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HETEROLOGOUS EXPRESSION OF MAMMALIAN PROTEINS IN YEAST FOR FUNCTIONAL, MOLECULAR AND PHARMACOLOGICAL STUDIES

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“Life is pleasant. Death is peaceful. It’s the transition that’s troublesome.”

Isaac Asimov

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ABSTRACT

Protein kinase C (PKC) isoforms, p53 tumour suppressor protein, and caspase family members are major regulators of cell proliferation and death. These proteins represent therefore key therapeutic targets in human diseases where these processes are dysregulated, such as cancer and neurodegeneration. A better understanding of their biology and pharmacology will certainly provide new therapeutic strategies against these pathologies. However, the high complexity of mammalian signalling pathways, particularly of those involving PKC isoforms, p53 and caspases, has highly hampered such a goal.

In the last years, yeast has emerged as a powerful model organism to unravel the molecular basis of complex human diseases. Besides its technical advantages, it presents a high level conservation between its cellular processes and those of mammalian cells. Additionally, the heterologous expression of human disease-related proteins in yeast has been successfully used to gain understanding of their functions and provide clues to the mechanisms of disease progression. Moreover, yeast expressing a disease-related target protein has widely contributed to the discovery of promising therapeutic agents. Based on this, in the present thesis we used the yeast model system to uncover major functional, molecular and pharmacological aspects of mammalian PKC isoforms, p53, caspase-3 and -7.

Though several works have already reported the phosphorylation and activation of p53 by PKCδ, the role of other PKC isoforms in the regulation of p53 activity was still elusive. Therefore, in order to discriminate the specific roles of PKCα, δ, ε and ζ, major PKC isoforms in carcinogenesis, in the regulation of p53 activity in cell proliferation and death, we reconstituted a mammalian PKC isoform-p53 network in yeast. With this work, we firstly confirmed the reported human wild-type (wt) p53-induced yeast growth inhibition, which was associated with a S-phase cell cycle arrest, and was regulated by a p53 transcription-dependent mechanism. This allowed the development of a yeast p53 phenotypic assay to be used in functional and pharmacological studies of p53. Using yeast cells co-expressing a PKC isoform and p53, it is shown a differential regulation of p53 activity in cell proliferation and death by PKC isoforms, with the identification of negative and positive regulators of p53 among the kinases tested. In fact, though PKCα does not interfere with the p53-mediated apoptosis, evidences are provided for a negative regulation of p53 activity in cell proliferation by this isoform. Most importantly, it is shown that PKCδ and ε phosphorylate and stimulate p53 activity in cell proliferation and apoptotic cell death. On the other hand, PKCζ has no effect on p53 activity. Another important outcome of this work is the reconstitution in yeast of the reported mammalian

transcription-dependent and -independent p53 apoptotic mechanisms, activated by PKCδ and ε, and which co-perate to ultimately cause an apoptotic cell death. Moreover, relevant insights about an unclear issue concerning the regulation of transcription-independent p53 apoptosis are provided. Together, this work represents an additional contribution to advance our knowledge about the role of PKC isoforms in apoptosis. Additionally, it reveals a new strategy of p53 regulation through modulation by PKCδ and ε, and provides the identification of these kinases as key therapeutic targets in tumours with a wt p53.

The elucidation of the molecular mechanism of action of coleon U, a diterpene compound isolated from *Plectranthus grandidentatus* reported to induce apoptosis in human cells, was another issue addressed in the present thesis. Using yeast cells individually expressing PKCα, βI, δ, ε or ζ, we reveal a potent and selective activator of PKCδ and ε with promising pharmacological applications as a probe in the PKC research field and as an anticancer agent. Moreover, with the comparison of the mechanism of action of coleon U with that of the standard PKC activator phorbol 12-myristate 13-acetate, we confirm in yeast that different stimuli can induce the translocation of a same PKC isoform to distinct cellular compartments, which is subsequently associated with different cellular responses.

An additional goal of this thesis was the development of yeast phenotypic assays for the screening of small molecule modulators of caspase-3 and -7. In fact, similarly to wt p53, the expression of active forms of human caspase-3 or -7 in yeast caused a marked growth inhibition that was proportional to the degree of activation of human caspases. With the established assays promising caspase-3 inhibitors were identified by testing a library of vinyl sulfones. Additionally, potent caspase-7 activators were identified by testing several prenylated flavonoids with antiproliferative effects on distinct human tumour cells. Besides the potential applications as pharmacological probes and therapeutic agents in cancer (caspase-7 activators) and neurodegeneration (caspase-3 inhibitors), the identified small molecules can also be used as lead compounds in the synthesis of new small molecules with improved potency and selectivity.

As a whole, in this thesis valuable data about the biology of major protein regulators of cell proliferation and death are provided using the yeast model system. Additionally, the exploitation of the yeast assays in the screening of small molecule modulators of these disease-related proteins led to the discovery of promising therapeutic agents against devastating diseases, such as cancer and neurodegeneration.

Keywords: Yeast; PKC isoforms; p53; Caspase-3; Caspase-7

RESUMO

As isoformas da família da cínase C de proteínas (PKC), a proteína supressora tumoral p53 e os membros da família das caspases desempenham um papel crucial na regulação da proliferação e morte celular. Desta forma, estas proteínas representam alvos terapêuticos importantes em doenças onde estes processos celulares estão desregulados, como o cancro e as doenças neurodegenerativas. Um conhecimento mais aprofundado sobre a biologia e a farmacologia destas proteínas irá certamente permitir o desenvolvimento de novas abordagens terapêuticas no tratamento destas doenças. No entanto, a elevada complexidade das suas vias de sinalização nas células de mamífero tem dificultado o alcance de tal objetivo.

Nos últimos anos, a levedura tem-se revelado um organismo modelo na elucidação da base molecular de doenças humanas complexas. Para além das vantagens técnicas da sua utilização, os processos celulares básicos encontram-se conservados entre a levedura e as células humanas. Adicionalmente, a expressão heteróloga de proteínas envolvidas em doenças humanas tem permitido o estudo das suas funções e dos processos celulares em que estão envolvidas, assim como a pesquisa de novos agentes terapêuticos. Com base no referido, nesta tese utilizou-se a levedura como modelo celular no estudo de aspetos funcionais, moleculares e farmacológicos cruciais das proteínas de mamífero, isoformas da PKC, p53, caspase-3 e -7.

Apesar de a fosforilação e ativação da p53 pela PKCδ ter sido demonstrada anteriormente, o papel de outras isoformas da PKC na regulação da atividade da p53 continua por esclarecer. Desta forma, com o objetivo de discriminar as funções especificas das PKCα, δ, ε e ζ, isoformas principais na carcinogénese, na regulação da atividade da p53, utilizaram-se leveduras a co-expressar cada uma das isoformas da PKC e a proteína p53 na forma nativa. Inicialmente confirmou-se a inibição do crescimento das leveduras causada pela expressão da p53, um efeito atribuído a uma paragem do ciclo celular na fase S e que se mostrou dependente da atividade transcricional da p53. Tal facto permitiu o desenvolvimento de um ensaio fenotípico com leveduras para estudos funcionais e farmacológicos da p53. De seguida, demonstrou-se uma regulação diferencial da atividade da p53 na proliferação e morte celular pelas isoformas da PKC, com a identificação de reguladores positivos e negativos da p53 entre as cínases testadas. De facto, apesar da PKCα não interferir com a morte apoptótica mediada pela p53, esta isoforma inibe a atividade da p53 na proliferação celular. Adicionalmente, mostrou-se que as PKCδ e ε fosforilam e estimulam os efeitos da p53 na morte e proliferação celular, ao passo que a PKCζ não afeta a atividade da p53.

Reconstituíram-se ainda na levedura os mecanismos apoptóticos dependentes e independentes da atividade transcricional da p53, que sendo ativados pelas PKCδ e ε, cooperam na indução de uma morte apoptótica. Estes trabalhos contribuíram, assim, para a elucidação da regulação da atividade da p53 independente da transcrição ainda por esclarecer. Com a identificação das PKCδ e ε como ativadores da p53, foi ainda reforçado o papel relevante destas isoformas como alvos terapêuticos, concretamente em cancros que expressam a forma nativa da p53.

Foi ainda objetivo desta tese elucidar o mecanismo molecular de ação do composto coleona U, um diterpeno isolado de *Plectranthus grandidentatus* e descrito como um indutor apoptótico em células humanas. Para tal utilizaram-se leveduras a expressar individualmente as PKCα, βI, δ, ε ou ζ. Os resultados obtidos revelaram que a coleona U é um ativador potente e seletivo das PKCδ e ε, e portanto um composto com aplicabilidade promissora em estudos farmacológicos experimentais destas cínases e como anticancerígeno. Comparando o seu mecanismo de ação com o do ativador padrão da PKC, 12-miristato 13-acetato de forbol, confirmamos na levedura que estímulos diferentes podem induzir a translocação da mesma isoforma da PKC para compartimentos celulares diferentes e, desta forma, respostas celulares distintas.

Nesta tese, implementaram-se ainda ensaios fenotípicos com leveduras para a pesquisa de pequenas moléculas moduladoras das caspases-3 e -7 humanas. Confirmou-se que a expressão de formas ativas destas caspases na levedura causava uma inibição do crescimento destas, proporcional ao grau de ativação das caspases humanas. Com estes ensaios identificamos inibidores da caspase-3, pesquisando uma biblioteca de vinil sulfonas, e ativadores da caspase-7, pesquisando vários flavonóides prenilados com atividade antiproliferativa em várias linhas celulares de tumores humanos. Para além do potencial terapêutico no tratamento do cancro (ativadores da caspase-7) ou de doenças neurodegenerativas (inibidores da caspase-3), os compostos identificados podem ser usados como precursores na síntese de novos compostos mais potentes e seletivos.

Em resumo, utilizando a levedura como modelo celular foi possível esclarecer vários aspetos da biologia de proteínas de mamífero com um papel central na regulação da proliferação e morte celular. Adicionalmente, a utilização deste modelo celular permitiu a identificação de pequenas moléculas moduladoras da atividade destas proteínas, com aplicações terapêuticas promissoras no tratamento de doenças humanas, como o cancro e as doenças neurodegenerativas.

Palavras-chave: Levedura; isoformas da PKC; p53; caspase-3; caspase-7

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LIST OF ABBREVIATIONS AND SYMBOLS

APAF1	Apoptotic protease activating factor 1
ASPP	Apoptotic specific regulator of p53
ATM	Ataxia-telangiectasia mutated
ATR	AT and Rad3-related
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CFU	Colony-forming units
Chk	Checkpoint kinase
cIAP	Cellular IAP
CDC	Cell division cycle
Coleon U	6,11,12,14-Tetrahydroxy-abieta-5,8,11,13-tetraene-7-one
DAPI	4,6-Diamidino-2-phenylindole
DD	Death domain
DHE	Dihydroethidium
DiOC6(3)	3,3'-Dihexyloxacarbocyanine iodide
DNA-PK	DNA-dependent protein kinase
DMSO	Dimethyl sulfoxide
GFP	Green fluorescent protein
IAP	Inhibitor of apoptosis protein
JNK	C-Jun N-terminal kinases
LS	Large subunit
$\Delta\psi_m$	Mitochondrial transmembrane potential
MDM2	Murine double minute 2
MOMP	Mitochondrial outer membrane permeabilization
mtDNA	Mitochondrial DNA
OD	Optical density
PAC-1	Procaspase activating compound 1
PAK1	p53 activating kinase
PFT	Pifithrin
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PKC	Protein kinase C
PS	Phosphatidylserine
PSE	Pseudosubstrate
PUMA	Upregulated modulator of apoptosis

RE	Responsive elements
ROS	Reactive oxygen species
SS	Small subunit
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
WCE	Whole cell extracts
WT	Wild-type
XIAP	X-linked IAP

CHAPTER 1

General Introduction

This chapter contains parts of the following publications:

New insights and therapeutic strategies emerging from yeast models of cancer and neurodegeneration

Clara Pereira, Isabel Coutinho, Joana Soares, Mariana Leão, Lucília Saraiva *(manuscript submitted for publication)*

Yeast as a powerful model system for the study of apoptosis regulation by protein kinase C isoforms

Rui Duarte Silva, Lucília Saraiva*, Isabel Coutinho, Jorge Gonçalves, Manuela Côrte-Real* *(*these authors contributed equally to this work) (manuscript submitted for publication)*

1.1. INTRODUCTION

Most of our knowledge about basic cellular processes has come from model organisms like yeast, which has given a major contribution to fields as diverse as cell metabolism, DNA replication, recombination, cell cycle, cell death, protein folding, trafficking, and organelle biogenesis (1, 2). Unpredictably, in the last years this knowledge has been applied and expanded to the understanding of human diseases. In fact, as a cell model of human diseases, yeast has given us insights into basic processes underlying pathogenesis. However, as a unicellular organism, the obvious limitation of this cell system for the study of human diseases is in the analysis of disease aspects that rely on multicellularity and cell-cell interactions. Additionally, as a less complex system, some relevant genes involved in the pathology may not be present in the yeast genome. While important aspects of human diseases lie beyond the reach of *Saccharomyces cerevisiae*, this cell system has already proven to be a valuable first-line tool in the discovery of mechanistic processes involved in the disease. This has been possible because many molecular processes are conserved from yeast to humans (1, 3). A remarkable example is the direct application of the fundamental knowledge of cell cycle regulation uncovered in yeast towards studies in human cancer biology (4). More recently, 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 the understanding of apoptosis-related diseases (5).

