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SEARCHING FOR NEW POTENTIAL SMALL MOLECULE MODULATORS OF PRO-APOPTOTIC PROTEINS USING THE YEAST-BASED SCREENING ASSAY

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Química Farmacêutica

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"Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional."

Brian Greene

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RESUMO

A pesquisa de novas terapias contra o cancro tem vindo a assentar na promoção da apoptose, bem como, em formas de ultrapassar a resistência a este processo nas células tumorais. As caspases são uma família de proteases com função crucial na apoptose. As caspases-3, -6 e -7 encontram-se na fase executora da apoptose, armazenadas como procaspases que uma vez ativadas por proteólise clivam um grande número de substratos, resultando na apoptose. Assim sendo, a pesquisa de ativadores destas procaspases merecem especial atenção na descoberta de novos potenciais fármacos anticancerígenos. Num trabalho anterior realizado pelo nosso grupo, utilizando o modelo de pesquisa da levedura *Saccharomyces cerevisiae* expressando as caspases-3, -6 e -7 humanas, levou à identificação de dois compostos ativadores da caspase-7: 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**). Estes resultados incitaram à realização da presente tese com o objetivo de descobrir novos ativadores das caspases-3, -6 e -7. Para isso, foram sintetizados análogos dos compostos **1a** e **2a**, e a sua atividade foi avaliada usando o modelo de levedura desenvolvido no trabalho anterior. Desta forma, foram identificados três potenciais ativadores da caspase-7: 7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one (**S1**), 6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one (**S2**) and 5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one (**S3**).

A proteína cinase C (PKC) é uma família de cinases de serina/treonina envolvidas na regulação da proliferação e morte celular, desempenhando assim um papel crucial no processo de carcinogénese. Desta forma, esta família representa um alvo promissor na terapia anticancerígena. Com base nisto, num trabalho prévio foi desenvolvido um ensaio de levedura para a pesquisa de moduladores isoforma-seletivos de PKCs. Utilizando este modelo foi identificado o composto **C4** como potencial ativador da PKCδ. Baseado nisto, na presente tese foi analisada a atividade antitumoral *in vitro* deste composto em linhas celulares HCT116 do adenocarcinoma do cólon. O composto **C4** exibiu uma potente inibição do crescimento nas células tumorais associada com a indução da apoptose.

Concluindo, foram identificados novos reguladores farmacológicos de proteínas pro-apoptóticas no presente trabalho, representando uma alternativa de estratégia personalizada no tratamento do cancro. Presentemente, mais trabalho está a decorrer de forma a confirmar o mecanismo de ação molecular dos mesmos em células tumorais humanas.

Palavras-chave: Cancro; Descoberta de novos fármacos; Modelo de pesquisa na levedura; Família de proteínas Caspases; Família de proteínas PKC.

ABSTRACT

The search for new therapies against cancer has been focused on devising ways to overcome resistance and to trigger apoptosis in tumour cells. Caspases are a family of proteases with a crucial role in apoptosis. Caspases-3, -6 and -7 are at the core of the execution phase of apoptosis, stored as procaspases that once activated cleave a large set of substrates, ultimately resulting in the hallmarks of apoptosis. Therefore, the search for activators of these procaspases has deserved particular attention in the field of anticancer drug discovery.

In a previous work from our group, the use of a yeast screening assay, based on the heterologous expression of human caspases-3 or -7 in the yeast *Saccharomyces cerevisiae*, led to the identification of two prenylated flavonoids as potential activators of caspase-7: 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**). These results prompted us to the present work aiming to discover new activators of caspases-3, -6 and -7. For that, analogues of flavonoids **1a** and **2a** were synthesised, and their activities on these proteins were evaluated, using the previously developed yeast-based screening assay. Three new potential selective activators of caspase-7 were identified: 7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one (**S1**), 6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one (**S2**) and 5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one (**S3**).

The protein kinase C (PKC) is a family of serine/threonine kinases involved in the regulation of cell proliferation and death, playing therefore a major role in the carcinogenesis process. Thus, this family represents a promising and appealing target in anticancer therapy. In this way, in a previous work, a yeast targeted screening assay was developed to search for PKC isoform-selective modulators. Using this yeast approach, the compound **C4** was identified as a potential selective activator of PKCδ. Based on this, in the present dissertation, the *in vitro* antitumor activity of **C4** was analysed in the human colon adenocarcinoma HCT116 cell lines. The tested compound exhibited a potent growth-inhibitory activity in tumour cells, which was associated with apoptosis induction.

In conclusion, in this dissertation, new potential pharmacological regulators of pro-apoptotic proteins were identified. Further work is underway to confirm their molecular mechanism of action in human tumour cells. Despite that, the data emerged from the present work demonstrated that these compounds may represent an alternative strategy for personalized cancer treatment.

Keywords: Cancer; Drug discovery; Yeast-based screening assays; Caspases family proteins; PKC family proteins.

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ABBREVIATIONS:

AIF - Apoptosis inducing factor;

ANT - Adenine nucleotide translocase;

Asp-X - Specific asparagines residues;

Apaf-1 - Apoptotic protease-activating factor 1;

Bcl-2 - B-cell lymphoma 2;

BID - BH3-interacting domain death agonist;

tBID - truncated BID;

CAMS - Cell-cell adhesion molecules;

CARD - Caspase recruitment domain;

CFU - Colony forming unit;

crmA - Cytokine response modifier A;

Cyt. *c* - Cytochrome *c*;

DAG - Diacylglycerol;

DED - Death effector domain;

DISC - Death-inducing signalling complex;

Endo G - Endonuclease G;

FADD - Fas-associated protein with death domains;

GI_{25} - 25% growth inhibition;

HTS – High-throughput screening;

IAP - Inhibitors of apoptosis family;

ILP2 - IAP-like protein 2;

LS - Large subunit;

ML-IAP - Melanoma IAP;

MOMP - Mitochondrial outer membrane permeabilization;

MW - Microwaves;

NAIP - Neuronal apoptosis inhibitory protein;

PAC-1 - Procaspase-Activating Compound 1;

PARP - Poly ADP ribose polymerase;

PB1 - Phox and Bem 1 domain;

PKC - Protein kinase C;

PS – Phosphatidylserine;

pRB – Retinoblastoma protein;

ROS - Reactive oxygen species;

Smac/DIABLO - second mitochondria-derived activator of caspases;

SS - Small subunit;

TLC - Thin-layer chromatography;

TNF - Tumour necrosis factor;

TRAIL - TNF-related apoptosis inducing ligand receptors;

UV - Ultraviolet radiation;

VDAC - Voltage dependent anion channel;

XIAP - X-linked IAP.

CHAPTER 1

Introduction

1. PRINCIPLES ABOUT CANCER

Cancer is a major public health problem worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012[1]. In fact, according to the World Health Organization, the number of new cases is expected to rise about 70% over the next 2 decades. Thus, the discovery of new therapeutic agents against this severe pathology is of enormous interest and urgently required.

Carcinogenesis is a multistep process reflecting a series of genetic and epigenetic alterations in normal human cells that drives their progressive transformation into highly malignant derivatives[2]. All cancers have many features in common at the molecular level. This suggests that the biochemical processes, including abnormalities in the genetic reading process leading to the development and progression of malignant transformation, follow a common pattern [reviewed in[3]].

According to Hanahan and Weinberg (2000) there are six hallmarks of cancer, comprising six biological capabilities acquired during the multistep development of human tumours. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Recently, in 2011, two emerging hallmarks were added to this list: the capability to reprogram the energy metabolism, in order to control the neoplasic transformation, and the capability of cancer cells to evade immunological destruction (**Figure 1**) [reviewed in[4]].

Sustaining proliferative signalling

Tumour cells have the capacity to stimulate its own growth and to resist to inhibitory signals to stop it, while normal cells require external growth factors to grow and divide, and if one of these signs is absent, they stop growing. In tumour cells, the presence of these external growth factors is not necessary to multiply, because they can generate their own growth factors, or the receptors are themselves overexpressed [reviewed in[5]].

Resisting cell death

Apoptosis is a form of programmed cell death that occurs in normal cells in response to cellular damages and is a crucial process for the maintenance of tissue homeostasis in multicellular organisms [reviewed in[6]]. Tumour cells are able to bypass this process. This cell death process is highly regulated by many proteins, such as the p53 tumour suppressor, caspases and Bcl-2 family members, among others. Any deregulation of this process lead to resisting cell death [reviewed in[7]].

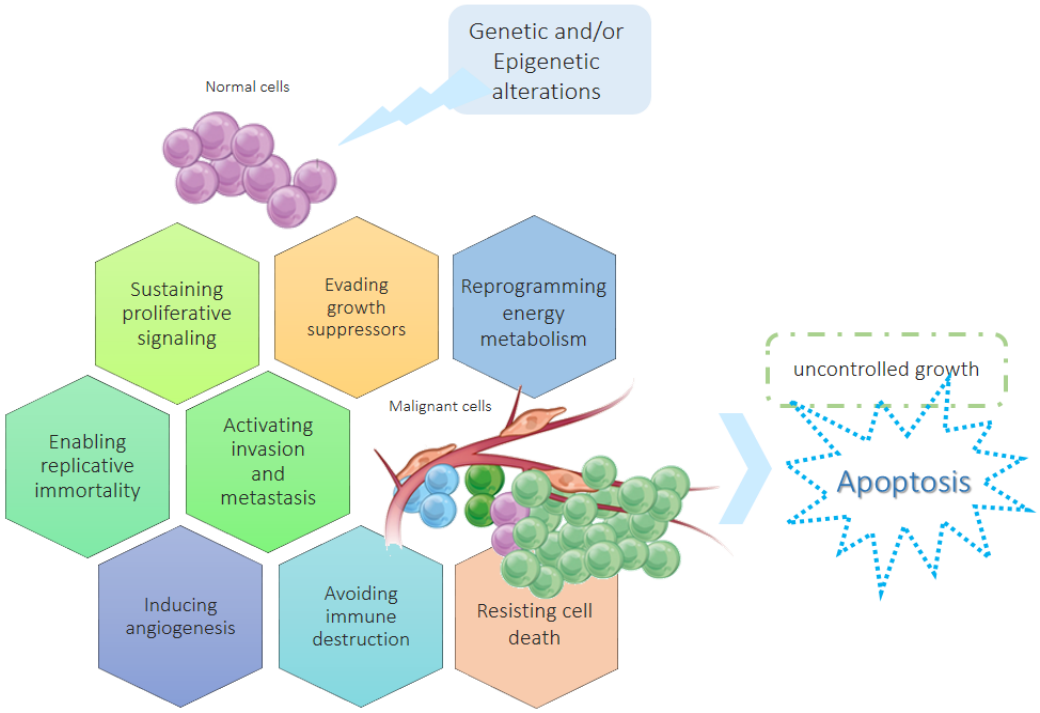


Figure 1 –The hallmarks of cancer acquired during the multistep development of human tumours lead to an uncontrolled growth. Apoptosis is required to control the development and progression of malignant transformation.

Enabling replicative immortality/evading growth suppressors

Normal cells can multiply about 60-70 doublings, and after this number of divisions they enter in senescence. Tumour cells, with damaged chromosomes, escape this limit, being capable of indefinite growth and division. This immortalization may be due to numerous processes, particularly, due to the disabling of retinoblastoma (pRB) and p53 tumour suppressor protein, which allow tumour cells to continue doubling. The counting device for cell doublings is the telomere, which loses DNA at the tips of every chromosome during each cell cycle. Many tumours involve the upregulation of telomerase, the enzyme that maintains telomeres [reviewed in[5]].

Inducing angiogenesis

Angiogenesis is the process by which new blood vessels are formed. Tumour cells initially do not have angiogenic ability, limiting their ability to expand. For instance, they must develop a blood supply in order to receive continual supply of oxygen and other nutrients. During tumorigenesis, the appropriate balance between proangiogenic and antiangiogenic molecules is lost [reviewed in[8]]. Tumour-angiogenesis therefore depends on tumour type, localization, growth, and stage of disease, and contributes to tumour growth, invasion, and metastasis [reviewed in[9]].

Activating invasion and metastasis

Tumour cells can spread to different parts of the body, in other words, they can metastasize. Metastatic cells must mimic normal cell-cell interactions, through cell-cell adhesion molecules (CAMs) and integrins. Integrins display substrate preferences, and changes in integrins are displayed by migrating cells. Matrix-degrading proteases are also necessary to facilitate invasion into stroma, across blood vessel walls, and through normal epithelial cell layers [reviewed in[10]].

Reprogramming energy metabolism

In the presence of oxygen, normal cells process glucose into pyruvate via glycolysis in the cytosol, and thereafter to carbon dioxide in the mitochondria. In the absence of oxygen, glycolysis is favoured and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. Tumour cells are able to reprogram their energy metabolism. Even in the presence of oxygen, tumour cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis [reviewed in[4]].

Evading immune destruction

Cells and tissues are constantly monitored by an immune surveillance that is responsible for recognizing and eliminating the wide majority of tumour cells and nascent tumours. However, it seems that the solid tumours are able to avoid detection by the immune system or have been able to limit the extent of immune killing, evading immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells, avoiding eradication [reviewed in[4]].

1.1. APOPTOSIS

Apoptosis is a type of programmed cell death that occurs when DNA damage is irreparable. This process is characterized by specific morphological and biochemical features, including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation [reviewed in[11]]. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events [reviewed in[12]].

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (**Figure 2**) [reviewed in[13]].

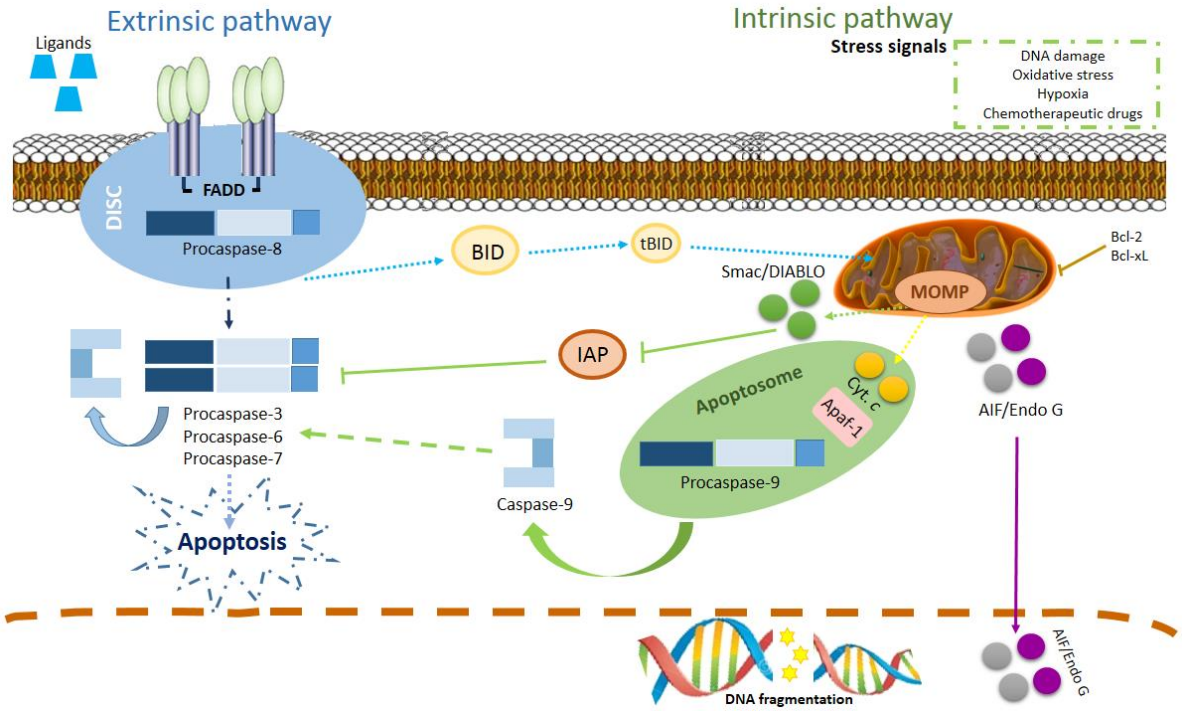


Figure 2 – The extrinsic and intrinsic pathways of apoptosis. Abbreviations: AIF - apoptosis inducing factor; Apaf-1 - apoptotic protease-activating factor 1; BID - BH3-interacting domain death agonist; tBID - truncated BID; Cyt. *c* - cytochrome *c*; DISC - death-inducing signalling complex; Endo G - endonuclease G; FADD - Fas-associated protein with death domains; IAP - inhibitors of apoptosis family; MOMP - mitochondrial outer membrane permeabilization; Smac/DIABLO - second mitochondria-derived activator of caspases.

