Many antitumoural compounds act by damaging cell structures, such as the cytoskeleton, in a way that compromises cell viability. Others act by inducing an arrest at a specific cell cycle stage; for instance, they may induce a mitotic arrest, preventing cells from dividing and proliferating. Cells may then remain arrested for an indefinite period of time, in the end of which they may die via apoptosis or re-enter the cell cycle. Many compounds may also induce cell death through autophagy.

In order to have a deeper insight into TXA1.HCl mechanism of action, its potential effects on cell morphology were studied. For this purpose, MCF-7 and HeLa cells were grown and exposed to the compound, at a final concentration of 5, 10 and 20 μM and for 14, 24, 48 and 72 hours. As in trypan blue assays, negative controls, in the presence or in the absence of water, were contemplated, leading to no detectable changes in cell morphology. Representative phase contrast microscopy images are depicted in Figure 13.

Figure 13A and Figure 13B show that TXA1.HCl effects on MCF-7 and HeLa are both time- and concentration-dependent. Evidences of cell death, such as cell shrinkage, detachment, vacuolization, round shape acquisition and membrane blebbing (Kroemer et al., 2009), become apparent for concentrations equal to or higher than 10 μM, for both MCF-7 and HeLa cell lines, in accordance to the results from trypan blue viability assays.

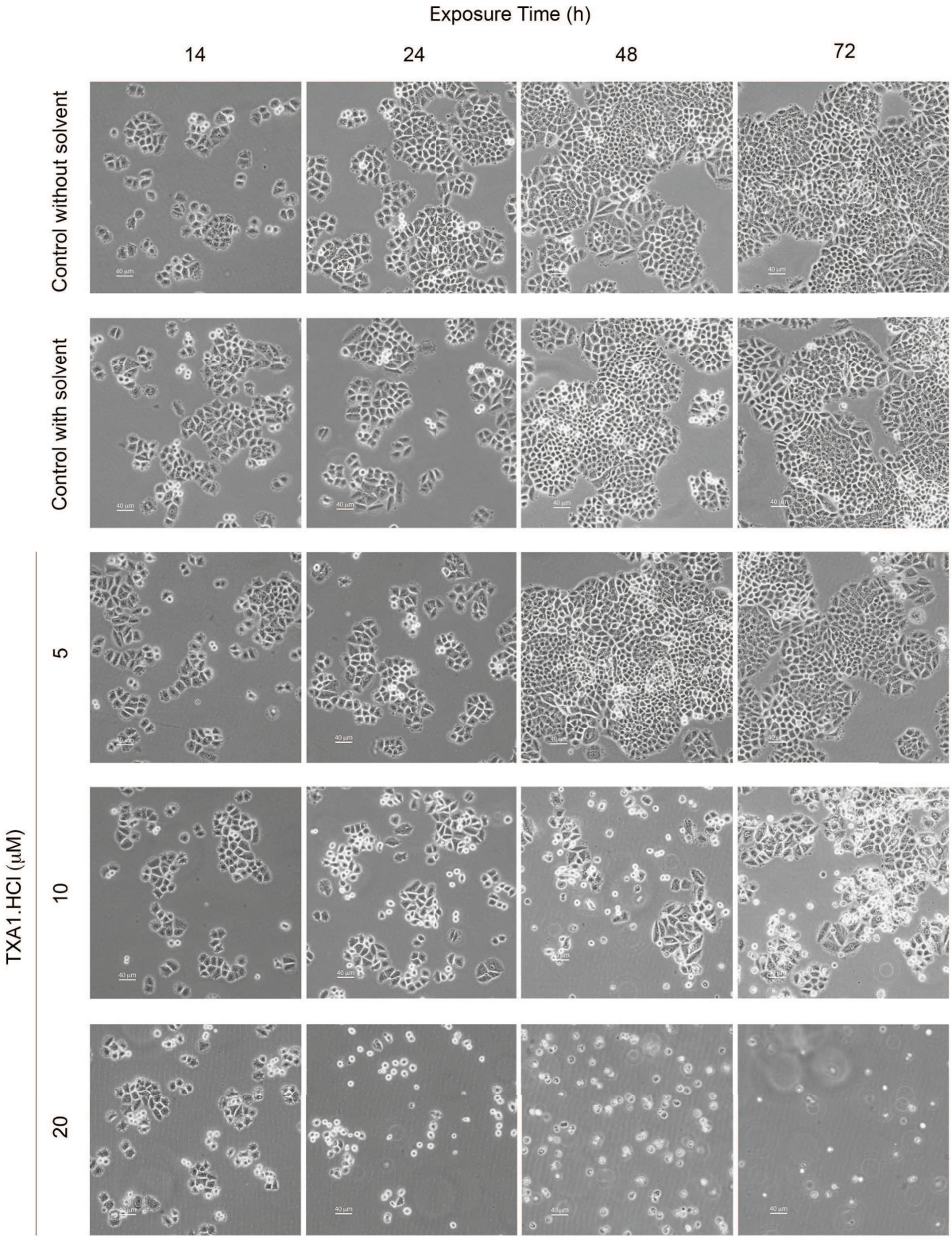
Regular monitoring of the cells revealed that these effects start to occur upon 10 to 12 hours of exposure to the compound, showing that this exposure time is enough to enable cell death.

Similar morphological alterations (cell shrinkage, detachment, round-shape acquisition and blebbing) have been described for xanthones obtained from *Garcinia hanburyi* that induced cancer cell death through apoptosis (Hahnvajanawong et al., 2010). The same alterations were observed on apoptotic breast cancer cells treated with α-Mangostin extracted from Mangosteen pericarp (Kurose et al., 2012). Moreover, Chao et al. have observed through transmission electron microscopy organelle containing vacuoles, which were later proven to be autophagic, in cells treated with α-Mangostin (Chao et al., 2011).