Yeast shows other undeniable advantages as a model organism as far as molecular studies are concerned. The genome of the budding yeast *Saccharomyces cerevisiae* was the first eukaryotic genome to be sequenced (6) and over the years this knowledge fuelled whole-genome scale screening methods including DNA and protein microarrays (7-9), two-hybrid analysis (10, 11) and the use of deletion and overexpression libraries (12, 13). Another advantage of the yeast model is the broad selection of easily accessible online dataset on genetic interactions, transcriptional changes, protein interactions and localization, further facilitating its widespread use by the scientific community [reviewed in (2)]. In addition, yeast presents many technical advantages over other systems, as a short generation time, ease of manipulation and a highly amenability to genetic modifications by gene deletion/replacement or by using recombinant DNA techniques. The ability to be maintained in a haploid or diploid state allows the study of

lethal mutations in heterozygous diploids and recessive mutations in haploids (14). Yeast is also one of the rare eukaryotes with a good fermenting capacity allowing the analysis of mitochondrial defects that would be lethal in other systems. This is of particular interest since mitochondrial dysfunction and oxidative stress have been linked to many human diseases (15).

All the advantages presented above contributed to the emergence of yeast models for a broad spectrum of human diseases. Different approaches are adopted when establishing these models, usually depending on the degree of conservation of the gene under study. If the gene implicated in the disease is conserved in yeast, it is possible to study directly its function. In fact, it became clear that yeast and human share many orthologues and pathways, what has allowed transposing the knowledge obtained in yeast to mammalian cells and vice-versa. On the other hand, even when the gene underlying the disease has no orthologue in yeast, the heterologous expression of the human gene in this organism (the so-called humanized yeast) has been also crucial to solve fundamental questions about the role of proteins and the molecular mechanisms of complex human disorders, such as cancer and neurodegenerative diseases (16). Yeast expressing the Parkinson´s disease associated protein α-synuclein (17) or the tumour suppressor p53 (18) protein are examples of this strategy. The absence of orthologues of a protein or of an entire pathway can be sometimes highly advantageous, since the protein can be studied in a simpler eukaryotic environment without the interference of other proteins with similar or overlapping functions and of its endogenous regulators. For instance, yeast has been widely used in the analysis of individual protein kinase C (PKC) isoforms (19, 20) or members of the caspase family (21, 22).

The unquestionable advantages exposed above also justify the extensive use of the yeast cell system in the drug discovery field (23-25). In fact, despite the presence of a cell wall, it is now well-known that it does not significantly interfere with the permeability of small molecules in yeast cells (23). Moreover, yeast cell-based assays have clear advantages over the *in vitro* biochemical drug screening assays. Particularly, the possibility to immediately select compounds having into account some relevant drug-like properties, such as permeability and stability in the intracellular environment; the ability to select against compounds that are cytotoxic; and the chance to examine a specific cellular process triggered by a define target are some of the relevant advantages. On the other hand, though mammalian cells provide a more physiological model system, yeast represents a more economical, rapid and simpler alternative for a first analysis of drug targets in a cellular environment. Additionally, in opposition to mammalian cells, where redundant processes often interfere with unambiguous measurement of a specific effect

on a given target, yeast allows a clean read-out in a null background environment for the expression of the human target protein. Yeast presents therefore potential benefits as a high-throughput first-line screening tool, accelerating the drug discovery process with concomitant economic advantages.

Commonly used experimental approaches to study mammalian proteins in yeast are summarized in Fig. 1.1.

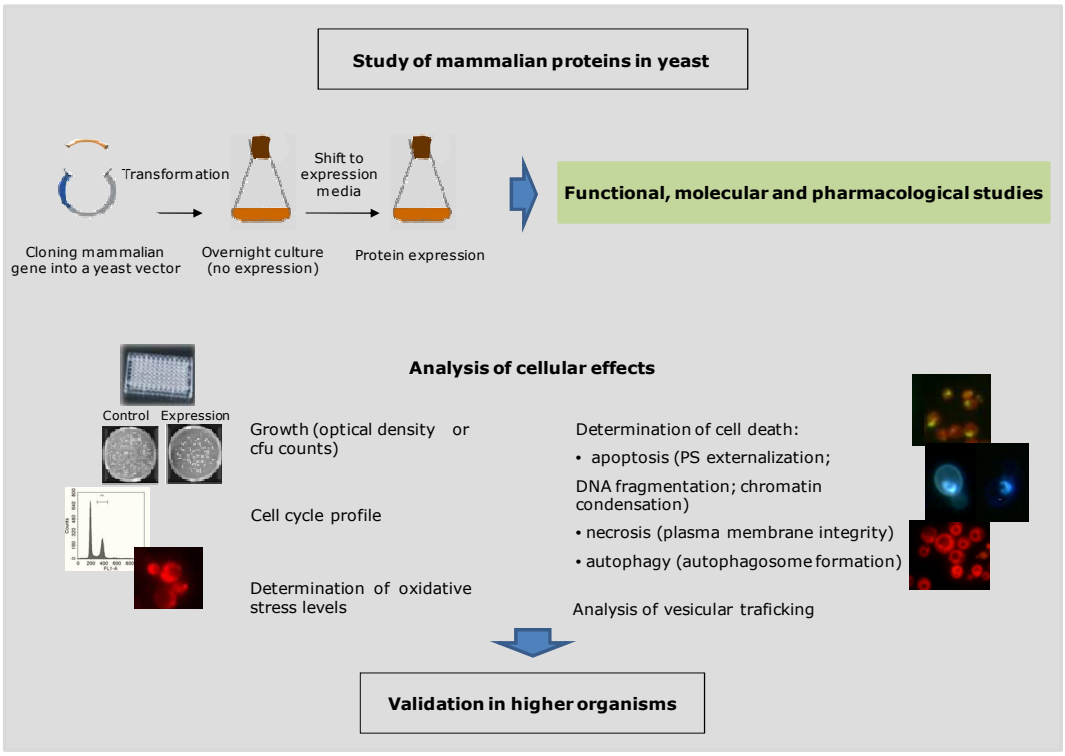

Fig. 1.1. Schematic illustration of commonly used experimental approaches for functional, molecular and pharmacological analysis of mammalian proteins in yeast. Firstly, the gene of interest is cloned into a yeast vector with a constitutive or more often regulatable promoter, since many mammalian disease-related proteins are cytotoxic in yeast. Secondly, the cytotoxicity of a mammalian protein is determined with growth assays by measuring the optical density (OD) or by counting the number of colony-forming units (cfu) in solid plates. The OD is a simple and fast method that can be used in high-throughput genomic or pharmacological screenings. The cfu counts allow distinguishing between decreased growth rates and increased cell death rates. Thirdly, it can be assessed whether the mammalian protein-induced yeast growth inhibition is due to a cell cycle arrest or cell death. Several techniques are available in yeast to distinguish between different types of cell death (apoptotic, necrotic or autophagic). Apoptosis can be identified for instance by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay that visualizes DNA fragmentation, by morphological analysis of chromatin condensation upon staining with 4',6-diamidino-2-phenylindole (DAPI), and by externalization of phosphatidylserine (PS) in the absence of membrane rupture. Propidium iodide, which only enters dead cells with ruptured cellular membranes, is used as a marker of necrosis. Autophagy can be assessed by monitoring the autophagy-associated increase of mature Atg8p or by monitoring the uptake of material by the vacuole. Since many proteins associated mainly to degeneration affect the vesicular trafficking, this can be also monitored by following the delivery of the lipophilic dye FM4-64 to the vacuole. As a first-line

model system all the discoveries made in yeast must be validated in more physiological model organisms (adapted from Pereira et al., 2011; manuscript submitted for publication).

The present chapter is an overview of the contribution of yeast to unravel structural, functional and molecular aspects of crucial mammalian regulators of cell proliferation and death. Moreover, the use of this cell system in the discovery of pharmacological modulators of these cancer and neurodegeneration-target proteins is also discussed.

1.2. YEAST AS A CELL MODEL TO STUDY PROTEIN KINASE C ISOFORMS

The protein kinase C (PKC) family consists of at least 10 serine/threonine protein kinases grouped into the classical (cPKC; α, βI, βII and γ), novel (nPKC; δ, ε, η and θ) and atypical (aPKC; ζ and λ\ι) sub-families, according to their primary structure and activating co-factors requirements (Fig. 1.2) (26).

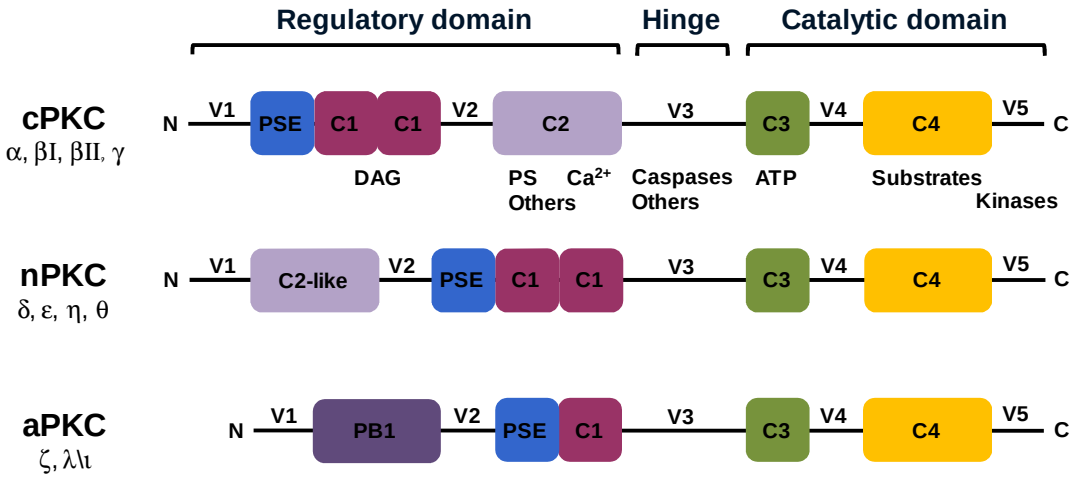

Fig. 1.2. Structural domains of PKC isoforms. PKC isoforms are composed by a regulatory domain at the N-terminus and a catalytic domain at the C-terminus, separated by a hinge region (V3) which is the site of caspase proteolytic cleavages, protein-protein interactions and tyrosine phosphorylations. The regulatory domain of cPKCs contains a C1 domain with two cysteine motifs, essential for binding diacylglycerol (DAG) and its analogs (e.g. phorbol esters), and a C2 domain that binds phosphatidylserine (PS) in a calcium-dependent manner. C2 domain is also responsible for the binding of receptors for activated C kinases (RACKS), a family of anchoring proteins that confers specificity to PKC isoforms by regulating the activation and subcellular localization and, consequently, the cellular functions of PKC isoforms. nPKCs contain a C1 domain and a C2-like domain that binds PS in a calcium-independent manner. aPKCs contain a C1 domain with only one cysteine motif, and therefore unable to bind DAG, and a Phox and Bem 1 (PB1) domain, responsible for protein-protein interactions. The auto-inhibitory pseudosubstrate (PSE) domain in the regulatory domain interacts with the substrate binding site/kinase catalytic centre (C4) in the catalytic domain, maintaining the enzyme in a catalytically inactive form. The catalytic domain of PKC isoforms contains an adenosine triphosphate (ATP) binding domain (C3) and a V5 C-terminal domain that is a site of protein-protein interactions and phosphorylation events determinant for PKC isoform-specific targeting and function [adapted from (26)].