The extrinsic pathway requires the activation of cell death receptors, namely tumour necrosis factor (TNF) superfamily, such as TNF, CD95 (Fas), and TNF-related apoptosis inducing ligand (TRAIL) receptors, found on the surface of the cell membranes, and their respective ligands transmitting the death signal from the surface to the intracellular signalling pathways. The activation of some receptors, as CD95 and TNF, leads to receptor clustering and intracellular recruitment of proteins such as the protein adaptor FADD (Fas-associated protein with death domains). In turn, FADD recruits the initiator procaspase-8, forming the death-inducing signalling complex (DISC). The formation of this complex leads to the activation of procaspase-8, an initiator caspase. This activation induces the execution phase of apoptosis via the activation of the executioner procaspases-3, -6 and -7 or induces the cleavage of the BH3-interacting domain death agonist (BID), leading to the generation of a mitochondrion-permeabilizing fragment, the truncated BID (tBID) [reviewed in[13-14]].

On the other hand, the mitochondrial pathway is activated by stress signals such as DNA damage, oxidative stress, hypoxia or chemotherapeutic drugs. These external stimuli lead to modifications in the inner mitochondrial membrane permeability. These alterations induce changes at the mitochondrial outer membrane permeabilization (MOMP), loss of

mitochondrial transmembrane potential ($\Delta\psi_m$) and to the release of pro-apoptotic proteins such as cytochrome *c* (cyt *c*), the second mitochondria-derived activator of caspases (Smac/DIABLO), the apoptosis inducing factor (AIF) and the endonuclease G (Endo G) [reviewed in[13]].

After release into the cytoplasm, cyt *c* binds to the apoptotic protease-activating factor 1 (Apaf-1) and to procaspase-9, stimulating the apoptosome formation. In turns, the apoptosome activates procaspase-9, which in turn activates the executioner procaspases-3, -6 and -7, resulting in an apoptotic cell death [reviewed in[15]]. Smac/DIABLO binds and inhibits several members of the inhibitor of apoptosis (IAP) family, preventing the IAPs from arresting the apoptotic process, thereby activating the executioner procaspases-3, -6, and -7, allowing apoptotic progression [reviewed in[13]].

In some models, the release of AIF and Endo G is a late event in apoptosis, which occurs once the cells are committed to die. Following the release of AIF and Endo G, these proteins are able to translocate to the nucleus, promoting DNA fragmentation through a caspase-independent manner [reviewed in[14]].The intrinsic pathway is mainly controlled by Bcl-2 family members, once they play a central role in the regulation of cyt *c* release from mitochondria. This family consists of anti-apoptotic members, such as Bcl-2 and Bcl-xL, and pro-apoptotic members, such as Bax, Bak and Bid. Bcl-2 and Bcl-xL are able to inhibit cyt *c* release by the protection of the membrane integrity through the interaction of these anti-apoptotic proteins with mitochondrial proteins such as the adenine nucleotide translocase (ANT) or the voltage dependent anion channel (VDAC), thus preventing them from forming mitochondrial pores. On the other hand, Bax can homodimerize or heterodimerize with Bak or tBib, leading to the disruption of the mitochondrial membrane integrity through the formation of mitochondrial pores, with the subsequent cyt *c* release [reviewed in[13]].

The apoptotic process is essential for the maintenance of tissue homeostasis in multicellular organisms. It is well-known that the deregulation of this process has been implicated in numerous pathological conditions such as in cancer [reviewed in[16]]. In this way, the search for new anticancer therapies has been focused on devising ways to overcome increasing tumour resistance and to trigger apoptosis in tumour cells. It is clear that a number of anticancer drugs induce apoptosis in tumour cells. However, the problem is that some of these drugs usually induce cell death in normal cells as well. Therefore, the goal is to selectively modulate genes/proteins involved in apoptosis in tumour cells. In this way, understanding how those genes/proteins work reveals to be a crucial task for the development of more potent and effective compounds [reviewed in[17]].

1.2. TARGET PROTEINS OF THE APOPTOTIC PATHWAY

1.2.1. CASPASES

It became evident that the cellular and biochemical features of apoptosis are a consequence of the cleavage of a subset of proteins by proteases of the caspase family [reviewed in[18]]. The central role in the regulation and in the execution of apoptotic cell death belongs to caspases. In fact, an inappropriate caspase activity can cause deficient cell death, potentially leading to carcinogenesis and other apoptotic-associated pathologies [reviewed in[19]].

Caspases are a conserved family of cysteine-dependent aspartate-specific proteases that are expressed in an inactive proenzyme form (zymogens). Structurally caspases consist of an N-terminal prodomain, followed by a large subunit with 20 kDa (p20) and a small subunit with 10 kDa (p10). The N-terminal prodomain contains structural motifs that belong to the death domain superfamily: the death effector domain (DED) and the caspase recruitment domain (CARD) [reviewed in[20]] (**Figure 3**). Depending on the structure and cellular functions, caspases are divided in inflammatory (Group I: caspases-1, -4, -5, -11, -12 e -13) and apoptotic (caspases-2, -3, -6, -7, -8, -9 e -10). In addition, apoptotic caspases are divided in initiator caspases (Group II: -2, -8, -9 e -10) and effector caspases (Group III: -3, -6 e -7) (**Figure 3**) [reviewed in[21]].

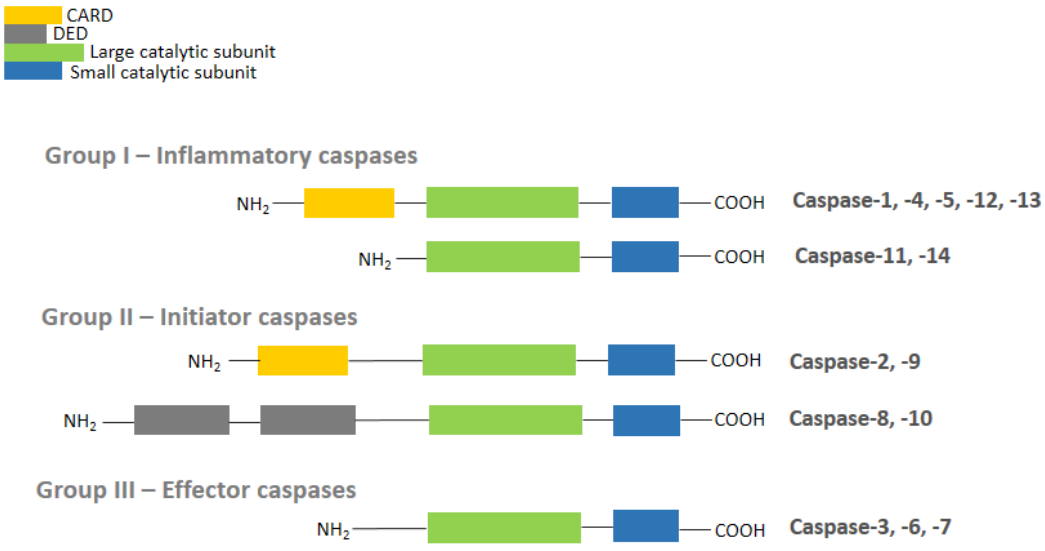


Figure 3 – Caspase family structure. Three major groups of caspases are presented: group I: inflammatory caspases; group II: initiator caspases; group III: effector caspases. Caspases consist of a single chain constituted by three domains: N-terminal prodomain (DED and CARD), large (p20) and small (p10) catalytic subunits. Adapted from[20].

In most cells, caspases are activated through proteolytic cleavage in specific asparagines residues (Asp-X). This results in the generation of mature caspases, allowing the initiation of a protease cascade that is implicated in the inflammatory process and in apoptosis [reviewed in[22]] (**Figure 4**). This strategy provides a control over the activity of caspases, in order to reduce the possibility of, inadvertently, they enter apoptosis [reviewed in[23]]. Initiator caspases are presented in cells as inert monomers, which require dimerization and inter-domain cleavage for their activation. In contrast, effector caspases present themselves as inactive dimers, requiring cleavage of its catalytic domain, leading to intermolecular rearrangements with consequent formation of enzymatically active dimer [reviewed in[24]].

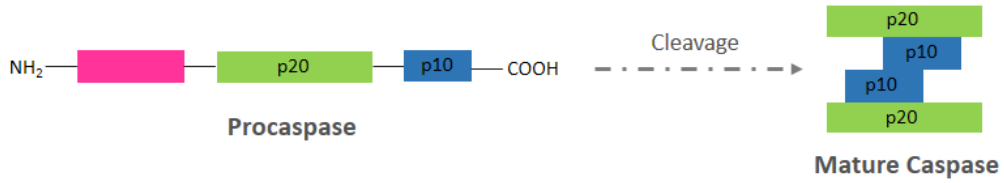


Figure 4 – Scheme of caspase activation. Cleavage of the procaspase leads to the formation of mature caspase, which comprises the heterotetramer $p20_2$–$p10_2$ without the prodomain. Adapted from[20].

There are two pathways through which the caspase family proteases can be activated: one is the signal-induced death receptor-mediated pathway and the other is the stress-induced, mitochondria-mediated pathway [reviewed in[24]]. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signalling pathway, leading to a rapid cell death. Basically, caspases may be activated in response to different pro-death stimuli through the formation of activating complexes in the presence of adaptor proteins [reviewed in[25]].

The outcome of death receptor--mediated activation of caspase-8 depends of the cell type [reviewed in[21]]. In type I cells, procaspase-8 activation can directly activate downstream effector caspases (caspases-3, -6, -7) to promote DNA damage and apoptosis without requirement of the mitochondria dependent pathway. On type II cells, the apoptotic process is mostly controlled by Bcl-2 family members. Both caspase-8 and -2 can cleave and activate the pro-apoptotic Bcl-2 family protein. Indeed, Bax can homodimerize or heterodimerize with Bak or tBib, leading to the disruption of the mitochondrial membrane integrity, release of cyt *c,* and to the subsequent induction of the intrinsic apoptosis pathway. Once released, cyt *c* binds to Apaf-1, which recruits procaspase-9 to form the apoptosome. Subsequently, caspase-9 is activated and cleaves the effector caspases-3,-6 and -7, leading to the induction of apoptosis in a caspase-dependent manner [reviewed in[26]].

Caspases activity can be suppressed by caspase inhibitor proteins, such as cowpox virus cytokine response modifier A (crmA), baculoviral p35/p49 and IAPS, a family of cellular proteins including, neuronal apoptosis inhibitory protein (NAIP), cellular IAP1 (cIAP1) and 2 (cIAP2), X-linked IAP (XIAP), survivin, bruce, melanoma IAP (ML-IAP) and IAP-like protein 2 (ILP2) [reviewed in[27]].

Mutation in *CASP* genes is not frequent in human tumour cells. In fact, it is believed that the inactivation of individual caspases is not usually sufficient to prevent the progression of caspase cascade. However, the reduced expression of pro-apoptotic caspases has been reported in manifold cancers, as well as specific inactivating mutations have been reported in many tumour types and stages of malignant transformation [reviewed in[21]].

Despite of the enormous relevance of initiator caspases, in this dissertation a particular attention will be given to effector caspases.

1.2.1.1. EFFECTOR CASPASES

Caspases-3, -6 and -7 are at the core of the execution phase of apoptosis [reviewed in[28]]. Structurally these proteases are composed by large and small catalytic subunits, existing in the cells as inactive homodimers. The effector caspases are activated through proteolytic cleavage mediated by an initiator caspase [reviewed in[19]]. Once activated, besides to lead to cell death through cleavage of specific target proteins, the executioner caspases will be able to activate other effector caspases, leading to the generation of a feedback loop of caspase activation [reviewed in[21]].

Caspase-3

Caspase-3 is encoded by the *CASP3* gene located in chromosome 4q35.1 and is the most well-characterized effector caspase, since exerts a key role in both extrinsic and intrinsic apoptotic pathways. Caspase-3 has both distinct and overlapping roles with caspase-6 and -7[29]. The divergent amino-terminal sequences between caspase-3 and -7 appear to contribute to different modes of regulation[30]. In fact, Slee et al. (2001) performed a study where caspases-3, -6 and -7 were silenced in order to analyse the impact of the absence of these proteases on the execution phase of apoptosis. Indeed, the authors demonstrated that caspase-3 is a key component of the apoptotic process by participating in several events among this process, while caspase-6 and -7 exert more specialized roles[31].

Many studies demonstrated that alterations in the *CASP3* gene might promote human tumorigenesis, suggesting that caspase-3 may play a crucial role in cancer prevention. In fact, *CASP3* polymorphism and their haplotypes help to define the individual's genetic susceptibility to cancer development [reviewed in[21]]. This may happen since the *CASP3* gene can give rise to an alternative splicing variant known as caspase-3s, which exert an anti-apoptotic function [reviewed in[32]].

Additionally, distinct works have demonstrated that procaspase-3 is overexpressed in a variety of human tumours, namely, colon cancer[33], lung cancer[34], melanoma[35], hepatoma[36], breast cancer[37], among others.

Caspase-6

Caspase-6 is encoded by the *CASP6 gene* located in chromosome 4q25. This executioner caspase are much more weakly apoptotic than caspase-3 and -7[38]. Nevertheless, caspase-6 also cleaves a different set of cellular substrates[39]. The substrates of this effector caspase include PARP, lamin and procaspase-3. In fact, caspase-6 can activate procaspase-3 by a positive feedback pathway [reviewed in[22]].

The way how caspase-6 is activated is still controversial. In 2009, it has been reported that caspase-6 is activated only following activation of caspases-3 and -7 and thus is not activated directly by an initiator caspase[29]. However, in contrast, previous works showed that this caspase can be activated in the absence of caspase-3 and -7[40]. More recently, it has been reported that, *in vivo*, caspase-6 can activate itself[41].

Expression of caspase-6 in gastric cancer is decreased, suggesting that loss of caspase-6 expression might be involved in the mechanism of gastric cancer development[42]. In fact, mutations in the *CASP6* gene have been found in 2% of human cancers of colonic and gastric origin[43].

Caspase-7

Caspase-7 is encoded by the *CASP7* gene located in chromosome 10q25.3. This caspase shares many similarities with caspase-3. Both proteases are effector caspases and are substrates of initiator caspases in the extrinsic or intrinsic apoptotic pathways. Despite that, caspases-3 and -7 exhibit distinct activities *in vivo* [reviewed in[32]]. This may be due to the presence in caspase-7 of an inhibitor of apoptosis binding motif (IBM), which is absent in caspase-3, that appears to be more efficiently ubiquitinated by IAPs, leading to a more readily proteasomal degradation of caspase-7 compared to caspase-3[44]. In fact, several substrates are specifically cleaved by caspase-7, such as cochaperone p23 [reviewed in[30a, 45]].

Mutations in the *CASP7* gene affect the pathogenesis of some human solid cancers, since the function of caspase-7 is compromised [reviewed in[21]]. In fact, in the analysis of multiple types of cancer, developed in 2003, somatic mutations in *CASP7* were detected in 2% of colon and esophageal carcinomas and 3% in head/neck carcinomas[46].

1.2.1.1.1. SMALL MOLECULE ACTIVATORS OF CASPASES FAMILY

The activation of effector caspases with small molecules could be important in the activation of these proteases and could have a direct impact on important physiological processes [reviewed in[28]]. Indeed, caspase activation can be particularly applied as an anticancer strategy by selective induction of apoptosis in cancer cells [reviewed in[47]]. Thus, the discovery of small molecule caspase activators has deserved particular relevance in the cancer field [reviewed in[32]]. However, to date, only few small molecule caspase activators have been reported [reviewed in[28, 47]].

Putt et al. (2006) discovered the Procaspase-Activating Compound 1 (PAC-1) (**Figure 5A**) as the first small molecule caspase activator that induces apoptosis in cancer cells through the activation of procaspases-3 and -7[33].