In order to assess if TXA1.HCl causes cell cycle arrest at a specific stage, slides were then mounted in DAPI. By staining DNA, DAPI allows an easier identification of the cell cycle stage. Cells were observed under fluorescence microscopy, however, no significant signals of cell cycle arrest at any stage were observed (data not shown), since there was not a prevalence of mitotic or interphase cells. Among mitotic cells, there was no trend towards an arrest at a specific mitosis stage. TXA1.HCl must then trigger cell death through other mechanism.

On the other hand, the following pictures evidenced the presence of apoptotic bodies-like structures, which suggested the occurrence of programmed cell death through apoptosis.

A


Exposure Time (h)
14
24
48
72
Control without solvent
Control with solvent
TXA1.HCl (μM)
5
10
20
40 μm

B

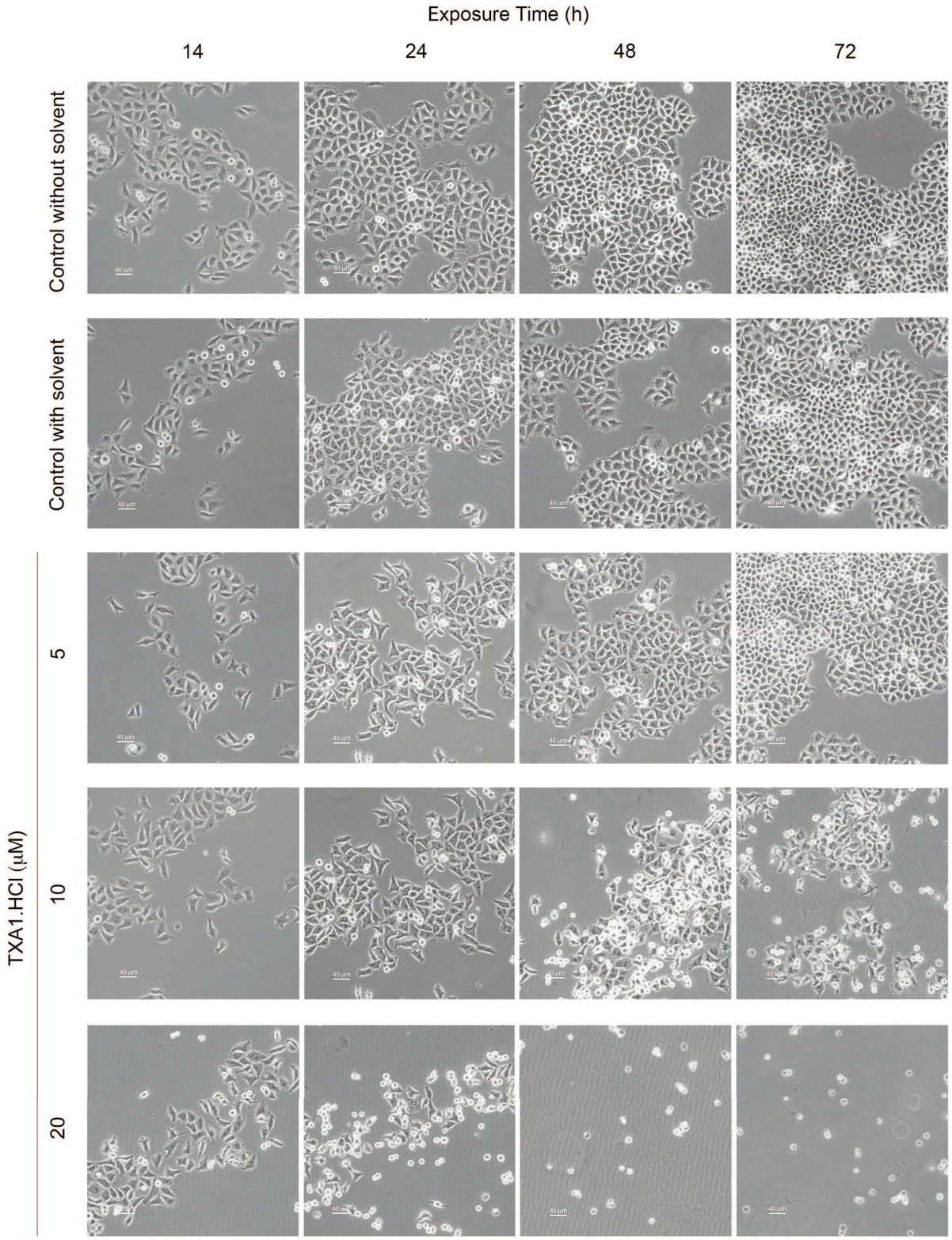


Figure 13: TXA1.HCl effect on MCF-7 (panel A) and HeLa (panel B) cell lines upon different exposure periods and different concentrations.

Results obtained through phase contrast microscopy. Photos of representative focus plan were taken. with a 100X magnification. Scale bar, 40 μm.

Reversibility studies

The evaluation of the reversibility of a compound's effect on cancer cells can be made by exposing two identical sets of cells to the same compound concentrations and for the same exposure period, in the end of which, one set of cells is assayed, while in the other the compound is removed and replaced by fresh medium. Following variable recovery periods, these cells are assayed for cell proliferation (Houghton et al., 2007).

In the morphology assays it was seen that a 10 hours exposure period to TXA1.HCl was sufficient to induce morphology changes on both MCF-7 and HeLa cells. Taking these results into consideration, and the possibility of non-visible metabolic effects with a shorter exposure time, MCF-7 cells were exposed to different TXA1.HCl concentrations for periods of 6 hours (in order to assay a shorter exposure time than 10 hours), 12, 24, and 48 hours. MTT assays were then performed immediately after these periods of exposure, as well as after 24 and 48 hours of recovery from the time that the compound was washed and replaced by fresh growth medium. The results are depicted in Figure 14.