PKC is responsible for regulating major cellular processes, such as cell proliferation and death, in an isoform-specific manner [reviewed in (27-29)]. 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 (Fig. 1.2) (26, 30). The activity of a PKC isoform is also highly dependent on the cellular background (27-29). This explains why distinct and even opposing functions are frequently ascribed to a same PKC isoform. For instance, PKCα and ε are frequently described as promoters of cell proliferation and survival (29, 31-34). However, these PKC isoforms have been also described as apoptotic promoters. For instance, PKCα meditates cisplatin-induced apoptosis in renal cells (35). Also, ethanol induces apoptosis in hepatocytes via activation of nPKC isoforms, including PKCε (36). Additionally, it was reported that, in a same cellular environment, the function of a given PKC isoform is dependent on the stimulus applied. Indeed, it was shown that distinct stimuli can induce the translocation of a specific PKC isoform to distinct subcellular compartments and the subsequent phosphorylation of distinct substrates involved in distinct cellular responses (37-39).

Not surprisingly, the activity and expression of some of these kinases are altered in human diseases where the cellular proliferation and death processes are dysregulated, such as in cancer and neurodegeneration [reviewed in (40, 41)]. PKC isoforms are therefore key pharmacological targets in these pathologies and their isoform-selective modulators promising therapeutic agents. However, the high complexity of the mammalian PKC family, namely the coexistence of several PKC isoforms in a same cell, has hampered the study of individual PKC isoforms, and particularly the discovery of isoform-selective PKC modulators [reviewed in (40-43)]. In this context, yeast arose as a promising cell system for an independent analysis of individual mammalian PKC isoforms in a simpler eukaryotic environment. In fact, in 1993, several works performed by Riedel and collaborators demonstrated that yeast, which endogenous PKC (Pkc1p in *S. cerevisiae*; Pkc1p and Pkc2p in *Schizosaccharomyces pombe*) is a structural but not a functional homologue of mammalian PKC isoforms (44-46), was a promising cell system to study individual mammalian PKC isoforms (47-49). These works showed that mammalian cPKCα and βI are functionally expressed in yeast leading to biological responses similar to those observed in mammalian cells. In fact, the activation of PKCα and βI by phorbol 12-myristate-13-acetate (PMA) caused PKC down-regulation, uptake of extracellular Ca^{2+}, Ca^{2+}-dependence of cell viability, changes in cell morphology, and a growth inhibition. Together, these findings indicated the conservation in yeast of a mammalian PKC signalling pathway. Several studies were performed to further explore the mechanisms behind PKC-induced yeast growth inhibition. Particularly, it was observed

that expression of PKCε in respiring yeast inhibited cell growth by delaying cell cycle progression through multiple mechanisms (e.g. delaying bud formation, altering cell morphology and impairing chromosome segregation and septum formation) (50). Additionally, activation of PKCα by PMA induced a G2/M cell cycle arrest through impairment of chromosome segregation, cytokinesis and septum formation, which was related to altered expression profile of genes regulating cell wall dynamics. The altered gene expression profile and phenotypic alterations observed were attributed to the phosphorylation and activation of the yeast cell division cycle 55 (Cdc55) by PKCα, which may therefore represent a PKCα substrate in yeast (51).

The observation that the degree of mammalian PKC-induced growth inhibition was proportional to the degree of its enzymatic activity led to the establishment of a yeast phenotypic assay for the assessment of PKC activity. Such method, based on yeast cell growth, revealed to be sensitive and fast to quantitatively measure PKC activity. Therefore, it was successfully explored by several groups in functional, molecular and pharmacological studies of several mammalian PKC isoforms, namely cPKCα, βI and γ, nPKCδ, η and ε and aPKCζ.

Indeed, the yeast PKC phenotypic assay was extensively used by several authors to establish PKC structure-function relationships. For instance, based on the phenotypic response of various PKCα mutants lacking amino acid sequences within the regulatory (V1, C1, V2 and C2) and catalytic (V5 and C4) domains, it was possible to analyse the function of these domains in the regulation of PKC catalytic activity and interaction with activating co-factors. These studies revealed that most of the C2 domain was not essential for phorbol ester stimulation and most of the regulatory domain was dispensable for Ca^{2+} regulation of PKC activity (52). These results were confirmed in mammalian cells and presently the structural basis for co-factor requirements of PKC isoforms is a well-established issue [reviewed in (26, 30)]. Additionally, carboxyl terminus truncated mutants of PKCα (49) and PKCβI (47) were expressed in yeast in order to map the carboxyl terminus sequence essential for their activity. The function of the carboxyl terminus was further analysed using a chimeric PKCα in which its carboxyl terminus had been replaced by the corresponding PKCβI sequence, PKCαβI. The PKCαβI chimera exhibited the same phenotypic characteristics observed with PKCα, confirming that the carboxyl terminus of distinct isoforms can complement each other (53). Later, it was observed that co-expression of PKCα regulatory domain with full-length PKCβI protein in yeast abolished the PKCβI-induced phenotype (54, 55). These results sustained the cross-talk observed in mammalian cells between some PKC isoforms (26). In a continuous effort to establish the

function of the PKC regulatory domain, another study suggested that, in addition to the PSE region, an amino acid segment in the C2 domain of PKCα, essential for the maintenance of the PKC inactive conformation, also regulates the PKC catalytic activity (56). The observation that PKCα activity was not strictly regulated by the PSE sequence led other authors to study the regions within the regulatory domain essential for the auto-inhibition of PKCα catalytic activity (57). The authors found multiple sequences in the regulatory domain of PKCα, outside the PSE sequence, with a direct role in the inhibition of PKCα activity. Together, in accordance with studies in mammalian cells (30), these works performed in yeast provided evidence to the existence of intra-molecular interactions between the regulatory and catalytic regions of PKCα responsible for the maintenance of its inactive conformation form.

Several works demonstrated the suitability of the yeast PKC phenotypic assay in pharmacological studies of individual mammalian PKC isoforms. In fact, it was shown that standard PKC activators (e.g. PMA, mezerein) induced growth inhibition in yeast cells expressing a mammalian PKC isoform that was proportional to the degree of PKC activation (58-60), while PKC inhibitors (e.g. chelerythrine and calphostin C) reverted the effects of PKC activators (58, 61). This was used for an *in vivo* characterization of the PKC regions required for the activity of small molecule modulators of mammalian PKC isoforms. Particularly, it was observed that distinct PKC activators displayed a differential ability to bind the C1 domain and activate cPKCα (62-64), βI and γ (63). In fact, while PMA and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA) activated PKC via any of the cysteine motifs in the C1 domain, indolactam V, ingenol-3 and 20-dibenzoate required both cysteine motifs. Additionally, whereas mezerein displayed a strong preference for the first cys-repeat of the C1 region, bryostatin 5 preferentially binds to the second cys-repeat. Guo and colleagues (65) further assessed additional sites within the PKCα regulatory domain necessary for phorbol esters activity. Curiously, they found that only some of the PKCα amino acid sequences necessary for phorbol ester activation were directly involved in phorbol ester binding. It was proposed that the other sequences functioned by providing the appropriate three-dimensional PKCα conformation for activation by phorbol esters. This approach was also used to characterize the potency and selectivity of small molecule modulators of PKC isoforms. For instance, the isoform-selectivity of several PMA analogues on cPKCα and βI, nPKCδ and ε and aPKCζ, was analysed using this assay (59). Surprisingly, two of the PMA analogues tested, namely PMA-4-O-methyl-ether (MPMA) and 4α–PMA displayed an effect similar to that exhibited by the PKC inhibitors NPC 15437 and chelerythrine, being therefore characterized as PKC inhibitors.

Subsequent studies performed with this yeast assay allowed the identification of new potent and selective small molecule modulators of individual mammalian PKC isoforms. Particularly, it was used to analyse the potency and isoform-selectivity of mezerein and its derivative daphnetoxin (60). Mezerein is a standard PKC activator with antiproliferative and antileukemic activities, while daphnetoxin is devoid of these effects. In this study, it was shown that mezerein was much more potent on PKCδ than on PKCα. Contrarily to mezerein, daphnetoxin showed to be much more potent on PKCα than on PKCδ. These results therefore suggested that the lack of antileukemic activity of daphnetoxin may be due to its higher activity on an anti-apoptotic isoform, PKCα, being its effect on the pro-apoptotic PKCδ almost negligenciable when compared to mezerein (60). Additionally, using yeast cells expressing PKCα, βI, δ, η or ζ, it was possible to ascertain the molecular mechanism of action of euxanthone, a natural compound that caused neuroblastoma cells differentiation(66). This work showed that euxanthone was a PKC activator with a remarkable selectivity for PKCζ, a PKC isoform reported to be involved in the differentiation of neuroblastoma cells. In a continuous effort to discover new PKC modulators more potent and selective towards individual PKC isoforms, the activity of several synthetic xanthones (67, 68) and xanthonolignoids (69) on PKCα, βI, δ, η and ζ was also evaluated using the yeast assay. Some of the tested xanthones showed to be more effective than the standard PKC activators (namely PMA for cPKCs and nPKCs, and arachidonic for aPKCζ). Most importantly, some of them showed to be highly selective for an individual PKC isoform as PKCδ, η and ζ (68). Additionally, two of the tested xanthones behaved as PKC inhibitors and presented differences on their potency towards the distinct PKC isoforms tested (67). A potent and selective inhibitor of PKCζ was also identified using the yeast PKC assay by testing a library of xanthonolignoid derivatives (69). Together, these works allowed the identification of several isoform-selective PKC modulators with promising applications as pharmacological probes in PKC-signalling pathway research field and/or as therapeutic agents. Additionally, they can be used as lead compounds for the synthesis of new compounds with improved potency and selectivity.

The finding that yeast can undergo apoptosis with similar characteristics of mammalian cells [reviewed in (5)] uncovered the possibility to exploit this model organism to unravel the role of PKC isoforms in the regulation of this cellular process. In fact, our group used the yeast model to study the ability of distinct PKC isoforms to modulate the activity of major apoptotic proteins of the Bcl-2 family, namely Bcl-xL (20) and Bax (70). Using yeast cells co-expressing an individual mammalian PKC isoform and the anti-apoptotic human Bcl-xL protein, it was shown that PKC isoforms differently interfered with

the cytoprotective effect of Bcl-xL in acetic acid-induced yeast cell death, by affecting the Bcl-xL phosphorylation state. Whereas PKCα phosphorylated Bcl-xL abolishing its anti-apoptotic activity, PKCε and ζ markedly inhibited Bcl-xL phosphorylation and stimulated its anti-apoptotic activity. Meanwhile, PKCδ had no effect on Bcl-xL phosphorylated state and anti-apoptotic activity (20). Du and colleagues (71) have proposed the existence in mammalian cells of a kinase and phosphatase system that may be operating in tandem, leading to a coordinated phosphorylation-dephosphorylation cycle that modulates Bcl-xL activity. However, the precise mechanisms for this modulation remain undetermined. Therefore, the study performed in yeast offered new insights into the role of phosphorylation on the modulation of the Bcl-xL function and identified individual PKC isoforms as key regulators of the phosphorylation-dephosphorylation state of Bcl-xL. In a later study, it was also demonstrated that PKCα was able to regulate the activity of Bax in yeast (70). The pro-apoptotic function of this Bcl-2 family protein depends on its ability to translocate, oligomerize and insert into the mitochondrial membrane following stress [reviewed in (72)]. Using yeast cells co-expressing an active form of Bax and PKCα, it was shown that PKCα increases the translocation and insertion of Bax into the outer mitochondrial membrane. This was associated with an increase in cytochrome *c* (cyt *c*) release, reactive oxygen species (ROS) production, mitochondrial network fragmentation and autophagic cell death. Curiously, this PKCα effect revealed to be independent of its kinase activity (70). This observation obtained with yeast supported the notion that PKCα can promote apoptosis by a kinase-independent way. In fact, non-traditional PKC activation mechanisms, such as kinase-independent actions of freed regulatory, are frequently reported for PKCs (26). Together, in agreement with several reports in mammalian cells (34), the studies performed in yeast identified the members of the Bcl-2 family as major apoptotic targets of PKC isoforms. Moreover, they represent an important contribution to the design of new strategies for apoptosis regulation through modulation of PKC isoforms activity.

Overall, valuable insights about the genetic, molecular and functional profile of individual PKC isoforms have been provided by the yeast PKC assay. Additionally, potent and selective pharmacological modulators of these kinases have been identified using this cell system. The suitability of this cell system to uncover many other aspects in the PKC research field is therefore undeniable.