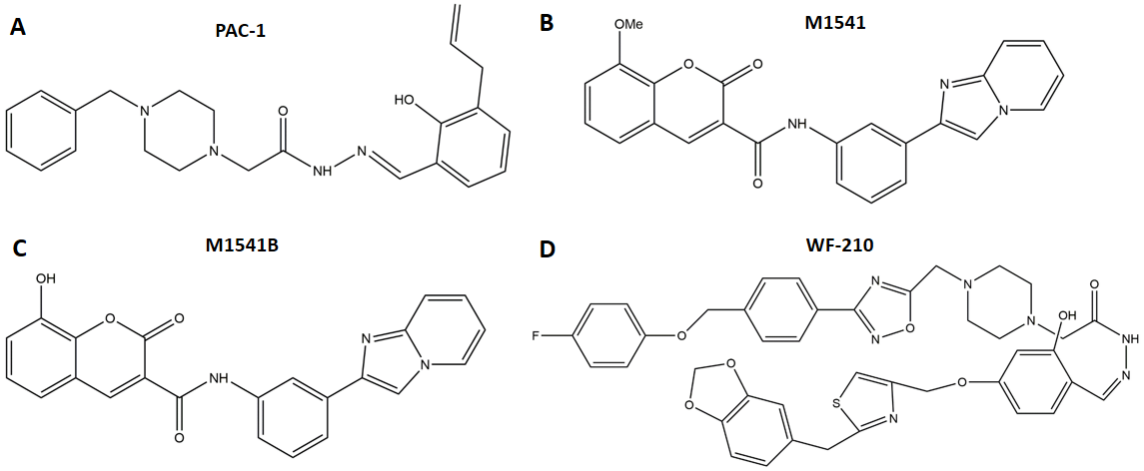


Figure 5 - Chemical structures of (**A**) PAC-1 which activates caspases-3 and -7, (**B**) M1541 which activates caspases-3 and -6 (**C**) M1541B, (**D**) WF-210 which activate caspase-3.

In fact, PAC-1 does not act by direct activation of executioner caspases, but through the zinc ion chelation, the more physiologically relevant metal in inhibiting procaspases activity (**Figure 6**)[48]. Despite that, some studies stated that PAC-1 is able to kill cerebellar granule neurons *in vitro* and causes neurotoxicity *in vivo*[49]. Therefore, these evidences led to intensive efforts in the development of novel procaspase activators with minimal side effects.

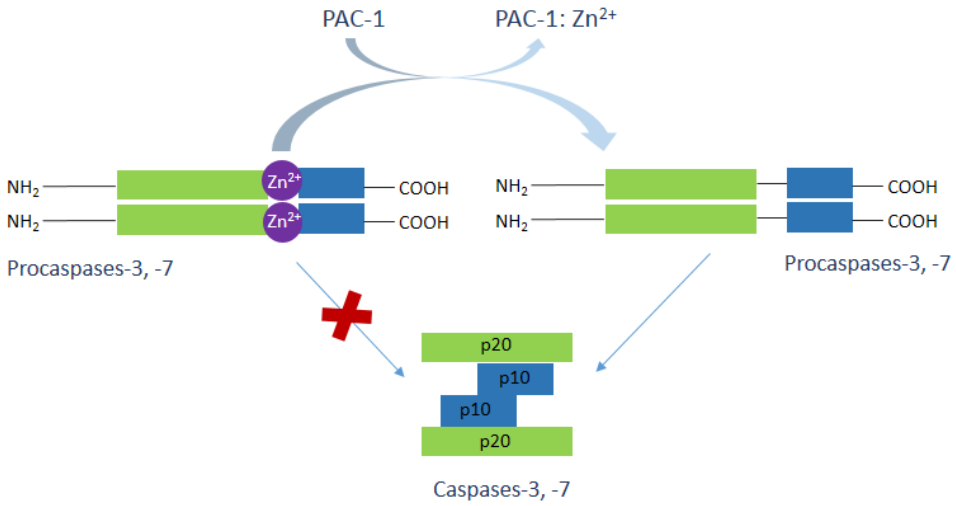


Figure 6 – Mechanism of action of compound PAC-1. Adapted from[33].

In 2009, Wolan and colleagues verified that compound M1541 (**Figure 5B**) was able to induce apoptosis in the p53-deficient breast cancer cell line BT549. In this study, it was reported that M1541 can bypass initiator caspase activation and directly induce apoptosis in a caspase-3/-6-dependent manner without requiring apoptotic stimuli or upstream factor[50]. This can be a promising strategy in cancer therapy where apoptosis of cancer cells can be directly triggered, resulting in cell death [reviewed in[28]]. In the same study, it was reported a potent activator of procaspase-3, compound M1545B (**Figure 5C**), an analogue of M1541[50]. Compound M1541B appears to activate procaspase-3 via a binding-induced shift in the on-off state equilibrium[50], or trough formation of nanofibriles[51].

More recently, a novel procaspase-3 activator, WF-210, (**Figure 5D**) was discovered. WF-210 induces apoptosis in human leukemia cell lines HL-60 and U-937 by activating procaspase-3. This compound showed higher *in vivo* efficacy and safety characteristics compared with PAC-1[52].

1.2.2. PROTEIN KINASE C FAMILY PROTEINS

The protein kinase C (PKC) is a family of serine/threonine kinases that are involved in relevant processes, including neoplastic transformation, carcinogenesis and tumour cell invasion. Hence, in these processes, the activity and expression of some of these kinases are altered. Therefore, they are potentially suitable targets for anticancer therapy [reviewed in[53]]. The PKC family consists of at least 10 isozymes with distinct and in some cases opposing roles in cellular processes, such as cell growth, differentiation, survival and apoptosis. This family is divided into three subfamilies according to their primary structure and cofactors required for activation: conventional or classic PKCs (cPKCs: PKCα, PKCβI, PCKβII and PKCγ), novel PKCs (nPKC: PKCδ, PKCε, PKCη and PKCθ) and atypical PKCs (aPCKS: PKCζ and PKCλ) (**Figure 7**) [reviewed in[53b, 54]].

The principal co-factors required for PKC activity are calcium, phosphatidylserine (PS) and diacylglycerol (DAG), a lipid second messenger transiently generated upon stimulation of membrane receptors such as tyrosine-kinase and G-protein coupled receptors [reviewed in[53b, 55]]. The differences between the distinct subfamilies reside in the regulatory domain. The C1 domain that comprises two cysteine-rich motifs forming the DAG binding site has only one cysteine-rich in aPKCs, and is not able to bind DAG. The C2 domain, that binds calcium and PS in cPKCs, is altered in nPKCs and aPKCs, losing the ability to bind calcium. In turn, C3 domain acts as an ATP binding site and C4 domain as a kinase catalytic centre [reviewed in[56]].

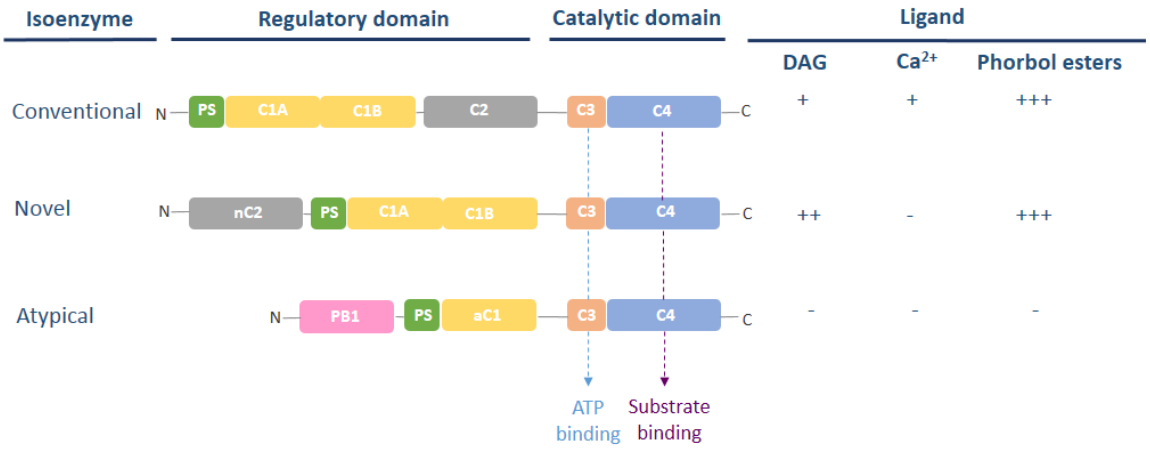


Figure 7 – PKC family structure. The three subfamilies are represented, as well as, their distinct bindings. The table on the right summarizes the second messengers that bind to each subfamily of PKC, with (+) representing binding, (++) representing binding with~100-fold higher affinity, (+++) representing binding with >100-fold higher affinity, and (−) representing lack of binding. Abbreviations: aC1 – atypical C1 domain; DAG – diacylglycerol; nC2 – novel C2 domain; PB1 - Phox and Bem 1 domain; PS – phosphatidylserine. Adapted from[57].

Individual PKC isoforms can exert either similar or opposite effects in cell proliferation and death, so an individual analysis of PKC isoforms would contribute to the identification of isoform-selective pharmacological modulator [reviewed in[58]]. The PKC isoform-specificity is basically a result of isoform-specific patterns of subcellular compartmentalization, protein-protein interactions and post-translational modifications. The specificity of each PKC isoform is therefore highly dependent on its primary structure, which determines the requirement for activating co-factors, regulatory phosphorylations, and intra- and inter-molecular interactions[59].

The existence of PKC isoforms with differential activation and tissue distribution raised the possibility to the development of PKC isoform-specific modulators with the potential to target specific intracellular pathways. However, the current PKC modulators that have reached the clinic are relatively non-specific [reviewed in[54a, 58, 60]]. This may be due to the highly similarity of the catalytic domain of PKC compared to many other protein kinases. Rottlerin is an example of a non-selective PKC modulator that is highly used to study the role of PKCδ. Nevertheless, rottlerin is a potent large conductance potassium channel opener that affects multiple other protein kinases [reviewed in[61]].

Loss-of-function mutations were observed in all PKC subgroups, and were associated with the prevention of second-messenger binding, phosphorylation, or catalysis[62]. PKC levels are aberrant in a variety of cancers and over 400 mutations in PKC have been identified in human cancers[63]. Recently, from 37 mutations studied, 24 reduced or completely abolished the PKC activity[63].

Altogether, based on the exposed above, isoform-selective pharmacological modulators are highly necessary, in order to target specific intracellular pathways to help in the prevention of cancer development. Despite the importance of all PKC-isoforms in cancer, this dissertation will be focused on the study of nPKC-δ.

Protein kinase C δ

PKCδ was the first identified member of the nPKC subfamily. PKCδ has been implicated in the regulation of both intrinsic and extrinsic apoptosis pathways. Indeed, the overexpression or activation of PKCδ results in the induction of apoptosis [reviewed in[64]]. During apoptosis, PKCδ is proteolytically cleaved by caspase-3 and translocates from the cytoplasm to the nucleus after treatment with genotoxic agents to generate a constitutively activated catalytic fragment, which amplifies apoptotic cascades. Other studies showed that PKCδ-mediated apoptosis involves the allosteric activation of the enzyme rather than proteolytic cleavage. Nevertheless, the complex role of PKCδ in apoptosis appears to be stimulus and cell type dependent [reviewed in[55b, 64]].

In fact, PKCδ can be activated by distinct apoptotic stimuli such as DNA damaging agents, ultraviolet (UV) radiation, and reactive oxygen species (ROS) [reviewed in[64]].

PKCδ is frequently associated with pro-apoptotic functions. Thus, this isoform has been largely implicated as a cell death mediator of chemotherapeutic agents and radiotherapy, since can be involved in both DNA damage and receptor-mediated cell death [reviewed in[55b]]. In fact, in 1993, it was reported that overexpression of PKCδ induced growth inhibition in NIH 3T3 cells[65], an effect also observed in other cell lines. For instance, it was demonstrated that the anti-proliferative effects of retinoic acid were dependent on PKCδ in leukemic and MCF-7 cells, indicating that the endogenously expressed PKCδ can suppress cell proliferation[66]. On the other hand, pro-survival properties of PKCδ in a number of tumour models, including breast, lung, pancreatic and liver cancer, were also described [reviewed in[67]].

Despite this, most studies around the effects of this isoform on cell proliferation indicate an inhibitory effect of this protein on cell proliferation. Nevertheless, it has been proposed that PKCδ has both tumour suppressor and proliferation capabilities that can be recruited as a backup kinase for both gatekeeper tumour suppression and as an activator of the Ras/Raf/MEK/MAP kinase signaling pathway in cell proliferation [reviewed in[68]]. Loss of PKCδ expression has been reported in some cancer types, such as, endometrial tumours[69], malignant fibrous histiocytoma[70] and human squamous cell carcinomas[71].

Hence, due to the relevant role of the PKCδ in the apoptotic process, the search for selective activators of this PKC isoform is an attractive and appealing therapeutic strategy in cancer therapy.

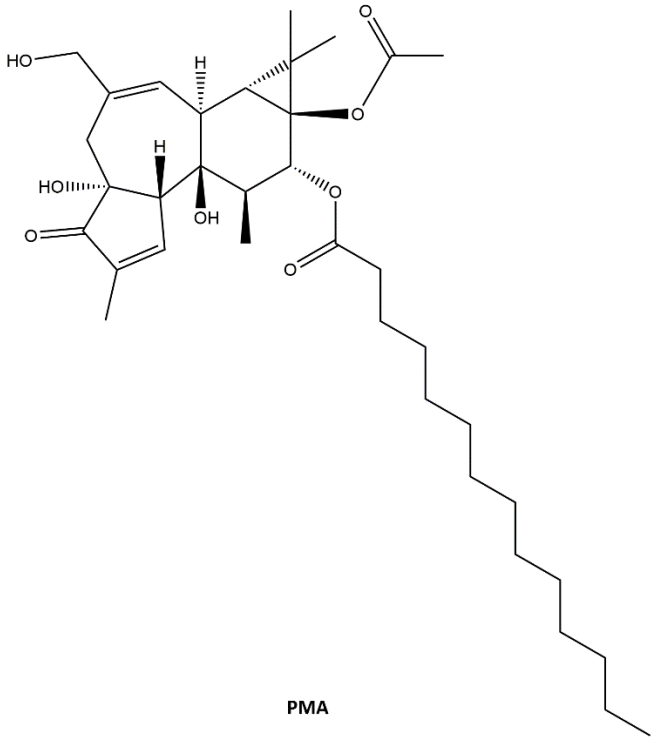
1.2.2.1. MODULATORS OF PKC FAMILY PROTEINS

A wide range of small molecule PKC activators and inhibitors (**Table 1**) have emerged to modulate the activity, subcellular distribution, protein interactions and life cycle of PKC [[72]; reviewed in[57]].

Table 1 – Mechanism of action and selectivity of PKC small molecule modulators.

Compounds	Mechanism of action	Selectivity to PKC	References
Small molecule activators			
PMA (Phorbol-12-myristate-13-acetate)	Activate PKC via any of the cysteine motifs in the C1 domain	Not well characterized on PKCε No effect on PKCζ	72-73
dPPA (12-deoxyphorbol 13-phenylacetate 20-acetate)	Activate PKC via any of the cysteine motifs in the C1 domain	No effect on PKCα, PKCδ, PKCε and PKCζ	72-73
Mezerein	Preferably active for the first cysteine repeat of the C1 region	Not well characterized on PKCε and PKCη No effect on PKCζ	72, 74
Bryostatin 1	Preferentially binds to the second cysteine repeat. Induces direct translocation to internal membranes	Selectively activates PKC-δ and PKC-ε.	72, 75
Coleon U	Induction of a Yca1p- and mitochondrial-dependent apoptosis	Selectively activates PKCδ and PKCε No effect on PKCα, PKCβI and PKCζ	74, 76
Small molecule inhibitors			
Chelerythrine	Induces apoptosis	None	57, 76
Calphostin C	Competes against DAG/phorbol ester binding C1 domain.	Not well characterized	57
PS168, PS171 Benzimidazole compounds	Inhibits allosterically	Selectively inhibits PKCζ	57
Rottlerin	Mitochondrial uncoupler	None No effect on PKCα	57, 76

In 1982, it was discovered a natural phorbol ester, the phorbol 12-myristate 13-acetate (PMA) (**Figure 8**), as a PKC activator. This compound mimicked DAG without generation of this unsaturated lipid. Moreover, it was established that phorbol esters competitively act with DAG for the same binding site and activate PKC in a similar manner[77]. These findings opened the way to the research of the PKC involvement in cell growth and tumour promotion. Additionally, they established the use of phorbol esters as crucial tools for the manipulation of PKC activity in cells, allowing the elucidation of a wide range of cellular processes regulated by these enzymes.



PMA

Figure 8 – Chemical structure of PMA.