From the analysis of Figure 14, it becomes apparent that TXA1.HCl effect is both time- and concentration-dependent. Although no changes in metabolic activity were detected immediately after 6 hours of exposure to the compound, a significant decrease in cell metabolic activity occurs upon 48 hours of recovery ($p \leq 0.05$). In other words, a 6 hour-exposure period is insufficient to decrease cell metabolic activity, but is somehow enough to interfere with mechanisms required for cell ability to survive and proliferate. In addition, a 12 hours exposure induced a more pronounced decrease in metabolic activity (comparing with a 6 hours exposure), and it was noticed a significant decrease upon 24 and 48 hours of recovery ($p \leq 0.001$). The same trend is observed when cells are initially exposed for 24 and 48 hours to the compound, although the reduction in cell metabolic activity is increasingly higher in these situations. These results show that cells are irreversibly affected by the TXA1.HCl effect.

In addition, these results are compatible with those from viability assays and morphology assays, which show that TXA1.HCl effects become detectable upon 10 to 12 hours of exposure.

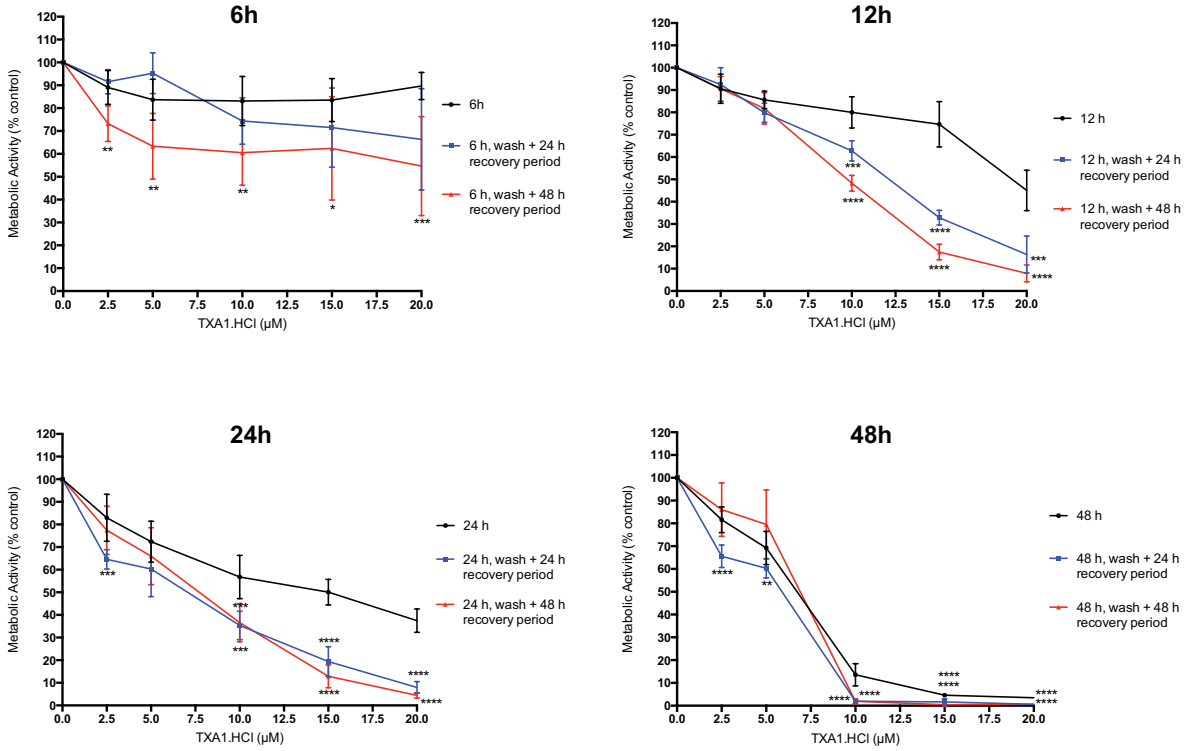


Figure 14: Metabolic activity data for MCF-7 cells, obtained through MTT assays.

Metabolic activity was expressed in percentual terms, as a fraction of the initial amount of cells (set as 100%). Cells were exposed to different TXA1.HCl concentrations for 6, 12, 24 and 48 hours, in the end of which they were assayed for cytotoxicity, followed by additional 24 and 48 hours of recovery. Results are expressed by means ± SD, from three assays run in triplicate. Statistical significance was tested by paired t-test, comparing results to exposure time with no recovery. *Indicates $p \leq 0.05$; **Indicates $p \leq 0.01$; ; *** indicates $p \leq 0.001$.****Indicates $p \leq 0.0001$.

The reversibility of the compound's effect on a cell line can also be assessed by the evaluation of their ability to form colonies after different exposure periods to different compound concentrations, as well as after a definite time of recovery.

The colony forming (or clonogenic) assay was originally described by Puck and Marcus, in 1956, in a study that generated the first radiation-dose response curve for X-ray irradiated mammalian (HeLa) cells in culture (Puck and Marcus, 1956). This assay assesses the differences in reproductive viability between control, untreated cells and cells that have been treated with ionizing radiation or chemical compounds (e.g. cytotoxic agents), for instance (Rafehi et al., 2011). Furthermore, it is commonly used in the evaluation of the efficacy of radiation modifying compounds, cytotoxic and other anti-cancer agents on colony forming ability, in different cell lines (Rafehi et al., 2011).

For this work, MCF-7 cells were exposed to 5, 10 and 20 μM TXA1.HCl for 6 and 24 hours. After this exposition to the compound, the compound-containing medium was removed, and the cells were allowed to recover with compound-free medium for 14 days. Results are depicted in Figure 15 and Figure 16.