1.3. YEAST AS A CELL MODEL TO STUDY P53

The p53 tumour suppressor protein acts as a major defence against cancer, and altered p53 activity is often observed in human cancers. In fact, about 50% of all reported cancers have a mutation in the *TP53* gene. Among the remaining cancers that retain a wild-type (wt) p53, the p53 pathway is often inactivated leading to multiple defects in p53 responses. Hence, restoring p53 function has been considered a promising strategy in anticancer therapy (73, 74). In this context, a better elucidation of the mechanisms of p53 regulation may contribute to the development of new approaches and therapies to restore the p53 function in human cancers. This has been therefore the focus of an intensive research.

The best-understood function of p53 is its transcriptional activity. In unstressed cells, the activity of p53 is normally held in check by its negative regulator MDM2, (Murine Double Minute 2), an E3 ubiquitin ligase, which binds to p53 and targets it for proteasomal degradation. In response to several stimuli, however, this inhibition is relieved, and p53, as a sequence-specific transcription factor, regulates the expression of several target genes to cause multiple cellular outcomes such as cell cycle arrest (e.g. p21 gene, growth arrest and DNA damage inducible gene 45 (*GADD45*), apoptosis (p53-inducible gene (*PIG*), *BAX*, p53 upregulated modulator of apoptosis (*PUMA*), and apoptotic protease activating factor 1 (*APAF1*) genes) and numerous other processes (75, 76). However, not all target genes are equally and coordinately responsive to p53. Instead, p53 transcriptional activity is regulated by several factors that contribute to this variable p53 response [reviewed in (75-77)]. After various types of genotoxic insults, p53 is stabilized, translocates to the nucleus, and binds to its response element (RE) most commonly found in the promoter at varying distances upstream from the transcription start site (TSS) of the target gene. It was shown that p53 binds with higher affinity to REs found in cell cycle arrest-related genes than to REs found in apoptosis-related genes (78). Hence, low levels of active p53 normally result in growth arrest, whereas high levels usually result in apoptosis (75-77). Moreover, multiple interactions with p53 binding proteins allow p53 to either promote or inhibit the transcription of different target genes. For instance, members of apoptotic specific regulator of p53 (ASPP) 1 and 2 (79) stimulate the p53 binding to REs from apoptotic genes, while the inhibitory ASPP (iASPP) (80) or the p53 family proteins, p63 and p73 (81) were described as inhibitors of the transactivation of pro-apoptotic genes. Finally, many covalent p53 post-translational modifications have shown to predispose p53 to selectively activate or repress certain target genes. In fact, p53

contains numerous conserved sites that can be regulated by a multitude of post-translational modifications including phosphorylation, acetylation, methylation, ubiquitination, sumoylation and neddylation. Among these, post-translational modification by phosphorylation, which can be brought about by several kinases, such as ataxia-telangiectasia mutated (ATM), AT and Rad3-related (ATR), DNA-dependent protein kinase (DNA-PK), checkpoint kinase (Ckh) 1 and Chk2, c-Jun N-terminal kinases (JNK) and PKC (Fig. 1.3), has been shown to play critical roles in the stabilization and activation of p53 (82, 83). In particular, several works underscored a major role of PKC in p53 phosphorylation and activation. In fact, it was demonstrated that PKCδ activated a p53-dependent apoptosis in mammalian cells through p53 phosphorylation (84-87). However, the role of other PKC isoforms in the regulation of p53 function is still elusive.

Fig. 1.3. Schematic representation of p53 domains and p53 phosphorylation sites. Human p53 protein consists of five well-defined functional domains: at the N-terminus, the transactivation domain (TAD), required for transcriptional activation, and the proline-rich domain (PRD) that enables protein–protein interactions; the central sequence-specific DNA-binding domain (DBD), required for DNA binding and where the vast majority of p53 cancer related-mutations occurs; and at the C-terminus, the tetramerization domain (TD), and the basic C-terminal regulatory domain (CTD), a non-specific DNA binding domain that binds to damaged DNA and is involved in the down-regulation of the DNA binding capability of the central domain. Major sites for p53 phosphorylation are indicated (red arrow). Major kinases involved in p53 phosphorylation are also listed, and include the ATM, ATR, DNA-PK, Chk1, Chk2, JNK and PKC kinases [adapted from (82)].

The high complexity of the mammalian p53-signalling pathway, associated with the absence of p53 orthologues in yeast (88, 89), led several researchers to explore yeast as a simpler eukaryotic cell system to study p53. In fact, it was earlier demonstrated that mammalian wt p53 was also a sequence-dependent transcription factor in yeast (18, 90). It was also found that p53 was predominantly localized in the nucleus of both *S. cerevisiae* (91) and *S. pombe* (92), a hallmark for p53 transcriptional activity in mammalian cells. Several yeast proteins affecting the p53 transcriptional activity were also identified. For instance, it was shown that the yeast p53 activating kinase (PAK1) regulated the p53 transcriptional activity in yeast, although this study did not evaluate a possible phosphorylation of p53 by PAK1 (93). The ability of p53 to be phosphorylated by

endogenous yeast kinases was confirmed in another study, although the kinases responsible for this effect were not revealed (94). Moreover, it was shown that the p53 transcriptional activity in yeast was strongly reduced in the absence of the gene encoding thioredoxin reductase, a redox enzyme (95, 96). Evidences that the p53 transcriptional activity in yeast was subjected to redox regulation and required thioredoxin reductase were thereafter provided in mammalian cells (97). A recent study also revealed remarkable similarities between co-activator requirements for p53 transcriptional activity in yeast and mammalian cells (89). Yeast has been also extensively used to study how p53 mutations affect its transcriptional activity and therefore the p53 response, an issue with a high clinical value. The functional analysis of separated alleles in yeast (FASAY), developed by Ishioka and colleagues (98), allows a rapid detection of p53 mutations in cancer cells (98). In this technique, the p53 status can be easily measured based on the ability of p53 to express a reporter gene. An enhanced version of this assay used *ADE2,* instead of *HIS3*, as a reporter gene and located it downstream of a p53 RE (99). This assay was based on the observation of the colour of the colonies, where white or red colonies indicated the presence of a wt p53 or a total loss-of-function mutant, respectively, and intermediate phenotypes (colonies exhibiting pink colour) indicated the presence of a partial inactivating p53 mutation. This assay was used in the analysis of the factors that regulate the differential p53 transcriptional activity. For instance, a study using 26 different p53 RE evaluated the effect on the p53 transcriptional activity of p53 levels and recognition and binding affinity of p53 to specific p53 REs (100). The results obtained showed a distinct ability of p53 to bind REs derived from genes involved in cell cycle, DNA repair and apoptosis. In fact, p53 had a weaker affinity to most of the apoptotic genes tested, requiring higher levels of p53 for their transactivation. This suggests that p53 levels, as well as p53 binding affinity to a RE, are important determinants of the p53 differential transcriptional activity (100). An extended study, to analyse all known p53 REs, revealed similar results in yeast and mammalian cells (101). Recently, an adaptation of the yeast transcriptional assay was performed for the high-throughput screening of factors that can influence the p53 activity, including mutations, co-factor proteins and small molecules (102). Using this assay it was shown for instance that MDM2 also led to a reduction in the p53-dependent transactivation in yeast. In fact, a study performed by Di Ventura and colleagues (91) showed that, as in mammalian cells, MDM2 can interact with endogenous yeast pathways to ubiquitinate and sumoylate p53. Ubiquitination led to p53 degradation, while sumoylation was essential for the localization of p53-MDM2 complexes to yeast nuclear bodies. Together, these studies established the yeast assay as an appropriate model system to elucidate the complex regulatory interplay between p53 and MDM2, and possibly other p53 regulatory proteins.

Despite the prominence of the p53 transcriptional activity in the induction of apoptosis, a transcription-independent p53 apoptotic activity, triggered by the p53 exported from the nucleus to the cytoplasm and/or mitochondria, has also been reported [reviewed in (103, 104)]. In fact, several evidences provide encouragement to further explore the potential of cancer therapeutics based on the activation of the p53 transcription-independent pathway, specially in those cancers carrying p53 mutants with no transcriptional activity. Moreover, unlike the transcriptional activity, transcription-independent p53 responses lead necessarily to apoptosis, therefore eliminating the cell cycle or other outcomes in tumour cell targets (103, 105). A better understanding of the mechanisms involved in the regulation of this p53 apoptotic pathway may therefore underscore new therapeutic strategies.

Although p53 transcription-dependent and -independent activities are independent pathways, several data indicate that they co-exist and complement each other for full apoptotic responses of p53. Apoptosis can be initiated by two convergent signalling pathways, extrinsic and intrinsic, both regulated by p53 (Fig. 1.4). The extrinsic pathway is initiated by the activation of death receptors at the cell surface, while the intrinsic mitochondria-mediated pathway is initiated by stress signals originated within the cell. In the intrinsic pathway mitochondria and, specifically, mitochondrial outer membrane permeabilization (MOMP), which is controlled by Bcl-2 family members, play a central role. These two pathways trigger a cascade of events that results in caspase activation and death (Fig. 1.4) [reviewed in (103, 104)]. Through its transcription-dependent activity, p53 regulates the expression of several proteins involved in both intrinsic and extrinsic pathways, including pro-apoptotic proteins, such as Bax, Bid, PUMA, Apaf1, the death receptor Fas, the adaptor protein p53-induced protein with a death domain (PIDD), and anti-apoptotic proteins, such as Bcl-2, Bcl-xL and the inhibitor of apoptosis protein (IAP) survivin (75, 76, 106) (Fig. 1.4). Outside the nucleus, p53 induces apoptosis in a transcription-independent manner through its interaction with several cellular proteins, such as Bcl-2 family members (Fig. 1.4). In fact, p53 has a dual action of neutralizing anti-apoptotic members (namely Bcl-xL and Bcl-2) and activating pro-apoptotic members (namely Bax and Bak) of the Bcl-2 family [reviewed in (103, 104)]. For instance, p53 can interact with Bak in the mitochondria, releasing it from an inhibitory complex formed with the anti-apoptotic protein myeloid cell leukemia sequence 1 (Mcl-1). This leads to the oligomerization and activation of Bak. At the cytosol, p53 can activate Bax by activating its translocation to mitochondria and oligomerization.

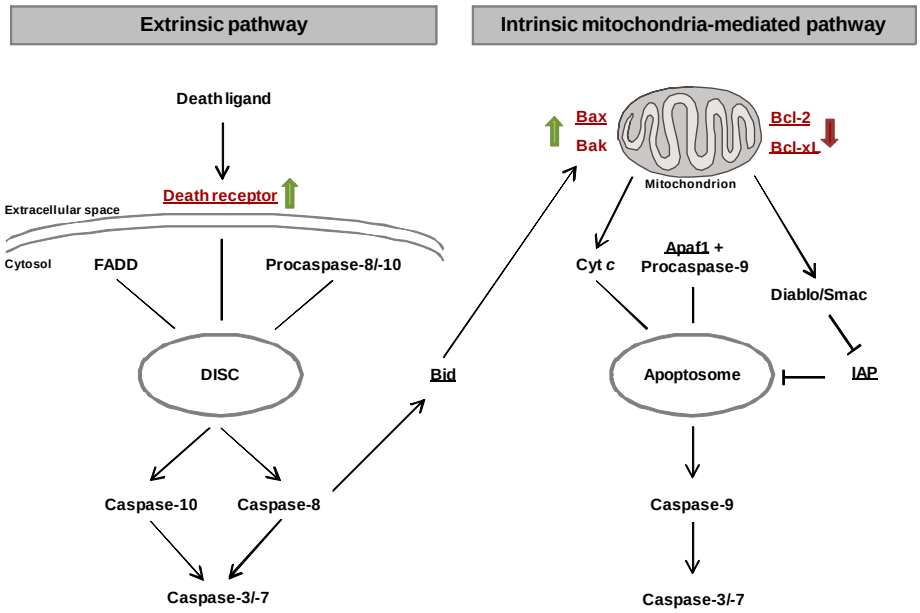

Fig. 1.4. Signalling pathways leading to caspase activation and apoptosis that are influenced by p53. In the extrinsic pathway, extracellular signals induce caspase-8/-10 recruitment to the death-inducing signalling complex (DISC), a typical transmembrane signalling receptor which includes a death receptor (e.g. Fas) and an adaptor protein (e.g. Fas-Associated protein with DD, FADD). Once activated, caspase-8 directly cleaves and activates procaspase-3/-7 and/or Bid, activating the intrinsic apoptotic pathway. Activated Bid (tBid) triggers oligomerization and activation of the pro-apoptotic proteins Bax/Bak, which lead to MOMP and to the release of mitochondrial signalling proteins into the cytosol, the most well-known being cyt *c*. Activation of Bax/Bak is also achieved indirectly when Bcl-2 anti-apoptotic members (namely Bcl-2 and Bcl-xL) are neutralized. At the cytoplasm, cyt *c* associates with Apaf-1 and procaspase-9 to form the apoptosome, which results in an activated caspase-9 and consequent procaspase-3/-7 proteolytic activation. Activation of the intrinsic pathway is also achieved independently of the extrinsic pathway, by other signals originated within the cell, such as DNA damage. In the figure, transcriptional targets of p53 are underlined (activation is indicated by a green arrow and repression by a red arrow), whereas proteins affected by transcription-independent activity of p53 are highlighted in red [adapted from (103)].