1.3. FLAVONOIDS AS PROMISING ANTICANCER AGENTS

Flavonoids are naturally occurring compounds that belong to a large group of plant secondary metabolites, which can be found in all plant parts, including in many fruits and vegetables [reviewed in[77b]]. These compounds have a C_6-C_3-C_6 carbon framework, that is, two aromatic rings (A and B) linked by a three carbon unity that may form a third ring (C). This basic structure occurs in a variety of structural forms, being subdivided into different classes, including flavanones, flavones, flavonols, isoflavones, dihidroflavonols, chalcones, dihidrochalcones, flavans, flavan-3-ol, isoflavones, and anthocyanidins, among others (**Figure 9**) [reviewed in[78]].

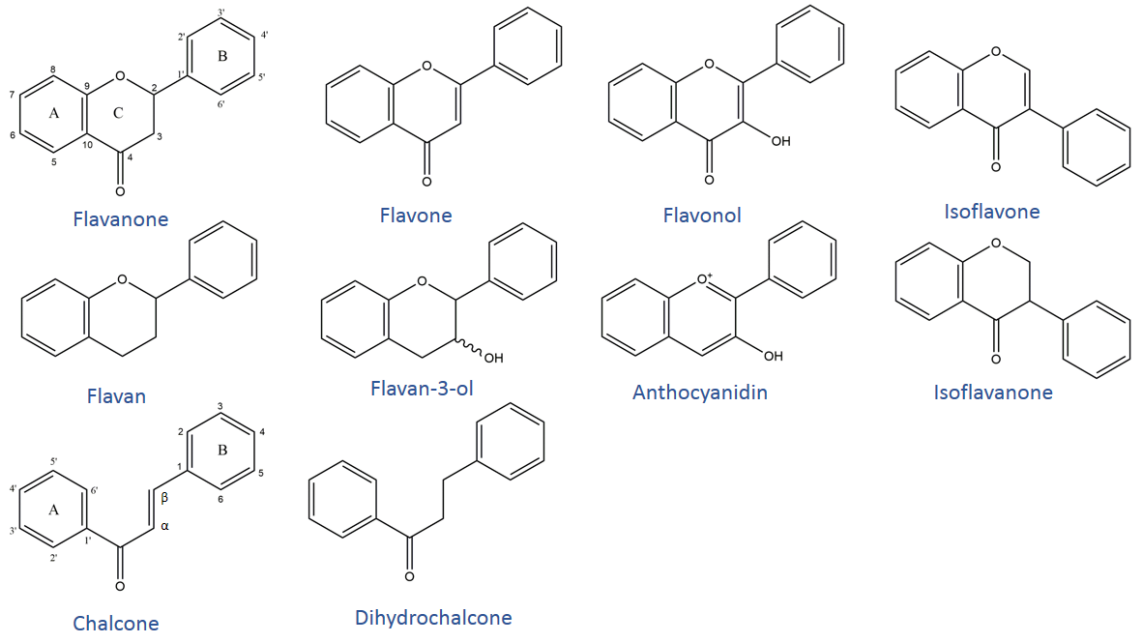


Figure 9 - Basic skeleton and numbering pattern of some flavonoid classes.

It is well-known that flavonoids and their synthetic analogues have therapeutic potential as anticancer agents, inhibiting various stages in the carcinogenesis process (**Figure 10**) [reviewed in[78]].

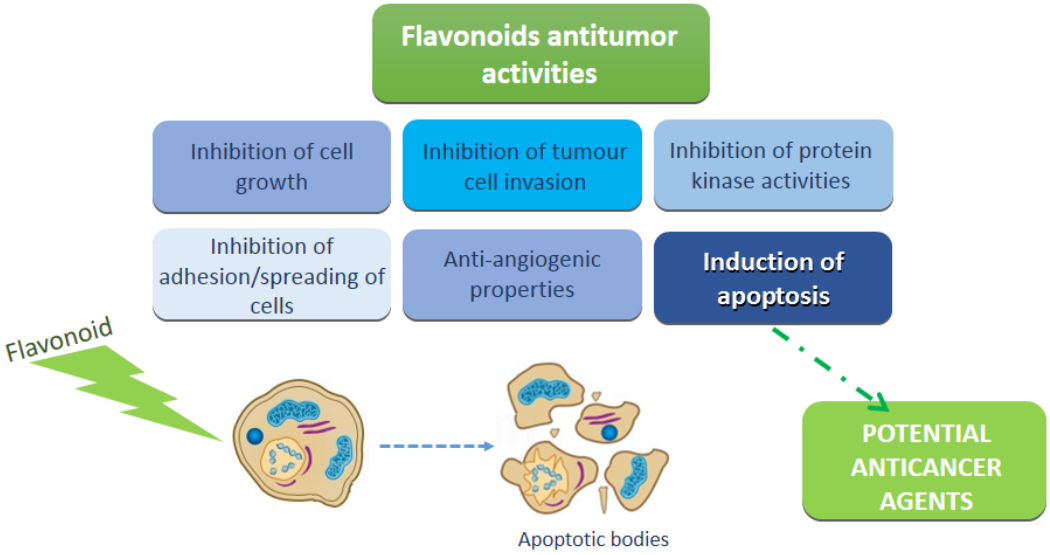
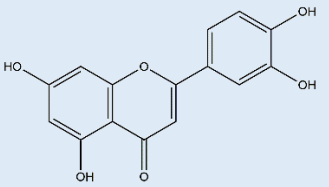
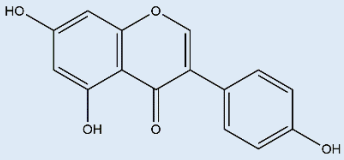
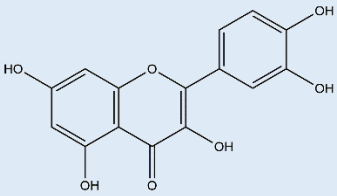
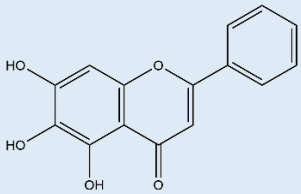
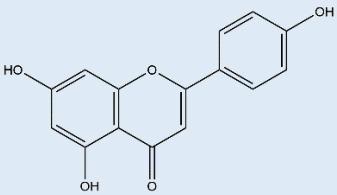


Figure 10 - Flavonoids antitumor activities.

In fact, these natural compounds are able to inhibit the tumour cell growth, an event associated with apoptosis induction (**Table 2**)[79]. However, in most of the tumour cells the molecular mechanism by which such compounds can induce apoptosis have not yet been clarified. Despite that, several mechanisms have been proposed, for example, decrease of ROS, release of cyt *c* with a subsequent activation of caspases-9 and -3, downregulation of Bcl-2 and Bcl-X(L) expression, and stimulation of Bax and Bak pro-apoptotic protein expression, among others [reviewed in[80]]. Another possible mechanism for the potential antitumor effects of flavonoids is their ability to inhibit various PKCs, thereby inhibiting signal transduction events of cell proliferation. In fact, some flavones and flavonols have shown to inhibit efficiently PKCs [reviewed in[77b]].

The potency of flavonoids for inducing apoptosis may be dependent on the number of hydroxyl groups in the 2-phenyl ring and on the absence of the 3-hydroxyl group [reviewed in[81]]. Actually, it is noted that the hydroxylation pattern of the B ring of the flavones and flavonols, such as luteolin and quercetin, seems to be critical for their activities, especially to the inhibition of the activity of protein kinases [reviewed in[78a]].

Table 2 – Flavonoids with antitumor activity in distinct human tumour cells.

Flavonoid	Antitumor activity	Human tumour cells	References
 **Luteolin**	Inhibits proliferation; Induces G2/M-phase cell cycle arrest; Induces Apoptosis.	Human esophageal squamous adenocarcinoma KYSE-510 cells; lung carcinoma CH27 cells and human colon cancer HT-29 cells.	82
 **Genistein**	Induces G2/M-phase cell cycle arrest; Induces apoptosis.	----	83
 **Quercetin**	Induces G2/M-phase cell cycle arrest; Induces apoptosis trough a p53-dependent mechanism and trough the release of cyt *c*.	Human leukaemia HL-60 cells.	84
 **Baicalein**	Induces apoptosis	Human colorectal adenocarcinoma HT-29 cells and human epithelial colorectal adenocarcinoma Caco-2 cells.	84b, 85
 **Apigenin**	Induces apoptosis	Human colorectal adenocarcinoma HT-29 cells; human epithelial colorectal adenocarcinoma Caco-2 cells and human leukaemia HL-60 cells.	84b, 86

In conclusion, flavonoids interact with a wide range of molecular targets involved in apoptosis and cell proliferation pathways by affecting their expression or activity. Accordingly, it is reasonable to consider this scaffold as a valuable basis for the development of new and more potent pro-apoptotic agents that could be relevant for cancer treatment [reviewed in[87]].

1.4. YEAST AS A MODEL SYSTEM

1.4.1. YEAST AS A MODEL TO STUDY HUMAN PROTEINS

In the last few years, the budding yeast *Saccharomyces cerevisiae* has provided a major contribution on the understanding of the pathobiology of some human diseases. In fact, the high degree of conservation of cellular and molecular processes between the yeast *S. cerevisiae* and higher eukaryotes have made this microorganism a valuable model system for numerous studies on devastating human disorders such as cancer [reviewed in[60, 76, 88]]. Easy manipulation, short generation time, a well-defined genetic system and a high amenability to genetic modifications represent advantages of yeast cells over other cell models. However, as a unicellular organism, the limitation of this cell system for the study of human diseases concerns in the analysis of disease aspects that rely on multicellularity and cell-cell interactions. In fact, some disease-associated genes may not be present in yeast [reviewed in[60, 89]]. In spite of this limitation, yeast has provided a valuable contribution in the discovery of mechanistic processes involved in the cancer disease. For instance, with the finding that yeast can undergo an apoptotic cell death exhibiting phenotypic features and basic molecular machinery similar to those found in higher eukaryotes, the mechanisms of apoptosis could also be intensively addressed in yeast and the knowledge obtained transposed to human cells, providing clues towards an understanding of apoptosis-related diseases, such as cancer [reviewed in[90]]. In fact, the yeast model system has been considered a useful tool to study cancer-related proteins. If a gene encoding a protein is conserved in yeast, it is possible to directly study its function in this organism. On the other hand, if the gene has no orthologues in yeast, its heterologous expression in this cell model can still be highly informative because yeast may conserve protein interactions that may give crucial clues to the gene function. In fact, the absence of orthologues of a protein or an entire pathway can sometimes be an advantage because the protein can be independently studied in a simpler eukaryotic system, without the interference of other proteins that can share similar or overlapping functions [reviewed in[60]].

Based on the exposed above, in the last few years, the yeast cell model has been used not only to study the biology of important molecular processes, but also to study the pharmacology of proteins involved in cancer.

1.4.2. YEAST AS A MODEL FOR TARGETED SCREENING ASSAYS

The simplicity of yeast genetics has made this cell model a promising system for identifying and testing target-specific drug therapies. Indeed, due to several advantages over cell-free and human cell-based assays (**Table 3**), yeast has emerged as a valuable tool for genetic and chemical large-scale screenings [reviewed in[89a, 91]].

Table 3 – Advantages and drawbacks of yeast targeted screening assays over other widely used assays. Adapted from[76].

Yeast target directed screening assays			
Advantages		Drawbacks	Remarks
Over cell-free assays	**Over human cell based assays**	Are not the most physiologically relevant model system; Tests in mammalian are crucial to validate the pharmacological relevance of the targets identified.	Cell wall does not limit compound permeability; Yeast pleiotropic drug resistance exporter proteins are structurally similar to the mammalian multiple drug resistance efflux pumps.
Purification of the target is not required; Target proteins are examined in a cellular environment; Selection against compounds that are generally cytotoxic, instable in a cell culture or that cannot permeate plasma membrane.	A clean read-out in a null background environment for the expression of the human protein; Less expensive to culture; Faster growth in defined media; Easier to propagate in HTS automated systems; Multitude of genetic tools and cellular selection systems readily converted to HTS formats.		

In the last years, humanized yeast cells have emerged as a useful model in the first-line drug screening, particularly in targeted screenings where the activity of a defined human protein is measured. Indeed, yeast can contribute to drug discovery through the identification of compounds that cause a desired physiological change through modulation of a specific protein [reviewed in[76]].

Most of the human proteins involved in cancer (e.g. caspases) are toxic when overexpressed in yeast, since are able to interfere with the growth-regulatory pathways of this microorganism. Based on this, the restoration of the yeast cell growth is the basis of several chemical and genetic screenings for inhibitors of human target proteins. In the same way, the increase of the growth arrest induced by the foreign protein is the basis of several screenings for activators of human target proteins[92]. Indeed, when used a yeast model in the first step of the drug discovery process, coupled with other complementary approaches in mammalian systems, it can have an important impact in the discovery of new therapeutic opportunities [reviewed in[93]].

The yeast targeted screening assays are developed based on the use of the gene overexpression technique. In the last years, genome-wide yeast overexpression libraries have been created, taking advantage of the high amenability of this microorganism to genetic alterations. In fact, due to this organism be easily transformed by extrachromosomal plasmids, the selection of transformed cells is quite straightforward, and there are several regulatable promoters available that allow a considerable control over gene expression [reviewed in[76]]. The gene overexpression technique consists on the use of a high-copy library, in which a cDNA from a library of human cDNAs, is expressed under a regulatable promoter, inserted in yeast expression vectors (**Figure 11**) [reviewed in[76]]. These overexpression libraries have been applied to studies concerning human disease-associated proteins that induce a screenable phenotype, like growth arrest, such as caspases [reviewed in[76]].

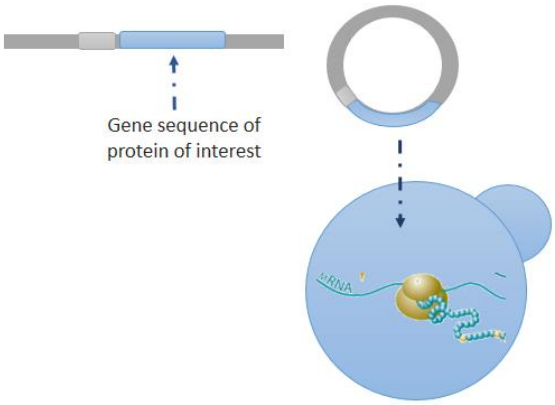


Figure 11 – Gene overexpression technique. The insert of a gene encoding a protein of interest in yeast results in an engineered yeast cell, which can be used to study the biology and the pharmacology of proteins that play a central role in human disorders.

1.4.3. HUMAN PROTEINS STUDIED IN YEAST

Based on the exposed above, during the last years, our research group has been focused on the development of yeast targeted screening assays to search for modulators of the activity of distinct cancer-related human proteins, such as caspases, PKC family proteins, among others [reviewed in[56, 60, 76, 94]].

1.4.3.1. CASPASE FAMILY PROTEINS

Based on the high complexity of caspases-signalling pathways, the yeast cell mode has been used as a simpler cell system for an independent analysis of human caspase family members in order to identify specific regulators and substrates [reviewed in[76]]. Indeed, several yeast assays have recently been developed for the HTS of modulators of human caspases, using either chemical or cDNA libraries. The exploitation of these assays to screen for activators and inhibitors of individual caspase family members may help in the discovery of new therapeutic agents against cancer [reviewed in[60]].

Yeast encodes a metacaspase (Yca1p)[95]. Metacaspases are found only in eukaryotes that are devoid of caspases, such as yeast. In fact, Yca1p shares structural homology and mechanical characteristics with mammalian caspases. However, metacaspases have a different cleavage specificity than mammalian caspases[96]. Although the controversy, recent studies have demonstrated that the activity of human caspases in yeast is independent of Yca1p[97].

The caspase activity in yeast has been frequently associated with the induction of a cell death phenotype. However, it was found that the phenotypic consequences of the expressed human caspase in yeast were highly dependent on the yeast strain used, probably a result of differences in the expression levels of endogenous caspase substrates or of other proteases with similar functions among the yeast strains [reviewed in[76]].

In fact, several aspects of the regulation of mammalian caspases could be recapitulated in yeast. For instance, it was reported in 2000 that IAPs in mammals, namely XIAP, suppressed the growth defect of *S. cerevisiae* expressing active human caspase-3[98].

Meanwhile, while the majority of mammalian caspases are auto-activated in yeast when expressed at high levels, downstream caspases, as caspases-3, -6 and -7, and the initiator caspase-9 cannot undergo auto-activation. In fact, these caspases require for their activation mammalian upstream proteins, such as initiator caspases or the Apaf-1, which are absent in yeast [reviewed in[18, 23]]. Indeed, this has been bypassed using engineered autoactivated variants that allow auto-activation of these caspases even in the absence of their upstream activators (**Figure 12**).