From the analysis of the results, it is evident that TXA1.HCl effect is both time- and concentration-dependent. The colony-forming ability decreased along with compound concentration and exposure period.

Treatment with 20 μM TXA1.HCl for 6 hours resulted in a significant loss of almost 70% in colony formation efficiency when compared with the control with solvent ($p \leq 0.0001$). In addition, a 24 hours exposition significantly decreased the colony forming ability in approximately 30% ($p \leq 0.05$), 95% ($p \leq 0.001$), and 100% ($p \leq 0.0001$), for 5, 10 and 20 μM concentrations, respectively.

Similarly to these results, α-mangostin isolated from the mangosteen fruit was shown to inhibit colony formation of prostate cancer cells (Johnson et al., 2012). In another study, Chen et al. concluded that gambogenic acid significantly inhibited the colony formation ability of glioblastoma cells (Chen et al., 2012).

All taken together, results obtained through the MTT assays and those from the colony forming assays demonstrate that, similarly to other xanthones, TXA1.HCl irreversibly acts as a cytotoxic, rather than as a cytostatic compound, being its effect irreversible after a 6 hours exposure.

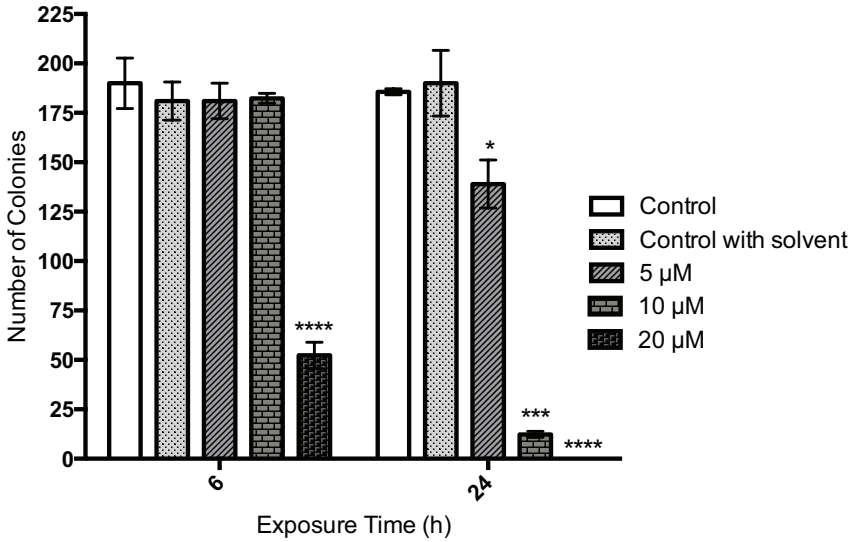


Figure 15: Compound effect on colony forming efficiency of MCF-7 cells.

Cells were exposed to different compound concentrations for 6 and 24 hours, washed and recovered in a drug-free medium for 14 days and assessed for their efficiency to form colonies. Results are expressed by means ± SD, from three assays carried out independently. Statistical significance was tested by paired t-test, comparing results to control with solvent. ; *Indicates $p \leq 0.05$; ***Indicates $p \leq 0.001$; ****Indicates $p \leq 0.0001$.

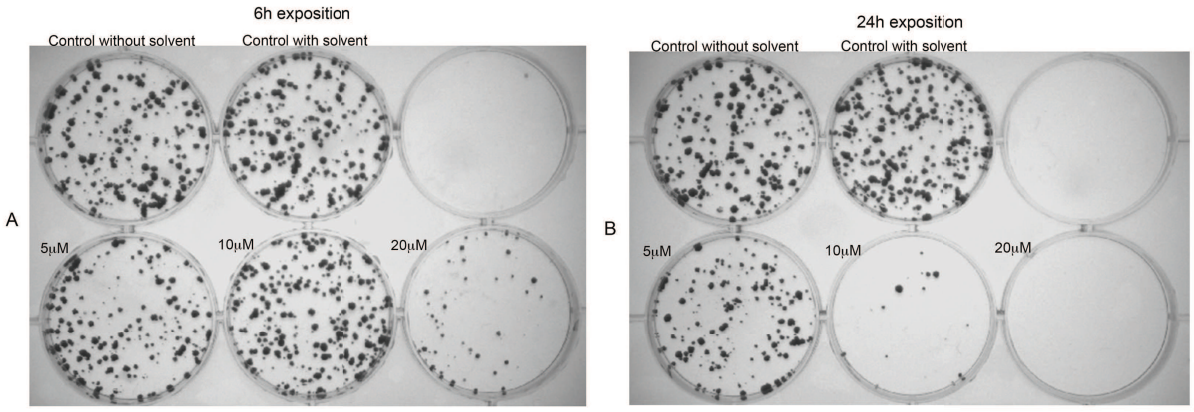


Figure 16: Compound effect on colony forming efficiency of MCF-7 cells – representative photos.

Representative photos taken to the plates obtained from the colony forming assay with MCF-7 for a 6 (A) and 24 hours (B) exposure period to TXA1.HCl, after 14 days of recovery in a drug-free medium.

Effect of TXA1.HCl on apoptosis

The mechanism through which xanthones and their derivatives trigger cell death seems to be very complex. Several mechanisms have been suggested (Han and Xu, 2009) and apoptosis seems to be one of the most studied. For instance, γ-mangostin was shown to induce apoptosis in human colon cells (Chang and Yang, 2012), while α-mangostin induced cell cycle arrest and apoptosis in breast cancer cells (Kurose et al., 2012). Johnson and co-workers reported the same findings in prostate cancer cells (Johnson et al., 2012). In turn, gambogenic acid induced cell cycle arrest and apoptosis in glioblastoma cells (Chen et al., 2012), while pyranocycloartobiloxanthone A, a xanthone isolated from *Artocarpus obtusus*, induced apotosis in MCF-7 cells (Mohan et al., 2012). Also, a xanthone isolated from *Garcinia oblongifolia* induced apoptosis in hepatocellular carcinoma cells (Fu et al., 2012).