Apoptosis can be also promoted by interaction of p53 with Bcl-xL and Bcl-2, which releases Bax and Bak from the inhibitory complexes formed with pro- and anti-apoptotic Bcl-2 family members. In a second step, PUMA, a p53 transcriptional product, releases p53 from the inactivating complex formed with BcL-xL, freeing p53 to activate pro-apoptotic proteins. These observations clearly evidence the co-operation between the p53 transcription-dependent and -independent pathways. In addition to its direct interaction with Bcl-2 family members, p53 was also found to regulate the activity of other proteins involved in the intrinsic pathway in a transcription-independent manner. For instance, p53 was found to interact with Ku70, a protein that binds to Bax preventing its translocation to mitochondria. Upon post-translational modification by acetylation, Ku70-Bax complex is

disrupted and Bax is activated resulting in apoptosis (107). In fact, besides their important role in the regulation of p53 transcriptional activity, post-translational modifications also appear to play a major role in the regulation of p53 transcription-independent apoptotic activity [reviewed in (103, 104)]. For instance, the export of p53 from the nucleus to the cytosol has been attributed to its post-translational mono-ubiquitination by MDM2. In addition, MDM2-mediated mono-ubiquitination of p53 greatly promotes its mitochondrial translocation. Once in the mitochondria, p53 is deubiquinated and free to induce apoptosis (108, 109). Although p53 phosphorylation has also been proposed as an obvious mechanism of regulation of p53 activities, this remains unconfirmed (103, 110).

The recognised involvement of p53 in the regulation of cell cycle and apoptosis (76, 77) along with the feasibility of the yeast cell model to study these cellular processes (4, 5) have prompted several researchers to use the yeast model to further explore the p53 functions in these cellular processes. In fact, similarly to that reported in mammalian cells, it was found that expression of wt p53 in *S. cerevisiae* caused growth inhibition associated with a G1/S cell cycle arrest, which was markedly increased by co-expression of human cell cycle-regulated protein kinase CDC2Hs (111). Curiously, it was shown that p53-induced growth arrest in *S. pombe* was abrogated by co-expression of the human cell cycle regulator protein phosphatase CDC25, indicating a functional interaction of both proteins (112). In a later study in mammalian cells, it was confirmed that p53 regulated cell cycle progression by interacting with CDC25 (113). Instead, Hadj Amor and colleagues (114) showed that expression of wt p53 in *S. cerevisiae* resulted in an apoptotic cell death. This work also revealed that p53 affected the yeast gene expression since a decrease of the expression of the gene that encodes the anti-apoptotic and anti-oxidant protein thioredoxin, was detected. Since this protein has a crucial function in the protection of yeast cells against ROS, these results suggested that p53 may induce apoptosis in part by down-regulation of anti-apoptotic proteins. The aforementioned distinct phenotypes achieved in yeast upon expression of p53 were attributed to different p53 expression levels due to the use of different yeast expression vectors (114). Altogether, these results revealed that yeast is a suitable model system to further elucidate p53-dependent activities in cell cycle and apoptosis.

In summary, the works referred above provided several evidences that highlight yeast as a powerful cell model system, which can be further explored for functional, molecular and pharmacological studies of p53.

1.4. YEAST AS A CELL MODEL TO STUDY CASPASE FAMILY MEMBERS

Caspases are a conserved family of cysteine-dependent aspartate-specific proteases composed by at least 15 members that are grouped into the inflammatory and apoptotic sub-families according to their major cellular functions. Apoptotic caspases can be further divided into two functional groups based on their position in the apoptotic cascade, the apical or initiator caspases (caspase-2, -8, -9 and -10) and the executioner or downstream caspases (caspase-3, -6 and -7) (115, 116). Besides its widely established roles in apoptosis, apoptotic caspases are also involved in other non-apoptotic processes, including cell survival, proliferation and differentiation. In fact, it has been assumed that high levels of caspase activity lead to a caspase-dependent apoptosis, while transient or low levels may lead to the induction of its non-apoptotic activities (117-119). Caspases are synthesized as catalytically inactive enzymes called procaspases, and possess distinct mechanisms of activation. Basically, initiator procaspases become active by proximity-induced dimerization at caspase activation complexes, which favours autoproteolytic processing, resulting in a mature caspase. Instead, downstream procaspases occur as inactive dimers in the cytosol, requiring proteolytic cleavage by upstream effectors (e.g. initiator caspases) to become active (see Fig. 1.4 for a schematic representation of the cascade of caspase activation during apoptosis) (115, 116). The final outcome of this activating cascade is the specific and irreversible cleavage of a wide variety of substrates that determine the cell fate (115, 120). Caspases activity can be suppressed by caspase inhibitor proteins, such as cowpox virus cytokine response modifier A (crmA), baculoviral p35/p49 and inhibitors of apoptosis proteins (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) (121, 122). Due to their key roles in coordinating major cellular processes, it is not surprising that dysregulation of caspase expression or activity has been associated with many human diseases, including cancer and neurodegeneration (123, 124). Caspases, especially caspase-3 and -7 due to their well-established contribution for executing the final processes of apoptosis (115, 116, 120, 125), therefore represent promising therapeutic targets in these human diseases. In fact, the identification of selective small molecule modulators of caspase family members, particularly of caspase-3 and -7, has been the focus of an intensive research (121, 126-128). Besides its potential as therapeutic agents, these compounds are highly required for the study of caspase-signalling pathways.

The high complexity of mammalian caspase-signalling pathways, particularly the co-existence of several caspase members and endogenous caspase regulators in mammalian cells, led several research groups to explore yeast for an independent analysis of caspase family members in a simpler eukaryotic cell system. Although yeast encodes a metacaspase (Yca1p) that shares structural homology and mechanistic features with mammalian caspases, major differences in the primary cleavage specificity have questioned its classification as a "true" caspase. This has been the subject of an intensive discussion (129, 130). In fact, Yca1p has been implicated in the same cellular processes as mammalian caspases, namely in apoptosis (131). However, while mammalian caspases specifically cleave their substrates after aspartic acid residues (132), metacaspases specifically cleave substrates after arginine or lysine (basic residues) (133). Though it is still being a controversial issue, recent reports have demonstrated that the activity of mammalian caspases in yeast (namely of caspase-2, -3, -4, -7, -8 and -10) is independent of Yca1p (22, 134). Based on this, yeast has been widely exploited to study several aspects of mammalian caspase family members, particularly to identify their regulators and substrates. However, while the majority of mammalian caspases are auto-activated in yeast when expressed at high levels, downstream caspases, namely caspase-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 caspase-9 adaptor protein Apaf-1, which are absent in yeast (115, 116). This has been surpassed using engineered auto-activated variants that allow auto-activation of these caspases even in the absence of their upstream activators. A commonly used strategy to induce caspase auto-activation in yeast is the generation of reverse caspases by rearrangement of their subunits (Fig. 1.5) (135-137). In fact, reverse caspases fold spontaneously into an active conformation inducing apoptosis in mammalian cells (138). Moreover, they exhibit similar intrinsic enzymatic activity to wt constructs, both in mammalian (138) and yeast (135) cells. Auto-activation in yeast has also been achieved using caspase variants lacking the N-terminal prodomain from the caspase coding sequence (139). This form of caspase was shown to induce apoptosis in mammalian cells due to the spontaneous interdomain cleavage, while the intrinsic enzymatic activity is not affected (140). Other strategies involved co-expressing the large and small subunits separately, for an intracellular assembling of the active caspase (141), and joining in-frame the caspase cDNA to the coding regions for bacterial β-galactosidase (lacZ), a fusion protein able to auto-activate probably due to multimer formation mediated by the β-galactosidase moiety (142, 143).

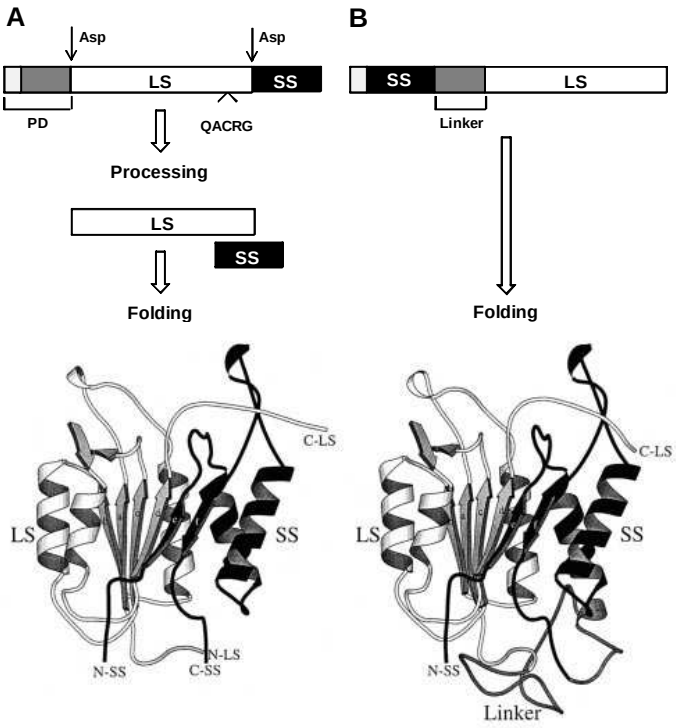

Fig. 1.5. Schematic representation of the structure, processing and folding of procaspase-3 and reverse caspase-3. (**A**) Similarly to other procaspases, procaspase-3 is composed by an N-terminal prodomain (PD) that is followed by a large subunit (LS or p20), containing the catalytic cysteine conserved site (QACR(X)G, where X varies according to the caspase members) and a small subunit (SS or p10). After proteolytic processing at specific aspartic acid residues (Asp; black arrow), the free SS and LS can fold properly into an active conformation. In this conformation, the N-terminus of the SS and the C-terminus of the LS are set apart, whereas the N-terminus of the LS and the C-terminus of the SS are close to each other. (**B**) Reverse caspase-3, with the SS before the prodomain and LS, folds spontaneously into an active conformation that simulates the three-dimensional structure of the processed procaspase. The linker region between SS and LS includes the prodomain [adapted from (138)].

An approach developed to monitor the activity of mammalian caspases in yeast used a modified yeast two-hybrid system, in which the bait vector contains the sequences for the two caspase-3 subunits and the LexA DNA-binding domain, and the prey vector contains a cDNA expression library fused to *Gal4* activation domain. In this system, the interaction of caspase-3 with its substrates was detected through the activation of a reporter gene (e.g. the bacterial lacZ) (141). Using this approach, gelsolin, an actin-regulatory protein cleaved during apoptosis in human cells, was identified as a substrate of caspase-3. This result was also confirmed *in vitro* using recombinant gelsolin and caspase-3, and in human cell lines (141). These experiments validated this yeast assay to identify caspase substrates. Later, a similar approach was developed to identify new caspase family members and their regulators. In this case, it was used a fusion protein in

which a transcription factor was linked to the intracellular domain of a transmembrane protein by caspase cleavage sites. The caspase activation resulted in the release of the transcription factor from the membrane, which drove the transcriptional activation of the reporter gene (139, 144). This assay was validated using the caspase-9 activating adaptor protein Apaf-1 that induced reporter gene expression, and mammalian caspase inhibitors (namely XIAP and baculoviral p35) that suppressed caspase-dependent reporter gene expression (139). Later, Hayashi and colleagues (144) adapted this yeast system to the high-throughput screening of caspase modulators. However, despite the advantages of this cell system as a first-line screening tool, so far no small molecule modulators of caspase family members have been identified using yeast.