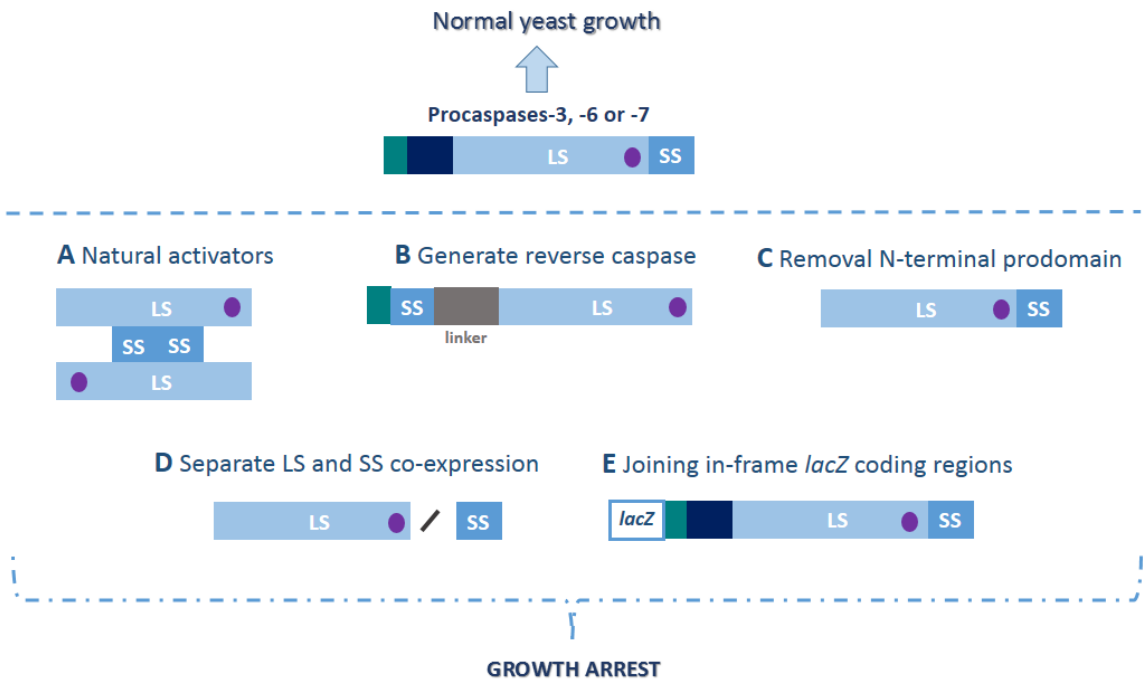


Figure 12 - Different strategies of human caspases-3, -6 or -7 activation in *S. cerevisiae.* (**A**) Natural caspase activators; (**B**) Generation of reverse caspase, in which the small subunit precedes its prodomain and large subunit; (**C**) Removal of the N-terminal prodomain from the caspase coding sequence; (**D**) Separately co-expressing the large and small subunits of an active caspase; (**E**) Joining in-frame the caspase cDNA to the coding regions for *Escherichia coli* β-galactosidase (*lacZ*). Activated caspases lead to yeast growth arrest. Catalytic cysteine represented in purple. Abbreviations: LS, large subunit; SS, small subunit. Adapted from[60].

The most commonly used strategy for studying human caspases in yeast has relied on the fact that high expression levels of a caspase lead to a pronounced yeast cell growth arrest. Based on this, yeast can be used as a system to search for pharmacological modulators of this family of proteins (**Figure 13**) [reviewed in[60]].

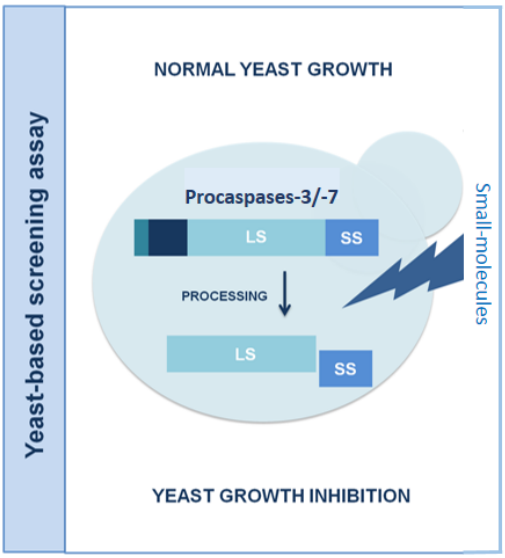


Figure 13 - The use of a yeast-based screening assay to search for modulators of procaspases-3 and -7, followed by an *in vitro* processing assay and assays in human tumour cell lines, led to the identification of activators of these proteins[99].

1.4.3.1.1. POTENTIAL PHARMACOLOGICAL MODULATORS OF CASPASES IDENTIFIED IN YEAST

In 2011, Glória and colleagues developed a yeast targeted screening assay to search for caspase-3 pharmacological activators. This assay is based on the measurement of yeast cell growth, particularly on the correlation established between the reversion of caspase-3-induced yeast growth inhibition by a caspase-3 inhibitor and the inhibition of caspase-3 activity. The use of this assay to screen for modulators of individual caspase family members may help in the discovery of new therapeutic agents, namely, against cancer. In fact, this assay led us to the discovery of new inhibitors of caspase-3 among a chemical library of vinyl sulfones[100].

More recently, the use of a yeast approach developed to search for caspase-7 modulators also led us to the identification of potential small molecule activators of caspase-7 with a flavonoid scaffold, being this activity also supported by *in vitro* processing assays and by assays in human tumour cell lines[99]. In this study, flavonoids **1a** (5,6-dihydroxy-7-prenyloxyflavone) and **2a** (3-hydroxy-7-geranyloxyflavone) (**Figure 14**) were identified as activators of caspase-7, without interfering with the activity of caspase-3. Indeed, the yeast model provided important data concerning structural requirements for the activity of flavonoids towards caspase-7[99].

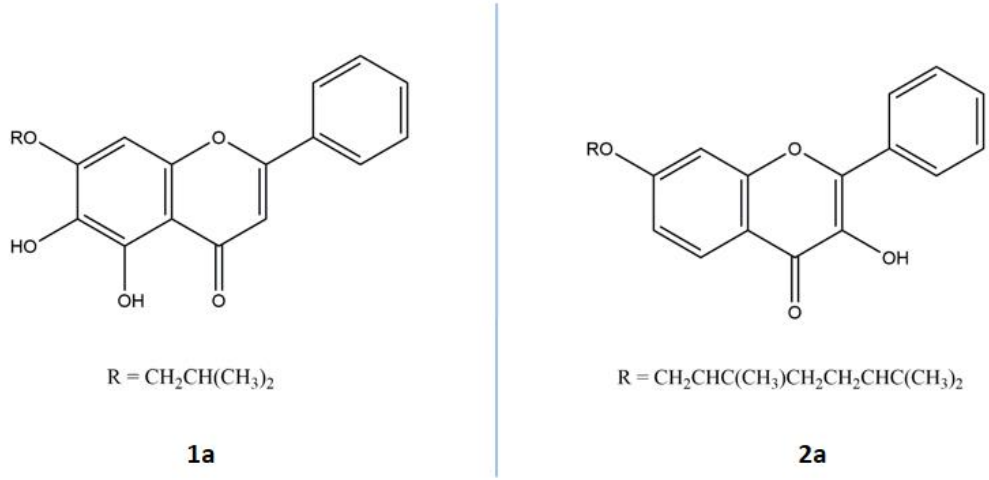


Figure 14 – Chemical structure of the flavonoids activators of caspase-7 identified using a yeast screening assay: compound **1a** and compound **2a**.

1.4.3.2. PKC FAMILY PROTEINS

In 2003 Riedel and collaborators showed, for the first time, that yeast was an efficient model system for the evaluation of the activity of specific mammalian PKC isoforms, due to the yeast Pkc1p be a structural but not a functional homologue of mammalian PKC isoforms[101]. Indeed, yeast arose as a promising cell system for an individual analysis of mammalian PKC isoforms expressed in a simpler eukaryotic cellular environment without the interference of other isoforms or endogenous regulators [reviewed in[76]].

The catalytic function of mammalian PKC isoforms expressed in yeast was confirmed by monitoring the phosphorylation of specific PKC substrates. Particularly, the ability of mammalian PKC isoforms (namely PKCα, δ and ζ) to modulate the phosphorylation state of human proteins, such as Bcl-xL[102] and p53[103] in yeast was also demonstrated.

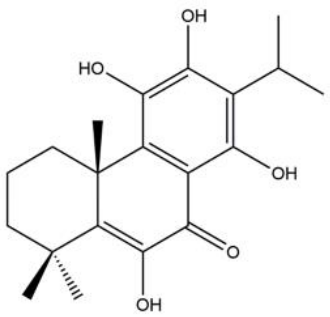
The activation of a mammalian PKC isoform in yeast induced a specific phenotype and an increase in the cell doubling time proportional to the level of its enzymatic activity. These features led to the establishment of a yeast PKC phenotypic assay [reviewed in[56]]. The use of this yeast assay as a tool for the screening of several chemical libraries has led to the identification of new potential PKC pharmacological modulators [reviewed in[56]].

In fact, in this assay the PMA-induced growth inhibition in yeast cells expressing a mammalian PKC isoform is proportional to the degree of PKC activation, whilst known PKC inhibitors, such as chelerythrine or NPC 15437, reverted the effects of PKC activators. Indeed, the use of the yeast-based PKC assay in the screening of several chemical libraries led to the identification of new potent PKC activators and inhibitors, some of them selective to a particular isoform, such as PCKδ, PKCη and PKCζ [reviewed in[56, 76]].

1.4.3.2.1. POTENTIAL PHARMACOLOGICAL MODULATORS OF PKC IDENTIFIED IN YEAST

In 2004, with yeast cells expressing PKCα, PKCβI, PKCδ, PKCη or PKCζ, the potency and selectivity of the known PKC activator, PMA and its analogues MPMA (phorbol-12-myristate-13-acetate-4-O-methyl-ether) and 4αPMA have been characterised[104]. Interestingly, MPMA and 4αPMA were identified as PKC inhibitors in contrast to their starting material[104]. In a previous work from the same group, the activity of daphnetoxin and its derivative mezerein has been compared, in yeast expressing PKCα, PKCβI, PKCδ or PKCζ, in order to investigate differences in their selectivity as PKC activators. In fact, daphnetoxin showed much lower potency to activate the PKCδ isoform and higher potency to activate PKCα, comparatively to mezerein[72]. Once again, using yeast, the molecular mechanism of action of the PKC activator euxanthone was ascertained, showing remarkable selectivity for PKCζ[105].

More recently, the activity of coleon U (6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one) (**Figure 15**), a diterpene compound with an antiproliferative effect on human tumour cells (e.g. MCF-7 and UACC62), was demonstrated on individual isoforms of the distinct PKC subfamilies, using a yeast PKC assay (**Figure 16**).



Coleon U

Figure 15 – Chemical structure of coleon U.

This study showed that coleon U induces the translocation of PKCδ and ε, in yeast expressing these proteins, to the nucleus, an effect associated with the induction of a Yca1p- and mitochondrial-dependent apoptosis. Additionally, it was also demonstrated that the PKC activator PMA was able to activate all PKCs tested except aPCKζ, while coleon U had no effect on aPKC and cPKCs. Besides, the effect of coleon U on nPKCs was higher than that of PMA. Therefore, using the yeast screening assay it was possible to determine that coleon U was a potent and selective activator of nPKCs, inducing an apoptotic yeast cell death only in yeast cells expressing PKCδ or ε isoforms (**Figure 16**)[74].

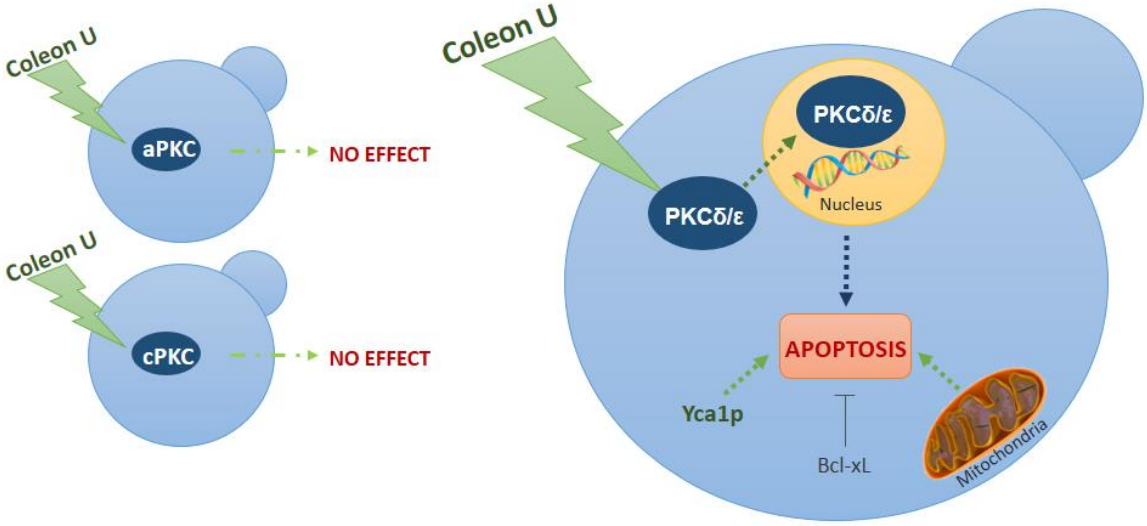


Figure 16 - Molecular mechanism of action of coleon U in yeast cells. Coleon U had no effect on yeast cells expressing aPKC and cPKCs. On the other hand, in yeast expressing PKCδ or ε, coleon U induced the translocation of these proteins to the nucleus and a Yca1p- and mitochondrial-dependent apoptosis, which was abolished by the co-expression in yeast of the human anti-apoptotic protein Bcl-xL.

Taken together, these results showed that the yeast PKC model can efficiently contribute not only to the discovery of new PKC isoform-selective modulator with promising therapeutic applications, but also to unravel the molecular mechanism of action underlying their pharmacological activities, as observed with coleon U [reviewed in[56]]. Additionally, important insights concerning the mechanisms of apoptotic function of PKC isoforms, and physiologically relevant substrates have been provided by the yeast PKC assays (**Table 4**) [reviewed in[60]].

Table 4 – Effects of mammalian PKC isoforms on distinct apoptotic stimuli and human apoptotic proteins expressed in yeast. Adapted from[60].

	cPKCα	nPKCδ	nPKCε	aPCKζ
Apoptotic stimuli				
Acetic acid	↑	↑	↑	↑
H_2O_2	*NE*	*NE*	↑	*NE*
Coleon U	*NE*	↑	↑	*NE*
Apoptotic proteins				
Bcl-xL	↓	*NE*	↑	↑
Bax	↑	-	-	-
p53	*NE*	↑	↑	*NE*

Legend: Up arrow (↑), increase of effect; Down arrow (↓), decrease of effect; *NE*, No effect; not determined.

In conclusion, the yeast PKC assays have provided valuable insights into the molecular and functional profile of individual PKC isoforms. Furthermore, potent and selective small molecule modulators of these kinases have been also identified. Indeed, the yeast screening assay represents an important contribution to the design of new therapeutic strategies against cancer through the identification of key apoptotic targets amongst the PKC family isoforms [reviewed in[60]].

1.5. SCOPE OF THIS DISSERTATION

The present dissertation in Pharmaceutical Chemistry aimed the search for new potential small molecule modulators of the pro-apoptotic proteins of the caspase and PKC family, using the yeast-based screening assay. Particularly, with this work, it was intended to identify new activators of caspases-3, -6 and -7 with a flavonoid scaffold and with improved pharmacological activities compared to those described by our group in previous work. Additionally, it was intended to analyse the *in vitro* antitumor potential of compound **C4**, previously identified in a yeast cell-based assay as a potential PKCδ isoform-selective pharmacological activator. Collectively, with this project, it was intended to provide new pharmacological alternatives to be explored as potential therapeutic strategies against cancer.