As already been said in the effect on morphology section, there was no visible trend towards an arrest at a specific mitosis stage after TXA1.HCl treatment. However, it was evidenced the presence of apoptotic bodies-like structures, which suggested the occurrence of programmed cell death through apoptosis.

Aiming at studying the mechanisms through which cells die in response to TXA1.HCl, the apoptosis hypothesis was tested through TUNEL assays that were performed with MCF-7 cells.

TUNEL is a method for detecting DNA fragmentation, a typical feature of apoptosis, by labeling the terminal ends of this nucleic acid. DNA is stained with an appropriate dye, such as DAPI. In turn, DNA nicks are detected by the rTdT enzyme, which catalyzes the addition of labeled dUTPs. The kit employed in the present work uses fluorescein-labeled dUTPs, which allows DNA nick-ends to be observed as green spots under fluorescence microscopy.

Similarly to other techniques employed in this work, cells were exposed to different compound concentrations and for different periods of time. Figure 17 depicts representative images from MCF-7 cells submitted to TUNEL assays. DNA was stained blue. Two different positive controls were used (data not shown). In one of them, cells were treated with DNase I, which catalyzes DNA degradation, leading to its fragmentation. In the other positive control, cells were treated with H_2O_2, a strong oxidative stress inducer, which endogenously triggers apoptotic cell death (Singh et

al., 2007, Xu et al., 2012). As expected, both positive controls led to DNA nick end labeling. On the other hand, in the negative controls, in which water or growth medium was added instead of the compound, no significant DNA fragmentation was observed.

Accordingly to what was observed in the results obtained from the trypan blue and the morphology assays, DNA fragmentation (and, thus, cell death) occurs in a time- and dose-dependent manner, as the amount of green spots increases with both.

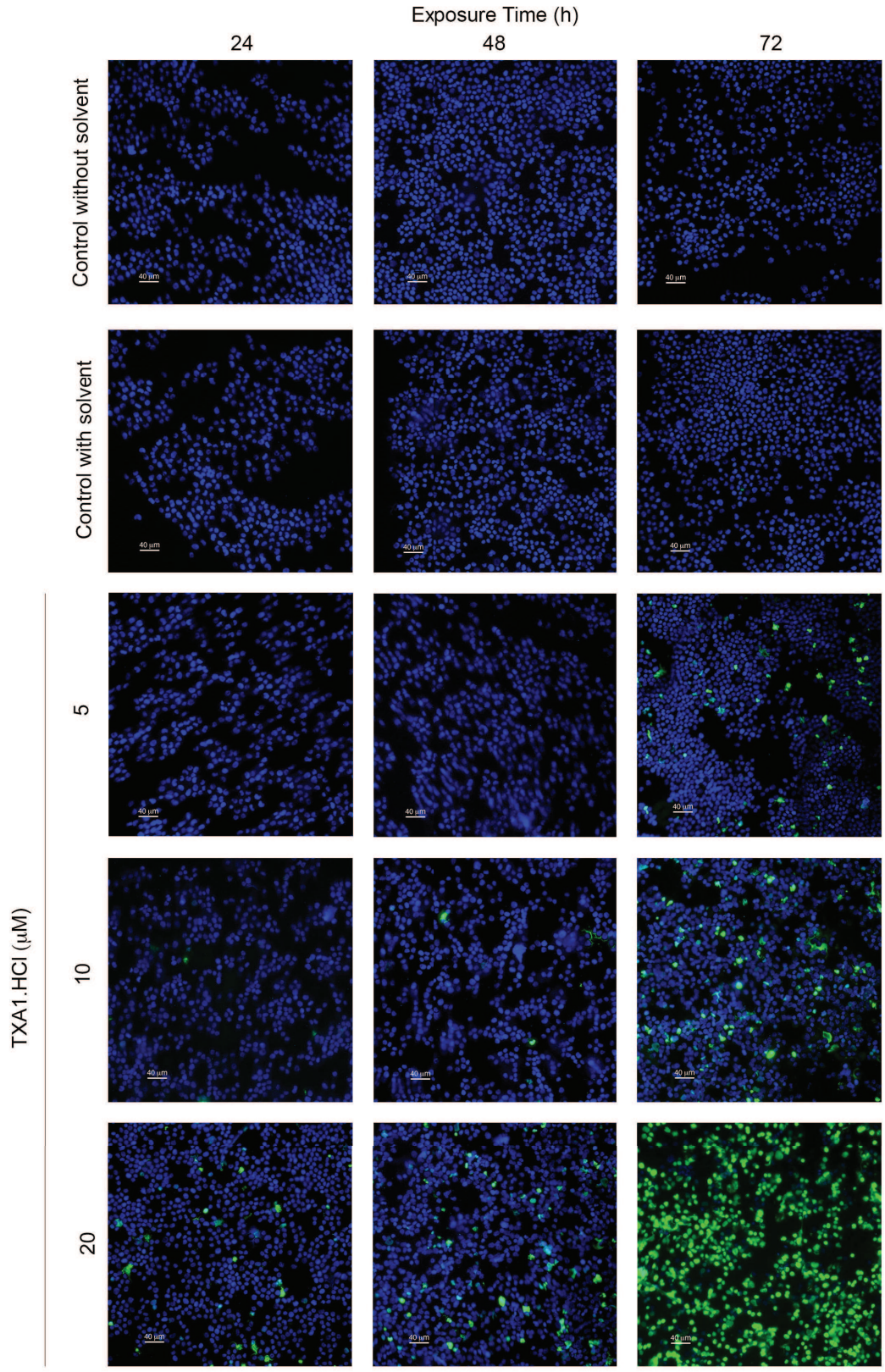


Figure 17: TXA1.HCl effect on apoptosis of MCF-7 cell line upon different exposure periods and to different concentrations.