The most commonly used strategy to study mammalian caspases in yeast relied on the fact that, in most cases, high expression levels of an active caspase induce yeast growth inhibition, which can be reverted or stimulated by caspase inhibitors or activators, respectively. For instance, it was shown that natural caspase-3 activators, such as initiator caspase-2 (145), -8 and -10 (137), stimulated caspase-3-induced yeast growth inhibition. Also, natural caspase-3 inhibitors, such as p35, p49 (146) and several members of IAPs (136, 143, 147), and the chemical caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (z-VAD-FMK) (148) reverted the observed phenotype. These observations led to the establishment of a yeast phenotypic assay suitable to study the mechanisms of mammalian caspase activation and to identify new regulators and substrates. In fact, this yeast phenotypic assay was used to reconstitute in yeast the mammalian cascade of caspase activation, namely the roles of caspase-8 and -10 in the activation of caspase-3 and -6. In this study, it was shown that activation of caspase-3 in yeast was successfully achieved by co-expression of caspase-8β or -10, whereas these caspases were insufficient to activate caspase-6. These results distinguished sequential modes of action for different caspases *in vivo* (149). Other authors used this yeast assay to reconstitute the Apaf-1-activated apoptotic mechanisms. In fact, as reported in mammalian cells (150), it was shown that Apaf-1 activated caspase-9, which in turn activated caspase-3 (136, 151). Also, in accordance with previous reports in mammalian cells [reviewed in (121)], XIAP was able to prevent this caspase activation cascade by inhibiting caspase-3 and/or caspase-9 activation (136, 151). Additionally, this effect was reverted by the IAP-binding antagonist Diablo (direct IAP-binding protein with low isoelectric point, also known as Smac, second mitochondria-derived activator of caspases), which disrupted the interaction between XIAP and caspase-9 (151). This yeast assay was also used to study the potency of some natural caspase activators and inhibitors on individual caspase members. For instance, it was shown that caspase-2

activated caspase-7 more effectively than caspase-3 (145). Furthermore, similarly to that described in mammalian cells (121), p35 and p49 exhibited a broad-spectrum inhibitory activity, inhibiting caspase-1, -2, -3, -4, -5, -7 and -8 in yeast, although with some different efficiencies (146). Also, a similar order of IAP activities found in mammalian cells expressing an auto-activating caspase-3, namely XIAP>cIAP2>cIAP1>survivin, was observed in yeast (152).

Distinct causes have been attributed to this caspase-induced yeast growth inhibition. In most studies, it has been associated with the induction of a cell death (22, 134). However, it was detected that this could vary depending on the yeast strain, possibly due to differences in the expression levels of endogenous caspase substrates and/or other proteases with similar functions (22, 151). Moreover, the strategy used to produce auto-activating forms of caspases was also referred as having some impact on the final outcome of caspase activation. For instance, while co-expression of the subunits of caspase-3 as separate proteins inhibited yeast growth without causing cell death (147, 148), expression of reverse caspase-3 induced yeast cell death (22). It was proposed that this can be related to different efficiencies on generating an active caspase by the two approaches, where the first strategy led to comparatively lower caspase activation sufficient to impair yeast growth but not to induce cell death. These results revealed that, as in mammalian cells where distinct levels of caspase activation lead to distinct cellular responses (117-119), different phenotypes can be obtained in yeast by manipulation of caspase activation levels. This cell system can therefore be further explored to reconstitute distinct phenotypes for functional, molecular and pharmacological studies of mammalian caspases.

1.5. SCOPE OF THIS THESIS

As addressed in previous section, the heterologous expression of disease-related proteins in yeast has been successfully used to gain understanding of the functions of these proteins, providing clues about the potential causes underpinning the associated disorders. Moreover, yeast expressing a disease-related target protein has widely contributed to the discovery of promising therapeutic agents.

The scope of this thesis was to use yeast for functional, molecular and pharmacological studies of major neurodegenerative and cancer-related proteins, particularly PKC isoforms, p53 and caspase family members. In fact, the high complexity of the signalling pathways associated to these proteins in mammalian cells has hampered the study of their biology, and especially the discovery of selective pharmacological modulators. Based on this, a mammalian PKC-p53 network was reconstituted in yeast and relevant insights about the regulation of p53 activity in cell proliferation (chapter 2) and death (chapter 3) by distinct PKC isoforms are provided. Also, a yeast PKC assay was used to elucidate the molecular mechanism of action of coleon U, a natural compound with an antiproliferative activity on several human tumour cell lines (chapter 4). With this assay, it was possible to identify a potent and selective activator of nPKCδ and ε with promising applications as a probe in the PKC research field and as an anticancer agent. Another goal of this thesis was the development of yeast phenotypic assays for the screening of small molecule modulators of caspase-3, caspase-7 and p53. With the established assays promising caspase-3 inhibitors (chapter 5) and caspase-7 activators (chapter 6) were identified.

The chapter 7 of this thesis is dedicated to a general discussion focusing on the major contributions of this work to advance our knowledge about the functional, molecular and pharmacological regulation of the studied proteins.

1.6. REFERENCES

(1) Botstein D, Fink GR. Yeast: an experimental organism for modern biology. Science 1988 Jun; 240 (4858): 1439-43.
(2) Khurana V, Lindquist S. Modelling neurodegeneration in Saccharomyces cerevisiae: why cook with baker's yeast? Nat Rev Neurosci 2010 Jun; 11 (6): 436-49.
(3) Smith MG, Snyder M. Yeast as a model for human disease. Curr Protoc Hum Genet 2006 Feb; Chapter 15: Unit 15 6.
(4) Hartwell LH. Nobel Lecture. Yeast and cancer. Biosci Rep 2002 Jun-Aug; 22 (3-4): 373-94.
(5) Carmona-Gutierrez D, Eisenberg T, Buttner S, Meisinger C, Kroemer G, Madeo F. Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ 2010 May; 17 (5): 763-73.
(6) Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, et al. Life with 6000 genes. Science 1996 Oct; 274 (5287): 546, 63-7.
(7) Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, Conway A, Wodicka L, et al. A genome-wide transcriptional analysis of the mitotic cell cycle. Mol Cell 1998 Jul; 2 (1): 65-73.
(8) Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, et al. Functional profiling of the Saccharomyces cerevisiae genome. Nature 2002 Jul; 418 (6896): 387-91.
(9) Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, et al. Global analysis of protein activities using proteome chips. Science 2001 Sep; 293 (5537): 2101-5.
(10) Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci U S A 2001 Apr; 98 (8): 4569-74.
(11) Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, et al. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 2000 Feb; 403 (6770): 623-7.
(12) Jones GM, Stalker J, Humphray S, West A, Cox T, Rogers J, et al. A systematic library for comprehensive overexpression screens in Saccharomyces cerevisiae. Nat Methods 2008 Mar; 5 (3): 239-41.
(13) Tong AH, Evangelista M, Parsons AB, Xu H, Bader GD, Page N, et al. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 2001 Dec; 294 (5550): 2364-8.
(14) Guthrie C, Fink G. Guide to yeast genetics and molecular biology. Methods Enzymol 1991; 194: 1-863.
(15) de Moura MB, dos Santos LS, Van Houten B. Mitochondrial dysfunction in neurodegenerative diseases and cancer. Environ Mol Mutagen 2010 Jun; 51 (5): 391-405.
(16) Petranovic D, Nielsen J. Can yeast systems biology contribute to the understanding of human disease? Trends Biotechnol 2008 Nov; 26 (11): 584-90.
(17) Outeiro TF, Lindquist S. Yeast cells provide insight into alpha-synuclein biology and pathobiology. Science 2003 Dec; 302 (5651): 1772-5.
(18) Fields S, Jang SK. Presence of a potent transcription activating sequence in the p53 protein. Science 1990 Aug; 249 (4972): 1046-9.
(19) Parissenti AM, Riedel H. Yeast as a host to screen for modulators and regulatory regions of mammalian protein kinase C isoforms. Methods Mol Biol 2003; 233: 491-516.
(20) Saraiva L, Silva RD, Pereira G, Goncalves J, Corte-Real M. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci 2006 Aug; 119 (Pt 15): 3171-81.
(21) Hawkins CJ, Wang SL, Hay BA. Monitoring activity of caspases and their regulators in yeast Saccharomyces cerevisiae. Methods Enzymol 2000; 322: 162-74.
(22) Puryer MA, Hawkins CJ. Human, insect and nematode caspases kill Saccharomyces cerevisiae independently of YCA1 and Aif1p. Apoptosis 2006; 11 (4): 509-17.
(23) Barberis A, Gunde T, Berset C, Audetat S, Lüthi U. Yeast as a screening tool. Drug Discovery Today: Technologies 2005 Summer 2005; 2 (2): 187-92.
(24) Tucker CL. High-throughput cell-based assays in yeast. Drug Discov Today 2002 Sep; 7 (18 Suppl): S125-30.
(25) Simon JA, Bedalov A. Yeast as a model system for anticancer drug discovery. Nat Rev Cancer 2004 Jun; 4 (6): 481-92.
(26) Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev 2008 Oct; 88 (4): 1341-78.
(27) Buchner K. The role of protein kinase C in the regulation of cell growth and in signalling to the cell nucleus. J Cancer Res Clin Oncol 2000 Jan; 126 (1): 1-11.
(28) Gutcher I, Webb PR, Anderson NG. The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci 2003 Jun; 60 (6): 1061-70.
(29) Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 2009; 14: 2386-99.
(30) Kheifets V, Mochly-Rosen D. Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. Pharmacol Res 2007 Jun; 55 (6): 467-76.

(31) Ahmad S, Mineta T, Martuza RL, Glazer RI**.** Antisense expression of protein kinase C alpha inhibits the growth and tumorigenicity of human glioblastoma cells. Neurosurgery 1994 Nov; 35 (5): 904-8; discussion 8-9.
(32) Dooley NP, Baltuch GH, Groome N, Villemure JG, Yong VW**.** Apoptosis is induced in glioma cells by antisense oligonucleotides to protein kinase C alpha and is enhanced by cycloheximide. Neuroreport 1998 Jun 1; 9 (8): 1727-33.
(33) Whelan RD, Parker PJ**.** Loss of protein kinase C function induces an apoptotic response. Oncogene 1998 Apr; 16 (15): 1939-44.
(34) Basu A, Sivaprasad U**.** Protein kinase Cepsilon makes the life and death decision. Cell Signal 2007 Aug; 19 (8): 1633-42.
(35) Nowak G**.** Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells. J Biol Chem 2002 Nov; 277 (45): 43377-88.
(36) Zhang Y, Venugopal SK, He S, Liu P, Wu J, Zern MA**.** Ethanol induces apoptosis in hepatocytes by a pathway involving novel protein kinase C isoforms. Cell Signal 2007 Nov; 19 (11): 2339-50.
(37) Shirai Y, Saito N**.** Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. J Biochem 2002 Nov; 132 (5): 663-8.
(38) Wang QJ, Lu G, Schlapkohl WA, Goerke A, Larsson C, Mischak H, et al. The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-delta and -epsilon. Mol Cancer Res 2004 Feb; 2 (2): 129-40.
(39) Ohmori S, Shirai Y, Sakai N, Fujii M, Konishi H, Kikkawa U, et al. Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C. Mol Cell Biol 1998 Sep; 18 (9): 5263-71.
(40) Ali AS, Ali S, El-Rayes BF, Philip PA, Sarkar FH**.** Exploitation of protein kinase C: a useful target for cancer therapy. Cancer Treat Rev 2009 Feb; 35 (1): 1-8.
(41) Cuny GD**.** Kinase inhibitors as potential therapeutics for acute and chronic neurodegenerative conditions. Curr Pharm Des 2009; 15 (34): 3919-39.
(42) Irie K, Nakagawa Y, Ohigashi H**.** Toward the development of new medicinal leads with selectivity for protein kinase C isozymes. Chem Rec 2005; 5 (4): 185-95.
(43) Mackay HJ, Twelves CJ**.** Targeting the protein kinase C family: are we there yet? Nat Rev Cancer 2007 Jul; 7 (7): 554-62.
(44) Perez P, Calonge TM**.** Yeast protein kinase C. J Biochem 2002 Oct; 132 (4): 513-7.
(45) Iwai T, Fujisawa N, Ogita K, Kikkawa U**.** Catalytic properties of yeast protein kinase C: difference between the yeast and mammalian enzymes. J Biochem 1992 Jul; 112 (1): 7-10.
(46) Goode NT, Hajibagheri MA, Warren G, Parker PJ**.** Expression of mammalian protein kinase C in Schizosaccharomyces pombe: isotype-specific induction of growth arrest, vesicle formation, and endocytosis. Mol Biol Cell 1994 Aug; 5 (8): 907-20.
(47) Riedel H, Hansen H, Parissenti AM, Su L, Shieh HL, Zhu J**.** Phorbol ester activation of functional rat protein kinase C beta-1 causes phenotype in yeast. J Cell Biochem 1993 Jul; 52 (3): 320-9.
(48) Riedel H, Parissenti AM, Hansen H, Su L, Shieh HL**.** Stimulation of calcium uptake in Saccharomyces cerevisiae by bovine protein kinase C alpha. J Biol Chem 1993 Feb; 268 (5): 3456-62.
(49) Su L, Parissenti AM, Riedel H**.** Functional carboxyl terminal deletion map of protein kinase C alpha. Receptors Channels 1993; 1 (1): 1-9.
(50) Parissenti AM, Villeneuve D, Kirwan-Rhude A, Busch D**.** Carbon source-dependent regulation of cell growth by murine protein kinase C epsilon expression in Saccharomyces cerevisiae. J Cell Physiol 1999 Feb; 178 (2): 216-26.
(51) Sprowl JA, Villeneuve DJ, Guo B, Young AJ, Hembruff SL, Parissenti AM**.** Changes in expression of cell wall turnover genes accompany inhibition of chromosome segregation by bovine protein kinase C alpha expression in Saccharomyces cerevisiae. Cell Biol Int 2007 Oct; 31 (10): 1160-72.
(52) Riedel H, Su L, Hansen H**.** Yeast phenotype classifies mammalian protein kinase C cDNA mutants. Mol Cell Biol 1993 Aug; 13 (8): 4728-35.
(53) Parissenti AM, Su L, Riedel H**.** Reconstitution of protein kinase C alpha function by the protein kinase C beta-I carboxy terminus. Mol Cell Endocrinol 1993 Dec; 98 (1): 9-16.
(54) Parissenti AM, Kim SA, Colantonio CM, Snihura AL, Schimmer BP**.** Regulatory domain of human protein kinase C alpha dominantly inhibits protein kinase C beta-I-regulated growth and morphology in Saccharomyces cerevisiae. J Cell Physiol 1996 Mar; 166 (3): 609-17.
(55) Parissenti AM, Kirwan AF, Kim SA, Colantonio CM, Schimmer BP**.** Molecular strategies for the dominant inhibition of protein kinase C. Endocr Res 1996 Nov; 22 (4): 621-30.
(56) Rotenberg SA, Zhu J, Hansen H, Li XD, Sun XG, Michels CA, et al. Deletion analysis of protein kinase Calpha reveals a novel regulatory segment. J Biochem 1998 Oct; 124 (4): 756-63.
(57) Kirwan AF, Bibby AC, Mvilongo T, Riedel H, Burke T, Millis SZ, et al. Inhibition of protein kinase C catalytic activity by additional regions within the human protein kinase Calpha-regulatory domain lying outside of the pseudosubstrate sequence. Biochem J 2003 Jul; 373 (Pt 2): 571-81.
(58) Keenan C, Goode N, Pears C**.** Isoform specificity of activators and inhibitors of protein kinase C gamma and delta. FEBS Lett 1997 Sep; 415 (1): 101-8.