CHAPTER 2

Material and Methods

2.1. CASPASE FAMILY PROTEINS

2.1.1. CHEMISTRY

Chemicals were purchased from Sigma-Aldrich (Sintra, Portugal). Three flavonoid derivatives were investigated: 7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one (**S1**), 5,6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one (**S2**) and 5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one (**S3**). These compounds were obtained under the master's dissertation of BSc Joana Patrícia Martins Moreira. Briefly they were synthesized by the MW irradiation of Baicalein with 1.6 eq. of alkylating agent (isopentyl, propyl or allyl iodide) in the presence of anhydrous potassium carbonate. MW reactions were performed using a glassware setup for atmospheric-pressure reactions and a 100 mL Teflon reactor (internal reaction temperature measurements with a fiber-optic probe sensor), and were carried out using an Ethos MicroSYNTH 1600 Microwave Labstation from Milestone (Australia). All the reactions were monitored by thin-layer chromatography (TLC). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR (software: WinFirst, v. 2.10) spectrophotometer in KBr microplates (cm^{-1}). Purification of compounds was carried out by flash chromatography using Macherey-Nagel silica gel 60 (0.04-0.063 mm), preparative TLC using Macherey-Nagel silica gel 60 (GF254) plates, and crystallization. ^{1}H and ^{13}C NMR spectra were taken in $CDCl_3$ at r.t., on Bruker Avance 300 instruments (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) used as an internal reference; ^{13}C NMR assignments were made by 2D (HSQC and HMBC) NMR experiments (long-range ^{13}C-^{1}H coupling constants were optimized to 7 Hz).

PAC-1 and M1541 were obtained from Calbiochem (VWR, Carnaxide, Portugal). All tested compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Sintra Portugal).

2.1.1.1. GENERAL PROCEDURES FOR THE PREPARATION OF BAICALEIN DERIVATES

A mixture of baicalein (0.20 g, 0.74 mmol), isopentyl/ propyl/ allyl iodide (1.18 mmol) and anhydrous K_2CO_3 (0.55 g, 3.7 mmol) in anhydrous acetone (60 mL) was submitted to successive 30 min of microwave irradiation at 200 W of potency. The final temperature was 60 °C and the total irradiation time was 2 h, except for the reaction with isopentyl iodide, for which the reaction time was 3 h. After cooling, the solid was filtered and the solvent removed

under reduced pressure to afford the crude product. The yellow-orange solid obtained was dissolved in acetone and purified as described below.

2.1.1.1.1. 7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one (S1). Purified by flash chromatography (SiO_2; n-hexane: EtOAc). Yield: 4.2% as yellow crystals; mp 160-162 ºC; IR (kBr) υmax: 3447, 2925, 2923,2854, 1663, 1576, 1489, 1449, 1252 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ= 13.04 (OH, s, H-5), 7.90-7.87 (2H, m, H-2',6'), 7.58-7.49 (3H, m, H-3',4',5'), 6.68 (1H, s, H-3), 6.63 (1H, s, H-8), 6.18-6.05 (1H, m, H-2''), 5.51-5.45 (1H, m, H-3''a), 5.41-5.37 (1H, m, H-3''b), 4.74 (2H, d, J=5.4, H-1''); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ= 182.6 (C4), 164.1 (C2), 151.7 (C7), 150.5 (C8a), 145.8 (C5), 131.8 (C2''), 131.4 (C1'), 129.1 (C3',5'), 126.2 (C2',6'), 131.8 (C4'), 129.8 (C6), 119.1 (C3''), 106.1 (C4a), 105.4 (C3), 91.6 (C8), 70.2 (C1'').

2.1.1.1.2. 5,6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one (S2). Purified by flash chromatography (SiO_2; petroleum ether: EtOAc). Yield: 6 % as yellow crystals; mp 167-170 ºC; IR (kBr) υmax: 3441, 2927, 2922, 2851, 1617, 1559, 1489, 1473, 1459, 1458 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ= 12.50 (OH, s, H-5), 7.91-7.88 (2H, m, H-2',6'), 7.55-7.52 (3H, m, H-3',4',5'), 6.69 (1H, s, H-3), 6.61 (1H, s, H-8), 5.37 (OH, s, H-6), 4.12 (2H, t, J=5.9, H-1''), 2.05-1.98 (2H, m, H-2''), 1.10 (3H, t, J=7.4, H-3''); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ= 182.7 (C4), 164.0 (C2), 152.3 (C7), 150.7 (C8a), 145.6 (C5), 131.7 (C4'),131.6 (C1'), 129.7 (C6), 129.1 (C3',5'), 126.2 (C2',6'), 106.0 (C4a), 105.4 (C3), 91.1 (C8), 71.0 (C1''), 22.3 (C2''), 10.4 (C3'').

2.1.1.1.3. 5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one (S3). Purified by flash chromatography (SiO_2; n-hexane: EtOAc). Yield: 5.6 % as yellow crystals; mp 175-178 ºC; IR (kBr) υmax: 3440, 2955, 2921, 2854, 1657, 1489, 1477, 1450, 1115 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ= 7.91-7.88 (2H, m, H-2',6'), 7.54-7.52 (3H, m, H-3',4',5'), 6.69 (1H, s, H-3), 6.62 (1H, s, H-8), 4.18 (2H, t, J=6.6, H-1''), 1.93-1.77 (2H, m, H-2''), 1.25 (1H, m, H-3''), 1.01 (6H, d, J=6.3, H-4'',5''); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ= 182.7 (C4), 164.0 (C2), 152.3 (C7), 150.7 (C8a), 145.6 (C5), 131.5 (C1'), 126.2 (C2',6'), 129.7 (C6), 129.1 (C3',5'), 131.8 (C4'), 106.0 (C4a), 105.4 (C3), 91.0 (C8), 68.0 (C1''), 37.5 (C2''), 29.7 (C3''), 22.7 (C4''), 22.6 (C5'').

2.1.2. BIOLOGICAL ACTIVITY

2.1.2.1. YEAST TARGETED SCREENING ASSAY

Sacharomyces cerevisiae (strain CG379 Matα, *ade*5, *his*2, *leu*2-112, *trp*1-289, *ura*3-52 [*Kil-O*]) (Yeast Genetic Stock Center, University of California, USA) expressing each human procaspase-3, -6 or -7 was obtained in a previous work[99]. For expression of human proteins (routinely grown in minimal selective medium), yeast cells were diluted to 0.05 optical density at 600 nm (OD_{600}) in induction selective medium with 2% (w/w) galactose (Sigma-Aldrich, Sintra, Portugal), 1% glycerol (Sigma-Aldrich, Sintra, Portugal), 0.7% (w/w) yeast nitrogen base without amino acids from Difco (Quilaban, Sintra, Portugal), and all the amino acids required for yeast growth (50 µg/ml) except leucine, and incubated at 30 ºC, under continuous orbital shaking (200 rpm). Yeast cells were incubated at 30 ºC under continuous orbital shaking (200 rpm) with 0.1-15 µM compounds **S1**-**3**, 0.1-50 µM PAC-1, 0.1-5 µM M1541 or 0.1% DMSO only, for approximately 24 h (time required by the yeast transformed with the empty vector, control yeast, to achieve 0.3 OD_{600}). Yeast growth was analyzed by counting the number of colony-forming units (CFU) after 2 days incubation at 30ºC on Sabouraud Dextrose Agar from Liofilchem (Frilabo, Porto, Portugal). For each culture, the percentage of drug-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with yeast incubated with DMSO only.

2.1.2.2. STASTICAL ANALYSIS

Data were analysed statistically using the SigmaPlot 12.00. Differences between means were tested for significance using the Student's *t*-test. Data are mean ± SEM of six independent experiment; *p=0.01, **p=0.001.

2.2. PROTEIN KINASE C FAMILY PROTEINS

2.2.1. COMPOUNDS

Phorbol 12-myristate 13-acetate (PMA) was obtained from Enzo Life Sciences (Group Taper, Sintra, Portugal) and 7α-acetoxy-6β-hydroxyroyleanone (**C4**) was synthesized as described in[106]. All tested compounds were dissolved in DMSO (Sigma-Aldrich, Sintra Portugal).

2.2.2. HUMAN TUMOUR CELL LINES AND GROWTH CONDITIONS

Human colon adenocarcinoma HCT116 cell line was kindly provided by Dr. A. Inga (CIBIO, University of Trento, Italy). HCT116 cell line was routinely cultured in RPMI-1640 with ultraglutamine medium from Lonza (VWR, Carnaxide, Portugal) supplemented with 10% fetal bovine serum from Gibco (Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37°C with 5% CO_2.

2.2.3. SULFORHODAMINE B (SRB) ASSAY

For the analysis of the effect of **C4** on the *in vitro* growth of human HCT116 cell line, cells were incubated in 96-well plates, at a final density of 5.0×10^3 cells/well, for 24 h. Cells were thereafter treated with serial dilutions of PMA (from 1.25 to 20.0 μM) and **C4** (from 0.23 to 3.6 μM). The effect of compounds on cellular integrity was analysed following 48 h incubation, using sulforhodamine B (SRB) assay. Briefly, following fixation with 10% trichloroacetic acid, plates were stained with 0.4% SRB both from Sigma-Aldrich (Sintra, Portugal), and washed with 1% acetic acid. The bound dye was then solubilized with 10 mM Tris Base and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA). The solvent of the compound (DMSO) corresponding to the maximum concentration used in this assay (0.1%) was included as control. The concentration of PMA and **C4** that causes a 50% reduction in the net protein increase in cells during treatment (GI_{50}, growth inhibition of 50%) was thereafter determined.

2.2.4. ANALYSES OF CELL CYCLE AND APOPTOSIS

HCT116 cells were plated in 6-well plates at a final density of 1.5×10^5 cells/well. After 24 h incubation, cells were treated with the GI_{50} concentration of PMA, **C4** or DMSO only for 24 h. For cell cycle analysis, cells were thereafter fixed in ice-cold 70% ethanol and incubated at 37ºC with RNase A from Sigma-Aldrich (Sintra, Portugal) at a final

concentration of 20 µg/mL for 15 min, and further incubated with 50 µg/mL propidium iodide (PI) from Fluka (Sigma-Aldrich, Sintra, Portugal) for 30 min, followed by flow cytometric analysis. For apoptosis analysis, cells were analysed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I from BD Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions.

2.2.5. WESTERN BLOT ANALYSIS

Whole protein extracts from HCT116 human tumour cell lines were obtained as described [107]. Protein extracts were quantified using the Coomassie staining Bradford from Sigma-Aldrich (Sintra, Portugal). Proteins (40 µg) were electrophoresed using a 10% SDS–PAGE and transferred to a Whatman nitrocellulose membrane from Protan (VWR, Carnaxide, Portugal). Membranes were blocked with 5% milk and probed with a mouse monoclonal anti-PARP (C2-10) followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody. For loading control, membranes were stripped and reprobed with a mouse monoclonal anti-GAPDH (6C5). All antibodies were purchased from Santa Cruz Biotechnology (Frilabo, Porto, Portugal). The signal was detected with the ECL Amersham kit from GE Healthcare (VWR, Carnaxide, Portugal) and with the Kodak GBX developer and fixer from Sigma-Aldrich (Sintra, Portugal).

2.2.6. FLOW CYTOMETRIC DATA ACQUISITION AND ANALYSIS

For the flow cytometric analysis an Accuri™ C6 flow cytometer and the included acquisition software from BD Biosciences (Enzifarma, Porto, Portugal) were used. Cell cycle phases were identified and quantified using the FlowJo software (TreeStar Inc., Oregon, USA).

2.2.7. STATISTICAL ANALYSIS

Data were analysed statistically using the GraphPad Prism 6.0. Differences between means were tested for significance using the Student's *t*-test. Data are mean ± SEM of tree independent experiment; *p<0.05, **p<0.01.

CHAPTER 3

Results

3.1. CASPASE FAMILY PROTEINS

IDENTIFICATION OF FLAVONOIDS S1, S2 AND S3 AS POTENTIAL ACTIVATORS OF CASPASE-7 USING A YEAST- BASED SCREENING ASSAY

In a previous work from our group, a yeast targeted screening assay was developed to search for small molecule modulators of human procaspases-3 and 7. Using this yeast approach, combined with *in vitro* assays and assays in human tumour cells, the prenylated flavonoids **1a** (5,6-dihydroxy-7-prenyloxyflavone) and **2a** (3-hydroxy-7-geranyloxyflavone) (**Figure 14**) were identified as potential activators of caspase-7[99].

In the present dissertation, this yeast screening approach was used to analyse the effect on the activity of human procaspases-3 and -7, of several baicalein derivatives [**S1** (7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one), **S2** (6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one) and **S3** (5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one)] (**Figure 17**), which are analogues of compounds **1a** and **2a**.

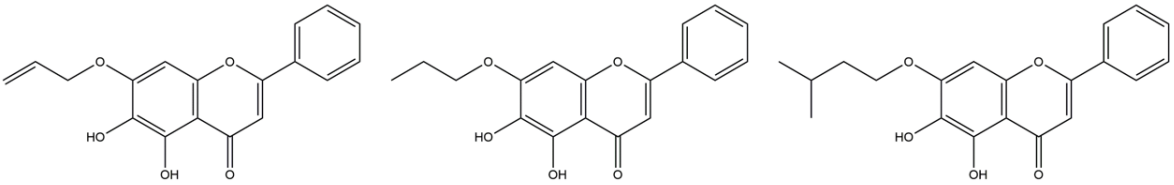


Figure 17 – Chemical structure of compounds **S1, S2** and **S3**.

The results obtained demonstrated that compounds **S1-3** induced growth inhibition in yeast cells expressing procaspase-7 at 1-15µM (**Figure 18A**), without interfering with the growth of control yeast (data not shown). Interestingly, for the same concentration range, the effect of these compounds was less pronounced in yeast cells expressing procaspase-3 (**Figure 18B**).

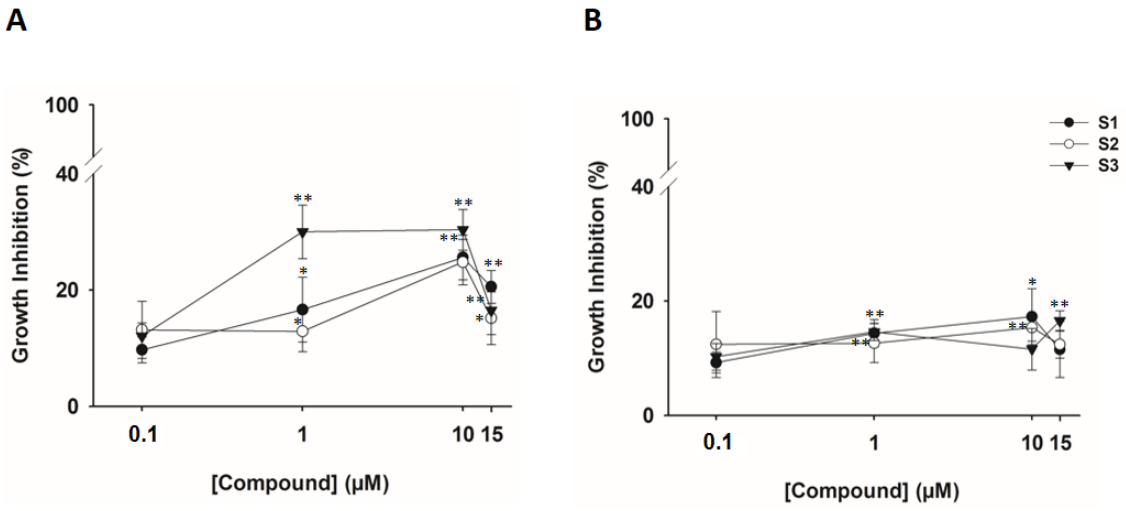


Figure 18 – **Effect of compounds S1-S3 on the growth of yeast cells expressing procaspases-3 or -7.** Concentration-response curves for the effects of flavonoids **S1-S3** on the growth of yeast cells expressing human (**A**) procaspase-7 or (**B**) procaspase-3, for 24 h treatment. The percentage of drug-induced growth inhibition was estimated considered 100% growth the number of CFU obtained with DMSO only. Data are mean ± SEM of six independent experiments; values significantly different from DMSO are indicated (**p*=0.01, ***p*=0.001; unpaired).

In addition, the GI_{25} values obtained from the concentration-response curves for the effects of 0.1-15 µM compounds **S1-S3** and 0.1-50 µM PAC-1 (a known activator of procaspases-3 and -7[33]) (data not shown), in yeast cells expressing procaspases-3 or -7, revealed that compounds **S1** and **S3** were significantly more potent than the positive control PAC-1, in yeast expressing procaspase-7 (**Table 5**). Despite the low GI_{25} value of compound **S2** compared to PAC-1, in yeast expressing procaspase-7, the difference between both compounds is not statistically significant. More experiments must be performed in a future work to better clarify the potency of compound **S2**.