Results were obtained through fluorescence microscopy. Photos of representative focus plan were taken with a 100X magnification. DNA nick-ends appear as green spots. Nucleus is stained with DAPI (blue). Scale bar, 40 µm.

Besides microscopy observation, TUNEL results were quantitatively analyzed. The number of cells presenting DNA nick-ends was determined using representative focus plans and cell populations comprising, at least, 1000 cells. The number of apoptotic cells was expressed in terms of the ratio between nick end-presenting cells and the total amount of cells considered. Resulting data are presented in Figure 18.

A 24 hours exposure to 20 μM TXA1.HCl significantly increased the percentage of TUNEL-positive cells in comparison to control with solvent (4.5 ± 1.4%; $p \leq 0.05$). The same trend could be seen for a 48 hours exposure with a more pronounced percentage of apoptotic cells for 20 μM TXA1.HCl (26.2 ± 3.2 %; $p \leq 0.001$). A 72 hours exposure notably increased the percentage of apoptotic cells submitted to 5 μM (8.8 ± 3.3%; $p \leq 0.05$) and 10 μM (22.1 ± 5.9%; $p \leq 0.01$). 20 μM TXA1.HCl drastically increased the percentage of death and destroyed cells.

Consistently with fluorescence microscopy observations, data presented in Figure 18 strongly suggest that apoptosis is the mechanism through which cells die when exposed to TXA1.HCl. The number of apoptotic cells is proportional to the compound's dose and to the extent to which cells have been exposed to it.

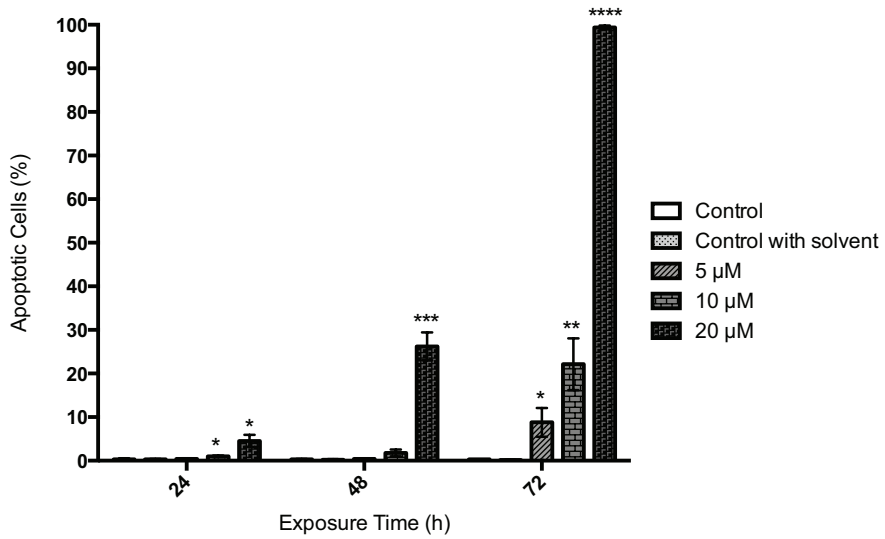


Figure 18: Apoptosis quantitative data for MCF-7 upon exposure to different TXA1.HCl concentrations and for different periods.

Apoptosis was expressed in percentual terms, as the ratio between the number of apoptotic, green spot-presenting cells and the total amount of cells. Results are means ± SD from, at least, 3 independent assays. Statistical significance was tested by paired t-test comparing results to control with solvent. *Indicates $p \leq 0.05$; **Indicates $p \leq 0.01$; ***Indicates $p \leq 0.001$; ****Indicates $p \leq 0.0001$.

However, these results are not totally consistent with data resulting from the SRB, MTT and trypan blue exclusion assays. The GI_{50} and IC_{50} values for MCF-7 were below 10 μM TXA1.HCl upon a 48h exposure. Nevertheless, the percentage of viable cells after a 48 hours exposure to 10 μM TXA1.HCl was approximately 80% (77.3 ± 9.1%, $p \leq 0.05$) for the same cell line. Results from TUNEL assays point out that the percentage of apoptotic MCF-7 cells resulting from a 48 hours exposure period to 10 μM TXA1.HCl was not significant, suggesting that another mechanism(s) should be involved in cell death besides apoptosis.

Furthermore, in order to confirm the results obtained from the TUNEL assay, TXA1.HCl effect on genomic DNA fragmentation, a hallmark of apoptosis, was also evaluated through electrophoresis. However, results were not conclusive (data not shown).

Effect of TXA1.HCl on autophagy

Previous data obtained by our research group (CEQUIMED-UP), has shown that TXA1.HCl induces programmed cell death through autophagy on A375-C5 (melanoma) cell line (unpublished data).

Yu and co-workers showed that gambogenic acid induces cell cycle arrest and triggers autophagy in lung cancer cells (Yu et al., 2012). α-Mangostin induced autophagic cell death in glioblastoma cell line but not apoptosis (Chao et al., 2011). In another work, α-mangostin induced autophagy activation *in vivo* and inhibited the growth of colon cancer in mouse transplant model (Kim et al., 2012b). Lucanthone, a thioxanthone, can also induce autophagy mechanisms (Yang et al., 2011).