(59) Saraiva L, Fresco P, Pinto E, Goncalves J. Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay. Eur J Pharmacol 2004 May; 491 (2-3): 101-10.
(60) Saraiva L, Fresco P, Pinto E, Portugal H, Goncalves J. Differential activation by daphnetoxin and mezerein of PKC-isotypes alpha, beta I, delta and zeta. Planta Med 2001 Dec; 67 (9): 787-90.
(61) Saraiva L, Fresco P, Pinto E, Goncalves J. Isoform-selectivity of PKC inhibitors acting at the regulatory and catalytic domain of mammalian PKC-alpha, -betaI, -delta, -eta and -zeta. J Enzyme Inhib Med Chem 2003 Dec; 18 (6): 475-83.
(62) Shieh HL, Hansen H, Zhu J, Riedel H. Differential protein kinase C ligand regulation detected in vivo by a phenotypic yeast assay. Mol Carcinog 1995 Mar; 12 (3): 166-76.
(63) Shieh HL, Hansen H, Zhu J, Riedel H. Activation of conventional mammalian protein kinase C isoforms expressed in budding yeast modulates the cell doubling time--a potential in vivo screen for protein kinase C activators. Cancer Detect Prev 1996; 20 (6): 576-89.
(64) Zhu J, Hansen H, Su L, Shieh HL, Riedel H. Ligand regulation of bovine protein kinase C alpha response via either cysteine-rich repeat of conserved region C1. J Biochem 1994 May; 115 (5): 1000-9.
(65) Guo B, Reed K, Parissenti AM. Scanning Mutagenesis Studies Reveal Multiple Distinct Regions within the Human Protein Kinase C Alpha Regulatory Domain Important for Phorbol Ester-dependent Activation of the Enzyme. Journal of Molecular Biology 2006; 357 (3): 820-32.
(66) Saraiva L, Fresco P, Pinto E, Kijjoa A, Gonzalez MJ, Goncalves J. Differential activation of protein kinase C isoforms by euxanthone, revealed by an in vivo yeast phenotypic assay. Planta Med 2002 Nov; 68 (11): 1039-41.
(67) Saraiva L, Fresco P, Pinto E, Sousa E, Pinto M, Goncalves J. Inhibition of protein kinase C by synthetic xanthone derivatives. Bioorg Med Chem 2003 Apr; 11 (7): 1215-25.
(68) Saraiva L, Fresco P, Pinto E, Sousa E, Pinto M, Goncalves J. Synthesis and in vivo modulatory activity of protein kinase C of xanthone derivatives. Bioorg Med Chem 2002 Oct; 10 (10): 3219-27.
(69) Saraiva L, Fresco P, Pinto E, Sousa E, Pinto M, Goncalves J. Inhibition of alpha, betaI, delta, eta, and zeta protein kinase C isoforms by xanthonolignoids. J Enzyme Inhib Med Chem 2003 Aug; 18 (4): 357-70.
(70) Silva RD, Manon S, Goncalves J, Saraiva L, Corte-Real M. Modulation of Bax mitochondrial insertion and induced cell death in yeast by mammalian protein kinase Calpha. Exp Cell Res 2011 Apr; 317 (6): 781-90.
(71) Du L, Lyle CS, Chambers TC. Characterization of vinblastine-induced Bcl-xL and Bcl-2 phosphorylation: evidence for a novel protein kinase and a coordinated phosphorylation/dephosphorylation cycle associated with apoptosis induction. Oncogene 2005 Jan; 24 (1): 107-17.
(72) Silva RD, Manon S, Goncalves J, Saraiva L, Corte-Real M. The importance of humanized yeast to better understand the role of bcl-2 family in apoptosis: finding of novel therapeutic opportunities. Curr Pharm Des 2011; 17 (3): 246-55.
(73) Wang W, El-Deiry WS. Restoration of p53 to limit tumor growth. Curr Opin Oncol 2008 Jan; 20 (1): 90-6.
(74) Cheok CF, Verma CS, Baselga J, Lane DP. Translating p53 into the clinic. Nat Rev Clin Oncol 2011 Jan; 8 (1): 25-37.
(75) Beckerman R, Prives C. Transcriptional regulation by p53. Cold Spring Harb Perspect Biol 2010 Aug; 2 (8): a000935.
(76) Millau JF, Bastien N, Drouin R. P53 transcriptional activities: a general overview and some thoughts. Mutat Res 2009 Mar-Jun; 681 (2-3): 118-33.
(77) Aylon Y, Oren M. Living with p53, dying of p53. Cell 2007 Aug; 130 (4): 597-600.
(78) Weinberg RL, Veprintsev DB, Bycroft M, Fersht AR. Comparative binding of p53 to its promoter and DNA recognition elements. J Mol Biol 2005 May; 348 (3): 589-96.
(79) Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, et al. ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell 2001 Oct; 8 (4): 781-94.
(80) Bergamaschi D, Samuels Y, O'Neil NJ, Trigiante G, Crook T, Hsieh JK, et al. iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. Nat Genet 2003 Feb; 33 (2): 162-7.
(81) Murray-Zmijewski F, Lane DP, Bourdon JC. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. Cell Death Differ 2006 Jun; 13 (6): 962-72.
(82) Dai C, Gu W. p53 post-translational modification: deregulated in tumorigenesis. Trends Mol Med 2010 Nov; 16 (11): 528-36.
(83) Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ 2006 Jun; 13 (6): 941-50.
(84) Ryer EJ, Sakakibara K, Wang C, Sarkar D, Fisher PB, Faries PL, et al. Protein kinase C delta induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. J Biol Chem 2005 Oct; 280 (42): 35310-7.
(85) Yoshida K, Liu H, Miki Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. J Biol Chem 2006 Mar; 281 (9): 5734-40.
(86) Lee SJ, Kim DC, Choi BH, Ha H, Kim KT. Regulation of p53 by activated protein kinase C-delta during nitric oxide-induced dopaminergic cell death. J Biol Chem 2006 Jan; 281 (4): 2215-24.

(87) Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Karino T, et al. Roles of protein kinase C delta in the accumulation of P53 and the induction of apoptosis in H2O2-treated bovine endothelial cells. Free Radic Res 2002 Nov; 36 (11): 1147-53.
(88) Smardova J, Smarda J, Koptikova J. Functional analysis of p53 tumor suppressor in yeast. Differentiation; research in biological diversity 2005; 73 (6): 261-77.
(89) Yousef AF, Xu GW, Mendez M, Brandl CJ, Mymryk JS. Coactivator requirements for p53-dependent transcription in the yeast Saccharomyces cerevisiae. International journal of cancer Journal international du cancer 2008; 122 (4): 942-6.
(90) Scharer E, Iggo R. Mammalian p53 can function as a transcription factor in yeast. Nucleic acids research 1992; 20 (7): 1539-45.
(91) Di Ventura B, Funaya C, Antony C, Knop M, Serrano L. Reconstitution of Mdm2-dependent post-translational modifications of p53 in yeast. PLoS One 2008; 3 (1): e1507.
(92) Bischoff JR, Casso D, Beach D. Human p53 inhibits growth in Schizosaccharomyces pombe. Mol Cell Biol 1992 Apr; 12 (4): 1405-11.
(93) Thiagalingam S, Kinzler KW, Vogelstein B. PAK1, a gene that can regulate p53 activity in yeast. Proc Natl Acad Sci U S A 1995 Jun; 92 (13): 6062-6.
(94) Nigro JM, Sikorski R, Reed SI, Vogelstein B. Human p53 and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae. Mol Cell Biol 1992 Mar; 12 (3): 1357-65.
(95) Pearson GD, Merrill GF. Deletion of the Saccharomyces cerevisiae TRR1 gene encoding thioredoxin reductase inhibits p53-dependent reporter gene expression. The Journal of biological chemistry 1998; 273 (10): 5431-4.
(96) Merrill GF, Dowell P, Pearson GD. The human p53 negative regulatory domain mediates inhibition of reporter gene transactivation in yeast lacking thioredoxin reductase. Cancer Res 1999 Jul; 59 (13): 3175-9.
(97) Hu JD, Ma XR, Lindner DJ, Karra S, Hofmann ER, Reddy SPM, et al. Modulation of p53 dependent gene expression and cell death through thioredoxin-thioredoxin reductase by the interferon-retinoid combination. Oncogene 2001 Jul; 20 (31): 4235-48.
(98) Ishioka C, Frebourg T, Yan YX, Vidal M, Friend SH, Schmidt S, et al. Screening patients for heterozygous p53 mutations using a functional assay in yeast. Nat Genet 1993 Oct; 5 (2): 124-9.
(99) Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, et al. A simple p53 functional assay for screening cell lines, blood, and tumors. Proc Natl Acad Sci U S A 1995 Apr; 92 (9): 3963-7.
(100) Inga A, Storici F, Darden TA, Resnick MA. Differential transactivation by the p53 transcription factor is highly dependent on p53 level and promoter target sequence. Mol Cell Biol 2002 Dec; 22 (24): 8612-25.
(101) Qian H, Wang T, Naumovski L, Lopez CD, Brachmann RK. Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. Oncogene 2002 Nov; 21 (51): 7901-11.
(102) Andreotti V, Ciribilli Y, Monti P, Bisio A, Lion M, Jordan J, et al. p53 Transactivation and the Impact of Mutations, Cofactors and Small Molecules Using a Simplified Yeast-Based Screening System. PLoS One 2011; 6 (6): e20643.
(103) Speidel D. Transcription-independent p53 apoptosis: an alternative route to death. Trends Cell Biol 2010 Jan; 20 (1): 14-24.
(104) Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature 2009 Apr; 458 (7242): 1127-30.
(105) Yu Q. Restoring p53-mediated apoptosis in cancer cells: new opportunities for cancer therapy. Drug Resist Updat 2006 Feb-Apr; 9 (1-2): 19-25.
(106) Lin Y, Ma W, Benchimol S. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat Genet 2000 Sep; 26 (1): 122-7.
(107) Sykes SM, Stanek TJ, Frank A, Murphy ME, McMahon SB. Acetylation of the DNA binding domain regulates transcription-independent apoptosis by p53. J Biol Chem 2009 Jul; 284 (30): 20197-205.
(108) Wade M, Wang YV, Wahl GM. The p53 orchestra: Mdm2 and Mdmx set the tone. Trends Cell Biol 2010 May; 20 (5): 299-309.
(109) Marchenko ND, Wolff S, Erster S, Becker K, Moll UM. Monoubiquitylation promotes mitochondrial p53 translocation. EMBO J 2007 Feb; 26 (4): 923-34.
(110) Nemajerova A, Erster S, Moll UM. The post-translational phosphorylation and acetylation modification profile is not the determining factor in targeting endogenous stress-induced p53 to mitochondria. Cell Death Differ 2005 Feb; 12 (2): 197-200.
(111) Nigro JM, Sikorski R, Reed SI, Vogelstein B. Human p53 and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae. Molecular and cellular biology 1992; 12 (3): 1357-65.
(112) Bureik M, Jungbluth A, Drescher R, Wagner P. Human p53 restores DNA synthesis control in fission yeast. Biol Chem 1997 Nov; 378 (11): 1361-71.
(113) Ruppenthal SL, Noll A, Gotz C, Montenarh M. Interference between p53 and cdc25C in cell cycle regulation. International journal of oncology 2007; 31 (2): 345-52.
(114) Hadj Amor IY, Smaoui K, Chaabene I, Mabrouk I, Djemal L, Elleuch H, et al. Human p53 induces cell death and downregulates thioredoxin expression in Saccharomyces cerevisiae. FEMS Yeast Res 2008 Dec; 8 (8): 1254-62.