Table 5 - GI_{25} values obtained for PAC-1 and flavonoids **S1**, **S2** and **S3**.

	GI_{25} (µM)	
	Procaspase-3	**Procaspase-7**
PAC-1	16.6 ± 2.87	24.5 ± 4.82
S1	*ND*	9.3 ± 2.23*
S2	*ND*	12.5 ± 4.91
S3	*ND*	1.2 ± 2.26**

GI_{25} values correspond to the concentration that caused 25% growth inhibition of yeast cells expressing procaspases-3 or -7 after treatment with 0.1-15 µM flavonoids **S1**-**S3**, and 0.1-50 µM PAC-1 (positive control). Data are mean ± SEM of six independent experiments; values significantly different from PAC-1 are indicated (**p*=0.01, ***p*=0.001; unpaired). *ND* – Not determined.

Interestingly, based on the GI_{25} values, in opposition to PAC-1, that showed to have activity in yeast cells expressing procaspases-3 or -7, compounds **S1-S3** appear to be selective for procaspase-7 (**Table 5**).

A similar yeast phenotypic assay was developed, by our group, for procaspase-6. The suitability of this yeast approach for the screening of activators of human procaspase-6 was validated, in the present dissertation, by testing the effect of a known activator of procaspase-6 in yeast cells, M1541[50]. As expected, for 1 and 5 µM, M1541 induced growth inhibition in yeast cells expressing procaspase-6 (**Figure 19A**), without interfere with the growth of control yeast (data not shown). These results demonstrated that as in human cells, M1541 was also able to activate procaspase-6 in yeast.

This yeast screening approach was thereafter used to analyse the effect of compounds **S2** and **S3** on procaspase-6 activity. The results obtained showed that, for the concentration range tested (0.1-15 µM), both compounds did not interfere with the growth of yeast cells expressing procaspase-6 (**Figure 19B**). These results suggest that flavonoids **S2** and **S3** were not activators of procaspase-6.

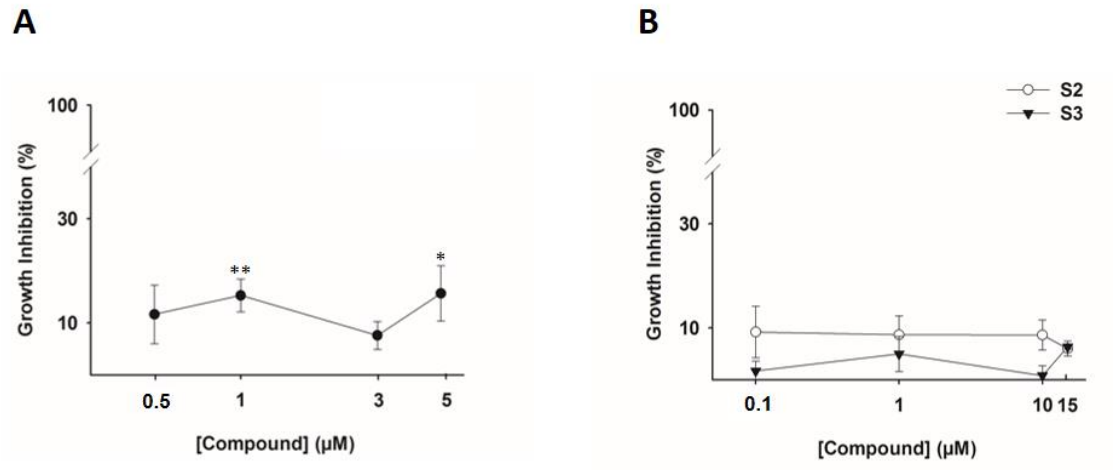


Figure 19 - **Effect of compounds M1541, S2 and S3 on the growth of yeast cells expressing procaspase-6.** Concentration-response curves for the effects of the (**A**) positive control M1541 and (**B**) flavonoids **S2** and **S3** on the growth of yeast cells expressing human procaspase-6 for 24 h treatment. The percentage of drug-induced growth inhibition was estimated considered 100% growth the number of CFU obtained with DMSO only. Data are mean ± SEM of six independent experiments; values significantly different from DMSO are indicated (*p=0.01, **p=0.001; unpaired).

3.2. PKC FAMILY PROTEINS

In a previous work from our group a yeast targeted screening assay was developed to search for modulators of PKC family proteins[74]. Using this approach a diterpene compound (coleon U) was identified as a potent and selective activator of the novel PKC δ and ε isoforms[74]. In this way, this yeast screening approach was used to analyse the effect of a coleon U derivative (compound **C4**) on the activity of several PKC isoforms. The results obtained in the yeast model indicated that **C4** was a potent and selective activator of PKC δ (unpublished results). Based on this finding, in the present dissertation, several assays were performing in human tumour cell lines in order to analyse the *in vitro* antitumor potential of **C4**.

C4 INDUCES A CYTOTOXIC EFFECT ASSOCIATED WITH APOPTOSIS IN HUMAN TUMOUR CELLS

The tumour cell growth-inhibitory potential of **C4** was ascertained using the human colon adenocarcinoma HCT116 cell lines (**Figure 20**). The GI_{50} value obtained for **C4** of 0.63 µM, in HCT116 cells, after 48 h treatment, showed that **C4** has a potent growth inhibitory effect in human tumour cells. Additionally, the results obtained in HCT116 cells showed that **C4** was significantly more potent than the known activator of classical and novel PKCs (used as positive control[77]), PMA with a GI_{50} value of 6.5 µM (*p<0.05) (**Figure 20A**).

Moreover, it was verified that the growth-inhibitory effect of compound **C4**, at the GI_{50} concentration, was not associated with cell cycle arrest (**Figure 20B**). Whereas, the growth-inhibitory effect of PMA, at the GI_{50} concentration, demonstrated to be associated with the induction of S-phase cell cycle arrest (**Figure 20B**). It was also observed that the growth-inhibitory effects of both PMA and **C4**, at the GI_{50} concentrations, were associated with the induction of late apoptosis (**Figure 20C**). By western blot analysis, it was verified that the growth-inhibitory effect induced by **C4** led to the induction o PARP cleavage, a product of caspase-3 activation, in HCT116 tumour cells (**Figure 20D**).

Altogether, these results showed that **C4** has a potent *in vitro* tumour growth-inhibitory activity associated with the induction of apoptosis.

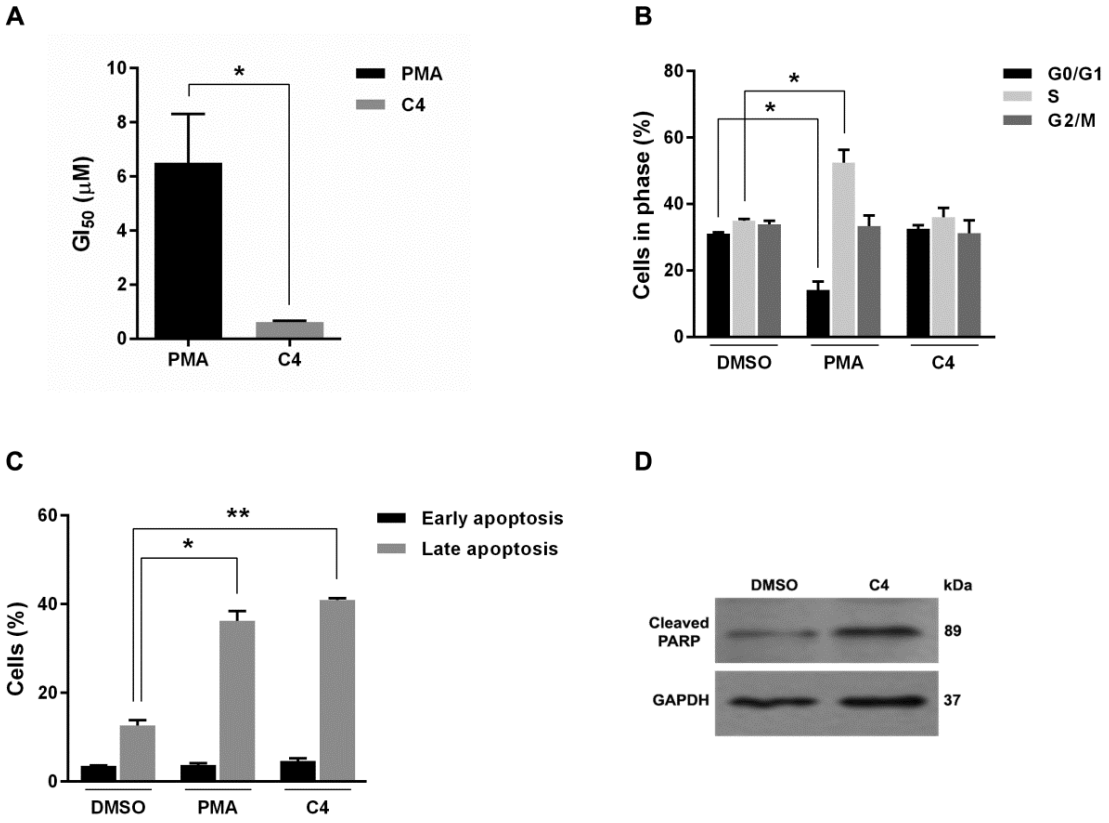


Figure 20 – C4 induces a potent growth-inhibitory effect associated with apoptosis induction, in human colon adenocarcinoma HCT116 tumour cells. (**A**) The GI_{50} concentration of PMA (positive control) and **C4** was determined in HCT116 cells, after 48 h treatment, using the SRB assay. Data are mean ± S.E.M. of three independent experiments; values significantly different from PMA (**p*<0.05) are indicated. (**B**) 0.63 μM **C4** does not induce cell cycle arrest in HCT116 cells, after 24 h treatment. Cell cycle phases were analyzed by flow cytometry using PI staining; data are mean ± S.E.M. of three independent experiments. (**C**) 0.63 μM **C4** induces late apoptosis in HCT116 cells, after 24 h treatment. Apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI; data are mean ± S.E.M. of three independent experiments. (**D**) 0.63 μM **C4** leads to PARP cleavage in HCT116 cells, after 24 h treatments. Immunoblots represent one of three independent experiments; GAPDH was used as loading control. In **B** and **C**, values significantly different from DMSO only: **p*<0.05, ***p*<0.01.

CHAPTER 4

Discussion

Conclusion and Future Prospects

Cancer is a major public health problem worldwide and the number of new cases is expected to rise significantly in the next decades. An additional concern is the increase of the resistance to the chemotherapy associated with many cancers.

Apoptosis is a crucial cellular process for the maintenance of tissue homeostasis in multicellular organisms. In fact, the mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. The deregulation of this process has been implicated in carcinogenesis [reviewed in[4]]. In this way, in the last years, the search for new anticancer therapies that selectively modulate proteins involved in the apoptotic process has been the focus of an intense research in order to overcome the resistance and to trigger tumour cells into apoptosis.

The yeast cell model has emerged as a powerful tool to study the biology and pharmacology of cancer-related proteins. In fact, yeast-based screening assays have already proven to be widely effective in the first-line screening of genetic and chemical libraries, leading to the successful identification of promising pharmacological modulators of human proteins involved in apoptosis, such as caspases and PKCs [reviewed in[56, 60, 76]].

Hence, with the present dissertation it was intended to use the yeast cell system to discover new small molecule modulators of pro-apoptotic proteins, with improved pharmacological properties. In fact, in the present work, promising modulators of caspases activity were identified. Additionally, the *in vitro* antitumor potential of a selective activator of PKCδ identified in yeast was confirmed.

4.1. IDENTIFICATION OF FLAVONOIDS S2 AND S3 AS POTENTIAL SELECTIVE ACTIVATORS OF CASPASE-7

At the core of the execution phase of apoptosis are the executioner caspases-3, -6 and -7. Like most proteases, these caspases are stored as procaspases that once activated, cleave a large set of substrates, ultimately resulting in the characteristic morphological and biochemical hallmarks of apoptosis. The reduced expression of pro-apoptotic caspases has been reported in manifold cancers and specific inactivating mutations in many tumour types and stages of malignant transformation [reviewed in[21]]. Indeed, the search for activators of these proteins can be considered as a novel strategy in cancer therapy by selective induction of apoptosis in cancer cells [reviewed in[21, 108]]. Despite that, to date, only few small molecule caspase activators have been reported. In fact, it has been shown that some of them have low antitumor activity and high toxicity[49a]. Therefore, these considerations led to intensive efforts to discover new executioner procaspase activators.

Since flavonoids and their synthetic analogues have revealed therapeutic potential as anticancer agents, inhibiting distinct stages of the carcinogenesis process [reviewed in[78a]], it is reasonable to consider this scaffold as a valuable tool for the development of new and more potent pro-apoptotic agents that could be helpful in the therapy against cancer. In fact, in several human tumour cells, it was demonstrated that the flavonoid baicalein induced a cell growth inhibitory effect associated with apoptotic cell death through a caspase-dependent manner[109]. Additionally, in a previous work from our group, two new potential small molecule activators of caspase-7, without interfering with the activity of caspase-3, were identified: the prenylated flavonoids **1a** (5,6-dihydroxy-7-prenyloxyflavone) and **2a** (3-hydroxy-7-geranyloxyflavone). These compounds were the first identified caspase activators with a flavonoid scaffold. In fact, these flavonoids showed to be potent growth inhibitors of the breast cancer (MCF-7) and leukaemia (HL-60) human tumour cells[99], by an increase of caspase-7 activity and procaspase-7 cleavage. This data demonstrated that prenylated flavonoids would be a promising scaffold to search for activators of executioner caspases key elements of the apoptotic process. Based on these previous results, derivatives of compounds **1a** and **2a** were synthesised [**S1** (7-(allyloxy)-5,6-dihydroxy-2-phenyl-4H-chromen-4-one), **S2** (6-dihydroxy-2-phenyl-7-propoxy-4H-chromen-4-one) and **S3** (5,6-dihydroxy-7-(isopentyloxy)-2-phenyl-4H-chromen-4-one) (**Figure 17**)], and their activity as activators of caspases-3, -6 and -7 was evaluated using the previous developed yeast-based screening assay[99].

With this approach, the flavonoids **S2** and **S3**, were identified as potential selective activators of procaspase-7. In fact, compounds **S2** and **S3** showed to be much less potent on procaspases-3 and -6 when compared to procaspase-7. Further assays are underway in order to analyse the impact of compound **S1** on the activity of procaspase-6 expressed in yeast. Despite this, as **S2** and **S3**, compound **S1** showed to be ineffective on procaspase-3 and exhibited a similar potency to **S2** as activator of procaspase-7.

Additionally, the results revealed that **S1** and **S3** were significantly more potent activators of procaspase-7 than the positive control PAC-1. In addition, in opposition to PAC-1 that activates both procaspase-3 and -7, **S1**, **S2** and **S3** appear to be selective for procaspase-7.

Together, with the present dissertation three potential activators of caspase-7 (**S1**, **S2** and **S3**) were identified. From these three small molecules, **S3** may be selected to be further explored in human tumour cells, since reveals to be a selective and potent activator of procaspase-7. Since **S3** have an isopentyl substituent, in a future work it would be interested to test if this substituent is a structural requirement for the selectivity of these compounds for procaspase-7. Promising pharmacological applications can be therefore

envisaged for these compounds, not only as probes in the study of caspases pathway, but also as anticancer agents. In fact, these flavonoids may represent an alternative strategy for personalized cancer treatment, namely against tumours without caspase-3 (e.g., breast adenocarcinoma) or with high levels of caspase-7 (e.g., prostate cancer).

4.2. COMPOUND C4 HAS A POTENT *IN VITRO* TUMOUR GROWTH INHIBITORY EFFECT ASSOCIATED WITH APOPTOSIS INDUCTION

PKC is a family of serine/threonine kinases that exert an important role in the carcinogenesis process. This family is composed by several isoforms considered crucial regulators of cell death and proliferation [reviewed in[54b]]. Once individual PKC isoforms can exert either similar or opposite effects in cell proliferation and death, an individual modulation of PKC isoforms effects is highly required. However, the current PKC modulators that have been currently described are relatively non-specific. Thus, the identification of PKC isoform-selective modulators has deserved great attention in drug discovery.