As it was already been said, the morphology assay revealed some vacuolization for concentrations equal to or higher than 10 μM TXA1.HCl, for both MCF-7 and HeLa cell lines. In order to characterize the cytoplasmic vacuoles and to assess its relationship to TXA1.HCl effect, two different assays were carried out in MCF-7 cell line.

Cells were exposed to different compound concentrations for a 48 hours period, in the end of which they were stained with monodansylcadaverine (MDC) and immediately analysed by fluorescence microscopy.

MDC is an autofluorescent *in vivo* marker that has been described as selective for autophagic vacuoles (Niemann et al., 2000, Biederbick et al., 1995). It has been used in the specific labelling of autophagosomes, autophagy hallmarks (Contento et al., 2005, Vazquez and Colombo, 2009). The specificity of its staining results from ion trapping, due to the acidic nature of autophagosomes, and from its interaction with lipids, whose content is very high in these structures (Contento et al., 2005). Cell fractioning and electron microscopy assays have shown that double membranes delimit MDC-stained autophagosomes (Contento et al., 2005). MDC labelling is prevented in the presence of autophagic inhibitors, which further substantiates its applicability as an autophagy marker (Contento et al., 2005). Also, a GFP-LC3 fusion protein was recruited to MDC-stained vesicles as a response to amino acid deprivation, confirming the autophagic nature of these structures (Contento et al., 2005).

This assay was used to assess rapamycin effect on autophagy induction in a melanoma cell line (M14) (Li et al., 2013a). It was also used to detect autophagic vacuoles induced by oridonin in human prostate cancer cells (Li et al., 2012). Vanderlaag and colleagues also used it to detect autophagic cell death induced by a methylene-substituted diindolylmethane in estrogen negative breast cancer cells (Vanderlaag et al., 2010). NF-κB nuclear role in autophagy activation and cell apoptosis in human gastric carcinoma cells was investigated using this assay (Zhu et al., 2011).

The same exposure scheme used in the MDC incorporation assay was adopted for another assay, in which cells were previously transfected with LC3 mCherry expression vector.

LC3 is the frequently name used for microtubule-associated protein 1 light chain 3, a ubiquitin-like molecule that is the mammalian homologue of the autophagy-related atg8 encoded product in yeast (Barth et al., 2010). It is a cytoplasmic protein that is processed into another form, LC3II, which associates with the autophagosome lipid membranes, being present both inside and outside autophagosomes (Munafo and Colombo, 2001). Furthermore, LC3-II is the only known protein that specifically associates with autophagosomes throughout the process from phagophore to lysosomal degradation, and not with other vesicular structures (Li et al., 2013b, Barth et al., 2010).

A recent review stated that LC3 is the most widely monitored autophagy-related protein (Klionsky et al., 2012). There are also several papers that describe the use of LC3 expression vectors. LC3 mCherry expression vector was used to evaluate the role of a viral protein (vFLIP) in autophagossome formation after apoptosis induction (Ritthipichai et al., 2012). A similar expression vector (mCherry-GFP-LC3) was used to quantify the autophagy induction by mTOR inhibitors (Nyfeler et al., 2012). The same expression vector was used to study the role of protein BNIP3 (a hypoxia-inducible pro-death protein) in autophagosome accumulation in cardiac myocytes (Ma et al., 2012).

Furthermore, members of CEQUIMED-UP have performed both MDC staining and LC3-mCherry transfection assays to successfully conclude on the effect of TXA1.HCl in A375-C5 cells (unpublished data).

MDC and LC3-positive cells were visualized in treated cells, for the correspondent assay, suggesting that these vacuoles have an autophagic origin.

Figure 19 shows that for the different assays the effect of TXA1.HCl on autophagy was dose-dependent as the positive-labelled cells increased along with them.

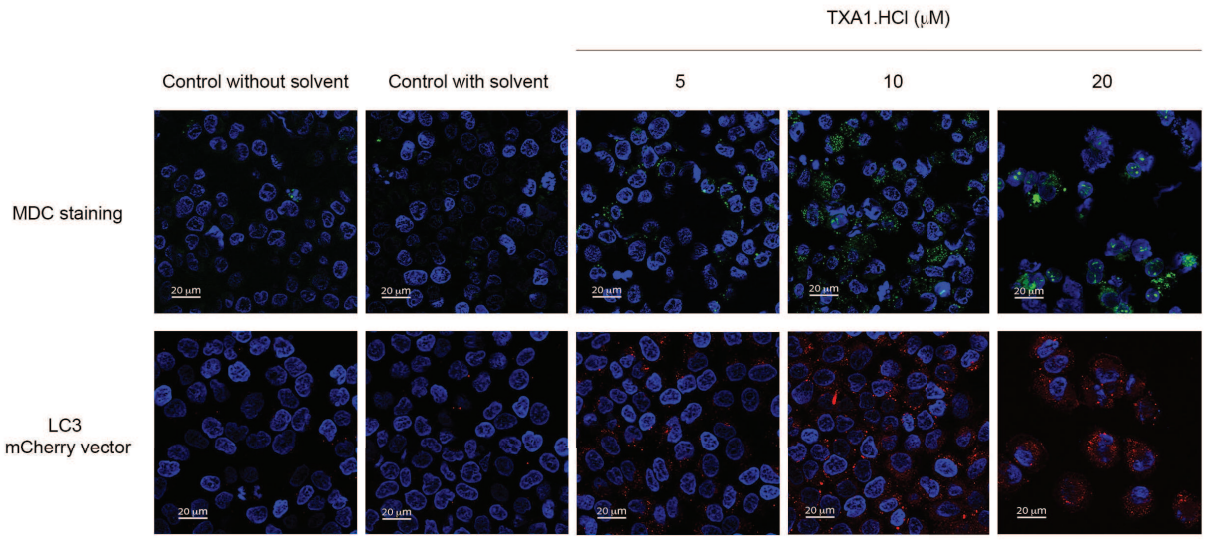


Figure 19: TXA1.HCl effect in autophagic vacuoles on MCF-7 cell line upon exposure to different concentrations.

Results were obtained through fluorescence microscopy. Images of representative focus plan were taken with a 400X magnification. Autophagic vacuoles appear as green and red spots for the MDC stained cells and for the LC3 mCherry vector transfected cells, respectively. Nucleus is stained with DAPI (blue). Scale bar, 20 μm.

He et al. demonstrated that cucurbitacin IIa induces apoptosis and simultaneously enhances autophagy in macrophages (He et al., 2013). In another work, Shaker and colleagues have shown that nilotinib induces apoptosis and autophagic cell death of hepatic stellate cells (Shaker et al., 2013). Triptolide was proven to be responsible for neuroblastoma cell death by both apoptosis and autophagy pathways (Krosch et al., 2013). Rapamycin also induced pancreatic cancer cells death by apoptosis and autophagy (Dai et al., 2012)

In fact, classical regulators of apoptosis, such as Bid, Bcl-2, and caspases, appear to crosstalk with Atg proteins and regulate autophagy (Esteve and Knecht, 2011). On the other hand, upstream these regulators of apoptosis are cytosol-released lysosomal cathepsins, which can induce apoptosis by activating pro-apoptotic proteins (Esteve and Knecht, 2011). Thus, although autophagy and

apoptosis may coexist as independent pathways, they are also interconnected processes that share some common proteins from their signalling routes (Esteve and Knecht, 2011).

All these findings, taken together with TUNEL assay data, offer new insights on this compound's mechanism of action that may not only target and activate apoptotic pathways, but also activate autophagic-programmed cell death.

Chapter V.
Conclusions

Cancer is a leading cause of mortality and morbidity throughout the world, and its incidence is predicted to increase in the next years. Many therapeutic approaches against cancer are currently available, from which chemotherapy stands out for the extent to which it has been studied and for its potential for optimization. Compound screening arises as a means to identify novel molecules with enhanced potency, selectivity and safety.

In the present work, both natural and synthetic compounds were screened for their cell growth inhibitory activity through the assay adopted by the National Cancer Institute – SRB assay.

From our results we can conclude that the methanolic crude extracts from *Cecropia catarinensis and from* macroalgae *Ulva rigida* and *Gelidium microdon* inhibited the growth of A375-C5, MCF-7, and NCI-H460 cell lines, which supports the potential role of nature on finding new anticancer compounds.

Furthermore, new synthetic xanthone derivatives, namely pyranoxanthones (XC20 and XC21) and thioxanthone (TXA1 and TXA1.HCl) did also successfully inhibit cell growth, revealing the potential use of the xanthonic scaffold to generate/improve anticancer compounds.

Herein, TXA1.HCl, the hydrochloride salt of TXA1, was studied for its chemotherapeutic potential. Its activity was studied using A375-C5 (melanoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and HeLa (cervical adenocarcinoma) cell lines. Moreover, both IC_{50} and GI_{50} values were shown to be below 10 μM, which represents a good indicator of the compound's potency.

TXA1.HCl mechanism of action was then further investigated at the molecular level, using MCF-7 cells.

Effects of the exposure to TXA1.HCl were both time- and concentration-dependent. There was a significant decrease in cell viability when MCF-7 cells were exposed to 20 μM TXA1.HCl for 24 hours. In fact, trypan blue assays confirmed that, in such conditions, cell viability was about only 40%.

TXA1.HCl effect was also proven to be irreversible. MTT assays performed after different exposure periods to the compound have shown that, even upon 6 hours of exposure, although no significant changes were observed in cell viability, cell survival and proliferation mechanisms were affected. Colony formation assays

also demonstrated a decrease in the ability to proliferate upon exposure to the compound. Again, exposure to 20 μM TXA1.HCl for 24 hours almost fully suppressed the ability to form new cell colonies.

In order to analyse the mechanisms through which cell death is mediated as a response to TXA1.HCl, specific methods were employed. TUNEL-positive results increased along with compound concentration and exposure period, thus showing that apoptosis is one possible fate for cells treated with the compound. In addition, assays with LC3 and MDC also originated positive signal upon a 48 hour-exposure to TXA1.HCl, in a concentration-dependent manner.

Taken together, these findings demonstrate that TXA1.HCl is a potent compound against breast cancer cells, presenting irreversible inhibitory effects on cell proliferation, exhibiting cytotoxicity and leading to apoptosis and autophagy. Such characteristics make it a promising lead compound for further studies.

Chapter VI.
Future
Perspectives

The work described in the previous sections sets the ground for the prosecution to additional assays, in order to complement and better clarify the present results.

Concerning TXA1.HCl effects, it would be interesting to elucidate the pathway through which TXA1.HCl triggers apoptosis on cancer cells. To this purpose, expression levels of apoptotic proteins from the intrinsic and extrinsic pathways could be analysed at the mRNA and protein level, through quantitative Real-Time PCR and Western blotting, respectively. In addition, Annexin V could be used for flow cytometric detection of apoptosis, as an assay to confirm the effect on apoptosis.

In order to better explain the interconnection between apoptosis and autophagy in TXA1.HCl-induced cell death, as well as the contribution of each phenomenon to the decrease in cell viability, assays could be performed in the presence of autophagy inhibitors. Furthermore, electron microscopy could be useful to confirm the TXA1.HCl effect on autophagy.

Finally, *in vivo* assays, already being pursued by members of the research group, will also add valuable information about TXA1.HCl potency, safety and feasibility as a chemotherapeutic agent.

Chapter VII.

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