Particularly, the PKCδ been implicated in cell death, in the regulation of both intrinsic and extrinsic apoptotic pathways [reviewed in[64]]. The loss of PKCδ expression has been reported in some cancer types[69-71]. As such, the search for selective activators of this isoform is an appealing strategy in cancer therapy.

Due to the high complexity of the mammalian PKC family, particularly the coexistence of several PKC isoforms in the same cell, yeast-based assays were previous developed by our group to study the biology and pharmacology of each isoform of the PKC family [reviewed in[56, 60, 76]]. In this way, using the previous developed yeast target directed screening assay to search for modulators of PKC isoforms[74] **C4** was identified as a promising potent and selective activator of PKCδ. Based on this, in the present dissertation the *in vitro* antitumor potential of **C4** was addressed in the human colon adenocarcinoma tumour cells HCT116. Compound **C4** was able to decrease the proliferation of HCT116 tumour cells, being more potent than the known activator of the PKC family, PMA. In fact, contrary to PMA, the antiproliferative effect of **C4**, in tumour cells, was not associated with a cytostatic effect (cell cycle arrest), but instead with the induction of apoptosis only.

Altogether, compound **C4** shows to be a promising compound to be further explore as potential anticancer agent.

4.3. FUTURE PROSPECTS

The results obtained in this dissertation may open the way to further works. Particularly, it would be interesting to:

i) Validate the molecular mechanism of action, in human tumour cells, of compound **S3**;

ii) Analyse the activity of the alkylated flavonoid **S1** on yeast cells expressing procaspase-6;

iii) Evaluate the activity of new synthesized derivatives of the alkylated flavonoids **S1**, **S2** and **S3** on procaspases-3, -6 and -7 activities, particularly of derivatives without the isopentyl substituent;

iv) Validate the molecular mechanism of action of compound **C4** in human tumour cells, and evaluate it antitumor properties *in vivo*.

CHAPTER 5

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CHAPTER 6

Attachments

Poster Communication in XX Encontro LusoGalego de Química

Porto, Portugal, November 26-28 2014

Discovery of new modulators of caspase family proteins using the yeast approach

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Accumulating evidence has shown the crucial role of the caspase family in both initiation and regulation of apoptosis [1]. Caspases-3 and -7 are at the core of the execution phase of apoptosis. Like most proteases, these “executioner” caspases are stored as procaspases that once activated by proteolysis cleave a large set of substrates, ultimately resulting in the characteristic morphological and biochemical hallmarks of apoptosis. Therefore, the search for activators of these procaspases has deserved particular attention in the field of anticancer drug discovery [2,3]. Nevertheless, to date, only few small-molecule caspase activators have been reported [2, 4]. A previous study from our group have reported the identification of two potential activators of caspase: 7, 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) by using a yeast target-based screening assays, based on the heterologous expression of these human proteins in *Saccharomyces cerevisiae* [5]. These results led us to the present project under the thesis for Masters in Pharmaceutical Chemistry aiming to discover new caspases -3 and 7 activators. In the present work, several derivatives of compounds **1a** and **2a** will be synthesised and the activity as activators of caspases-3 and -7 will be evaluated using the yeast approach followed by validation of the molecular mechanism of action in human tumor cells. With this work it is intended to identify new activators of caspases-3 and -7 with improved pharmacological activities.

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XX ENCONTRO LUSO-GALEGO DE QUÍMICA

Faculdade de Farmácia UP
26 a 28 de novembro de **2014**

DISCOVERY OF NEW MODULATORS OF CASPASE FAMILY PROTEINS USING THE YEAST APPROACH

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INTRODUCTION

Cancer is among the leading cause of death in developed countries [1]. One of the hallmarks of cancer is the resistance to apoptosis [2]. It became evident that the cellular and biochemical features of apoptosis are a consequence of the cleavage of a subset of proteins by proteases of the caspase family. At the core of the execution phase of apoptosis are the effective procaspases-3, -6 and -7 [3]. Activation of these effective procaspases is considered a promising strategy in cancer therapy by a selective induction of apoptosis in cancer cells [4] (**Figure 1**). Thus, the discovery of small-molecule activators of these caspases has deserved particular relevance in anticancer research [5]. In spite of this, to date, a limited number of small-molecule activators of procaspases are known, such as the procaspase activating compound-1 (PAC-1) that activates caspase-3 and -7 [6], the compound "1541" that activates procaspases-3 and -6, and its analogue 1541B that activates procaspase-3 [7].

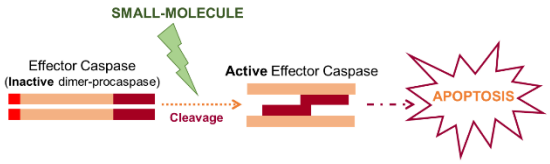
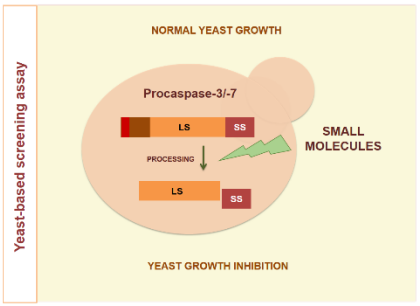


Figure 1 – Activation of effector procaspases by small-molecules leads to apoptosis.

PREVIOUS WORK

In the last years, yeast has emerged as a valuable tool for genetic and chemical large-scale screenings [8]. Based on this, recently, our group used the yeast model expressing individual human procaspases-3 or -7 for the development of a screening cell system to search for activators of these procaspases (**Figure 2**). Using this approach, two potential activators of procaspase-7, 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) (**Figure 3**), were identified [9]. The activity of these compounds was further confirmed in *in vitro* processing assays, using recombinant human procaspases, and in human tumor cell lines (**Figure 2**). With this work, a proof of concept was provided for the effectiveness of the yeast assays in the discovery of executioner procaspase activators. Additionally, the identified compounds paved the way for a new class of executioner procaspase activators with potential anticancer properties [9].



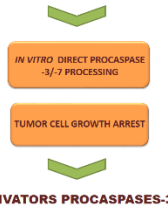


Figure 2 – Yeast model as a screening cell system to search for activators of effector procaspases.

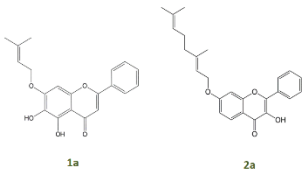


Figure 3 - Activators of procaspase-7 with a flavonoid scaffold, 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**).

Table 1 -Maximal increase in yeast growth inhibition induced by PAC-1 and flavonoids 1a and 2a.

	Drug-induced growth inhibition (%)	
	Procaspase-7	**Procaspase-3**
PAC-1	33.3 ± 6.0*	32.0 ± 6.1*
1a	20.8 ± 6.5*	9.0 ± 0.4
2a	26.2 ± 5.4*	-8.3 ± 8.2

Values correspond to the maximal increase achieved at 0.1 - 50 μM compounds. The percentage of drug-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with yeast incubated with DMSO only. Values are mean ± SEM of six independent experiments; values significantly higher than control yeast, *P< 0.05.

FUTURE WORK

These previous results led us to the present project aiming to discover new procaspases-3 and 7 activators. In the present work, several derivatives of flavonoids **1a** and **2a** will be synthesized and the activity as activators of procaspases-3 and -7 will be evaluated using the yeast approach followed by validation of the molecular mechanism of action in *in vitro* processing assays and in human tumor cells (**Figure 2**).

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This work is funded through national funds from FCT – Fundação para a Ciência e a Tecnologia under the project projects REQUIMTE-Pest-C/EQB/LA0006/2013 and CEQUIMED—PEst-OE/SAU/UI4040/2014.

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Poster Communication in

8.ª edição do Encontro de Investigação Jovem da Universidade do Porto

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Discovery of new modulators of caspases 3 and 7 using a yeast-based screening assay

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Caspases are a family of proteases with a crucial role in the initiation and regulation of apoptosis [1]. At the core of the execution phase of apoptosis are the executioner caspases 3 and 7. Like most proteases, these caspases are stored as procaspases that once activated by proteolysis, cleave a large set of substrates, ultimately resulting in the characteristic morphological and biochemical hallmarks of apoptosis. Therefore, the search for activators of these procaspases has deserved particular attention in the field of anticancer drug discovery [1]. Nevertheless, to date, only few small molecule caspase activators have been reported [1].

In a previous work from our group, the use of a yeast screening assay, based on the heterologous expression of human caspases 3 or 7 in the yeast *Saccharomyces cerevisiae*, led us to the identification of two activators of caspase 7: 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) [2]. These results prompted us to the present work aiming to discover new activators of caspases 3 and 7. For that, several analogues of compounds **1a** and **2a** were synthesized, and their activities on caspases 3 and 7 were evaluated, using the previously developed yeast-based screening assay [2]. Potential activators of caspases 3 and/or 7 were therefore identified with improved pharmacological properties compared to their starting analogues. Further work is underway to confirm their molecular mechanisms of action in human tumor cells.

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ENCONTRO INVESTIGAÇÃO JOVEM
DA UNIVERSIDADE DO PORTO
13,14 E 15 DE MAIO 2015

Discovery of New Modulators of Caspases 3 and 7 using a Yeast-based Screening Assay

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INTRODUCTION

Cancer is among the leading cause of mortality worldwide. Indeed, although the advances in therapeutic medicine against this disease, the search for novel drugs to overcome increasing tumour resistance and to trigger apoptosis in tumour cells, is largely required [1]. Thus, caspase activation can be particularly applied as a novel strategy in cancer therapy by selective induction of apoptosis in cancer cells [2]. Caspases 3 and 7 are at the core of the execution phase of apoptosis. Like most proteases, these caspases are stored as procaspases that once activated by proteolysis, cleave a large set of substrates, ultimately resulting in the morphological and biochemical hallmarks of apoptosis. Therefore, the search for activators of these procaspases has deserved particular attention in the field of anticancer drug discovery [3,4]. Nevertheless, to date, only few small-molecule caspase activators have been reported, namely the procaspase activating compound-1 (PAC-1) that activates caspase-3 and -7 [4], the compound 1541 that activates procaspases-3 and -6, and its analogue 1541B that activates procaspase-3 [2,3]. In a previous work from our group [5], the use of a yeast screening assay, based on the heterologous expression of human procaspases-3 or -7 in the yeast *Saccharomyces cerevisiae*, led us to the identification of two activators of procaspase-7: 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) (**Fig. 1**). These results prompted us to the present work aiming to discover new activators of procaspases-3 and -7. For that, several analogues of compounds **1a** and **2a** were synthesized, and their activities on procaspases-3 and -7 were evaluated, using the aforementioned screening assay.

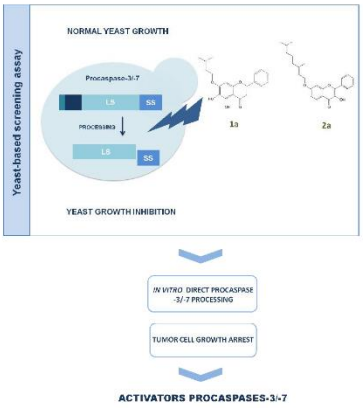

Figure 1 - The use of a yeast-based screening assay, to search for modulators of procaspases-3 and -7, followed by an *in vitro* processing assay and assays in human tumour cell lines, led to the identification of two activators of procaspase-7, compounds **1a** and **2a** [5].

METHODS

Transformation of *Saccharomyces cerevisiae* with pGALL-*LEU* encoding human procaspase-3 or -7 was performed by the lithium acetate method in a previous work [5]. Transformed yeast cells expressing procaspase-3 or -7 were incubated in induction selective medium, containing 2% galactose and without leucine, at 30ºC with continuous orbital shaking, for approximately 24 hours (time required by yeast transformed with the empty vector, control yeast, to achieve 0.30 OD_{600}), with compounds or DMSO only. Effects of procaspases expression on cell growth were evaluated along time by colony-forming units (CFU) counts. Differences between means were tested for significance using the unpaired Student´s *t*-test.

RESULTS

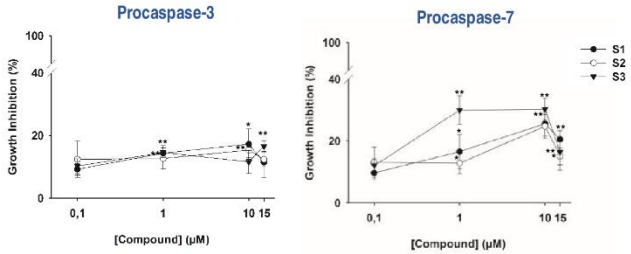

Figure 2 - Concentration-response curves for the effects of flavonoids **S1**, **S2** and **S3** on the growth of yeast expressing human procaspase-3 or -7, for 24 hours treatment.. The percentage of drug-induced growth inhibition was estimated considered 100% growth the number of CFU obtained with DMSO only. Data are mean ± SEM of six independent experiments; values significantly different from DMSO are indicated (*p=0.01, **p=0.001; unpaired).

Table 1 – GI_{25} values obtained for PAC-1 and flavonoids **S1**, **S2** and **S3**.

	GI_{25} (µM)	
	Procaspase-3	Procaspase-7
PAC-1	16.6 ± 2.87	24.5 ± 4.82
S1	*ND*	9.3 ± 2.23*
S2	*ND*	23.9 ± 4.91
S3	*ND*	1.3 ± 2.26**

GI_{25} values correspond to the concentration that caused 25% growth inhibition of yeast cells expressing procaspase-3 or -7 after treatment with 0.1 - 15 µM flavonoids **S1**, **S2** and **S3** (Fig.2), and 0.1 - 50 µM PAC-1 (positive control). Data are mean ± SEM of six independent experiments.; values significantly different from PAC-1 are indicated (*p=0.01, **p=0.001; unpaired). *ND* – Not determined.

The previous developed yeast-based screening assays [5] were used to analyse the effect of analogues of the compounds **1a** and **2a** (**Table 1**; **Fig.2**.) on the activity of human procaspases-3 and -7. Among the tested flavonoids, the compounds **S1**, **S2** and **S3** induced growth inhibition in yeast cells expressing procaspase-7 at 1 - 15 µM (**Fig. 2**), without interfering with the growth of control yeast (data not shown). Interestingly, the effect of these compounds was less pronounced in yeast cells expressing procaspase-3. The GI_{25} values obtained from 0.1 - 15 µM concentration-response curves in yeast cells expressing procaspase-7 revealed that compounds **S1** and **S3** were more potent than the positive control PAC-1, while compound **S2** was as potent as PAC-1. Additionally, in opposition to PAC-1, the GI_{25} values obtained demonstrated that compounds **S1**, **S2** and **S3** were selective for procaspase-7.

CONCLUSIONS

Using a yeast approach, it was shown that flavonoids S1, S2 and S3 are potential selective activators of procaspase-7.

FUTURE WORK

Validation of the molecular mechanism of action of compounds selected in yeast in human tumour cell lines.

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Oral Communication in

II SIMPOSIO DE JOVENES INVESTIGADORES DE LA SEQT

Madrid, Espanha, June 12 2015

NEW POTENTIAL SELECTIVE ACTIVATORS OF CASPASE-7 IDENTIFIED USING THE YEAST SCREENING ASSAY

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The search for new anticancer therapies has been focused on devising ways to overcome resistance and to trigger apoptosis in tumour cells [1]. Caspases are a family of proteases with a crucial role in apoptosis [2]. At the core of the execution phase of apoptosis are the executioner caspases-3 and -7. These caspases are stored as procaspases that once activated by proteolysis cleave a large set of substrates, ultimately resulting in the morphological and biochemical hallmarks of apoptosis. Thus, the search for procaspase activators has deserved particular attention in anticancer drug discovery [2]. Yet, to date, few small-molecule caspase activators have been reported [3,4]. Our previous study reported the identification of two potential activators of caspase-7: 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) using a yeast screening assay, based on the heterologous expression of these human proteins in *Saccharomyces cerevisiae* [5]. These results prompted us to discover new activators of caspases-3 and -7. For that, several analogues of compounds **1a** and **2a** were synthesized, and their activities on these caspases were evaluated, using the developed yeast assay [5]. Potential selective activators of caspase-7 were identified with improved pharmacological properties compared to their starting analogues. Further work is underway to confirm their molecular mechanisms in human tumor cells.

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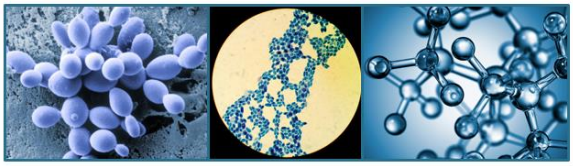

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