(115) Chowdhury I, Tharakan B, Bhat GK. Caspases - an update. Comp Biochem Physiol B Biochem Mol Biol 2008 Sep; 151 (1): 10-27.
(116) Logue SE, Martin SJ. Caspase activation cascades in apoptosis. Biochem Soc Trans 2008 Feb; 36 (Pt 1): 1-9.
(117) Feinstein-Rotkopf Y, Arama E. Can't live without them, can live with them: roles of caspases during vital cellular processes. Apoptosis 2009 Aug; 14 (8): 980-95.
(118) Lamkanfi M, Festjens N, Declercq W, Vanden Berghe T, Vandenabeele P. Caspases in cell survival, proliferation and differentiation. Cell Death Differ 2007 Jan; 14 (1): 44-55.
(119) Hashimoto T, Yamauchi L, Hunter T, Kikkawa U, Kamada S. Possible involvement of caspase-7 in cell cycle progression at mitosis. Genes Cells 2008 Jun; 13 (6): 609-21.
(120) Luthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. Cell Death Differ 2007 Apr; 14 (4): 641-50.
(121) Callus BA, Vaux DL. Caspase inhibitors: viral, cellular and chemical. Cell Death Differ 2007 Jan; 14 (1): 73-8.
(122) Dubrez-Daloz L, Dupoux A, Cartier J. IAPs: more than just inhibitors of apoptosis proteins. Cell Cycle 2008 Apr; 7 (8): 1036-46.
(123) Bulat N, Widmann C. Caspase substrates and neurodegenerative diseases. Brain Res Bull 2009 Oct; 80 (4-5): 251-67.
(124) Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and cancer: mutations within caspase genes. Journal of Medical Genetics 2009; 46 (8): 497-510.
(125) Walsh JG, Cullen SP, Sheridan C, Luthi AU, Gerner C, Martin SJ. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci U S A 2008 Sep; 105 (35): 12815-9.
(126) Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. J Clin Invest 2005 Oct; 115 (10): 2665-72.
(127) Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland MS, Kwon JT, et al. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nat Chem Biol 2006 Oct; 2 (10): 543-50.
(128) Wolan DW, Zorn JA, Gray DC, Wells JA. Small-molecule activators of a proenzyme. Science 2009 Nov; 326 (5954): 853-8.
(129) Carmona-Gutierrez D, Frohlich KU, Kroemer G, Madeo F. Metacaspases are caspases. Doubt no more. Cell Death Differ 2010 Mar; 17 (3): 377-8.
(130) Enoksson M, Salvesen GS. Metacaspases are not caspases--always doubt. Cell Death Differ 2010 Aug; 17 (8): 1221.
(131) Madeo F, Herker E, Maldener C, Wissing S, Lachelt S, Herlan M, et al. A caspase-related protease regulates apoptosis in yeast. Mol Cell 2002 Apr; 9 (4): 911-7.
(132) Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, et al. Human ICE/CED-3 protease nomenclature. Cell 1996 Oct; 87 (2): 171.
(133) Vercammen D, van de Cotte B, De Jaeger G, Eeckhout D, Casteels P, Vandepoele K, et al. Type II metacaspases Atmc4 and Atmc9 of Arabidopsis thaliana cleave substrates after arginine and lysine. J Biol Chem 2004 Oct; 279 (44): 45329-36.
(134) Lisa-Santamaría P, Neiman AM, Cuesta-Marbán Á, Mollinedo F, Revuelta JL, Jiménez A. Human initiator caspases trigger apoptotic and autophagic phenotypes in Saccharomyces cerevisiae. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 2009; 1793 (3): 561-71.
(135) Araya R, Takahashi R, Nomura Y. Yeast two-hybrid screening using constitutive-active caspase-7 as bait in the identification of PA28gamma as an effector caspase substrate. Cell Death Differ 2002 Mar; 9 (3): 322-8.
(136) Hawkins CJ, Silke J, Verhagen AM, Foster R, Ekert PG, Ashley DM. Analysis of candidate antagonists of IAP-mediated caspase inhibition using yeast reconstituted with the mammalian Apaf-1-activated apoptosis mechanism. Apoptosis 2001 Oct; 6 (5): 331-8.
(137) Kang JJ, Schaber MD, Srinivasula SM, Alnemri ES, Litwack G, Hall DJ, et al. Cascades of mammalian caspase activation in the yeast Saccharomyces cerevisiae. J Biol Chem 1999 Jan; 274 (5): 3189-98.
(138) Srinivasula SM, Ahmad M, MacFarlane M, Luo Z, Huang Z, Fernandes-Alnemri T, et al. Generation of constitutively active recombinant caspases-3 and -6 by rearrangement of their subunits. J Biol Chem 1998 Apr; 273 (17): 10107-11.
(139) Hawkins CJ, Wang SL, Hay BA. A cloning method to identify caspases and their regulators in yeast: identification of Drosophila IAP1 as an inhibitor of the Drosophila caspase DCP-1. Proc Natl Acad Sci U S A 1999 Mar; 96 (6): 2885-90.
(140) Denault JB, Salvesen GS. Human caspase-7 activity and regulation by its N-terminal peptide. J Biol Chem 2003 Sep; 278 (36): 34042-50.
(141) Kamada S, Kusano H, Fujita H, Ohtsu M, Koya RC, Kuzumaki N, et al. A cloning method for caspase substrates that uses the yeast two-hybrid system: cloning of the antiapoptotic gene gelsolin. Proc Natl Acad Sci U S A 1998 Jul; 95 (15): 8532-7.
(142) Ekert PG, Silke J, Vaux DL. Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. EMBO J 1999 Jan; 18 (2): 330-8.

(143) Silke J, Ekert PG, Day CL, Hawkins CJ, Baca M, Chew J, et al. Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP. EMBO J 2001 Jun; 20 (12): 3114-23.
(144) Hayashi H, Cuddy M, Shu VC, Yip KW, Madiraju C, Diaz P, et al. Versatile assays for high throughput screening for activators or inhibitors of intracellular proteases and their cellular regulators. PLoS One 2009; 4 (10): e7655.
(145) Ho P-k, Jabbour AM, Ekert PG, Hawkins CJ**.** Caspase-2 is resistant to inhibition by inhibitor of apoptosis proteins (IAPs) and can activate caspase-7. FEBS Journal 2005; 272 (6): 1401-14.
(146) Jabbour AM, Ekert PG, Coulson EJ, Knight MJ, Ashley DM, Hawkins CJ**.** The p35 relative, p49, inhibits mammalian and Drosophila caspases including DRONC and protects against apoptosis. Cell Death Differ 2002 Dec; 9 (12): 1311-20.
(147) Wright ME, Han DK, Hockenbery DM**.** Caspase-3 and inhibitor of apoptosis protein(s) interactions in Saccharomyces cerevisiae and mammalian cells. FEBS Lett 2000 Sep; 481 (1): 13-8.
(148) Wright ME, Han DK, Carter L, Fields S, Schwartz SM, Hockenbery DM**.** Caspase-3 inhibits growth in Saccharomyces cerevisiae without causing cell death. FEBS Lett 1999 Mar; 446 (1): 9-14.
(149) Kang JJ, Schaber MD, Srinivasula SM, Alnemri ES, Litwack G, Hall DJ, et al. Cascades of mammalian caspase activation in the yeast Saccharomyces cerevisiae. The Journal of biological chemistry 1999; 274 (5): 3189-98.
(150) Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 1999 Jan; 144 (2): 281-92.
(151) Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL**.** DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. J Cell Biol 2001 Feb; 152 (3): 483-90.
(152) Wright ME, Han DK, Hockenbery DM**.** Caspase-3 and inhibitor of apoptosis protein(s) interactions in Saccharomyces cerevisiae and mammalian cells. FEBS letters 2000; 481 (1): 13-8.

CHAPTER 2

Differential regulation of p53 function by protein kinase C isoforms revealed by a yeast cell system

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DIFFERENTIAL REGULATION OF P53 FUNCTION BY PROTEIN KINASE C ISOFORMS REVEALED BY A YEAST CELL SYSTEM

2.1. ABSTRACT

The complexity of the mammalian p53 pathway and protein kinase C (PKC) family has hampered the discrimination of the effect of PKC isoforms on p53 activity. Using yeast co-expressing the human wild-type p53 and a mammalian PKC–α, –δ, –ε or –ζ, we showed a differential regulation of p53 activity and phosphorylation state by PKC isoforms. Whereas PKC-α reduced the p53-induced yeast growth inhibition and cell cycle arrest, PKC–δ, –ε enhanced the p53 activity through p53 phosphorylation, and PKC–ζ had no effect on p53. This work identified positive and negative p53 regulators which represent promising pharmacological targets in anti-cancer therapy.

Keywords: p53, PKC isoforms, Cell growth, Cell cycle, p53 phosphorylation, Yeast

2.2. INTRODUCTION

The p53 tumour suppressor protein is mutated in about half of all human tumours and in many others that retain a wild-type (WT) p53, p53-dependent pathways leading to cell cycle arrest or cell death are often deficient. Restoring p53 function represents therefore a promising approach for cancer therapeutics. Among the different mechanisms of p53 regulation, post-translational modification by phosphorylation has been shown to play a critical role in the stabilization and activation of WT p53 (1).

One of the key enzymes involved in p53 phosphorylation is protein kinase C (PKC) (2,3). PKC is a family of serine/threonine kinases with at least 10 isoforms grouped into three subfamilies based on their primary structure and cofactors required for activation: classical (α, βI, βII and γ), novel (δ, ε, η and θ) and atypical (ζ and λ/ι). PKC isoforms are important regulators of several cellular processes, such as cell proliferation and death, and a striking feature is that individual isoforms can exert either similar or opposite effects in these processes (4,5).

Due to the high complexity of the mammalian p53 pathway and PKC family, particularly the coexistence of several PKC isoforms in the same cell, discrimination of the role of each PKC isoform in the regulation of p53 activity has been hampered. To circumvent this limitation, in this work, we exploited yeast cells co-expressing the human WT p53 and an individual mammalian PKC isoform.

The remarkably high degree of conservation of many pathways and cellular processes among yeast and human has allowed transposing the knowledge obtained in yeast to mammalian cells and vice versa. Additionally, the data obtained with the expression of human proteins that lack a yeast orthologue have been crucial to elucidate the role of proteins and the molecular mechanisms of complex human disorders, such as cancer and neurodegenerative diseases (6). Since the yeast PKC (Pkc1p in *Saccharomyces cerevisiae*) is a structural but not a functional homologue of mammalian PKC isoforms, yeast was considered a well-suited organism to study individual mammalian PKC isoforms (7). Indeed, the yeast PKC assay has been used by us to search for isoform-selective PKC modulators (8) and to study the regulation of human apoptotic proteins by PKC isoforms (9). Similarly, though no orthologues of human p53 have been identified in yeast, *S. cerevisiae* has been extensively used to score several functional properties of human p53 (10).

The yeast expression systems established in this study allowed investigating the existence in yeast of a molecular mechanism underlying the regulation of cell proliferation through specific PKC isoforms and WT p53. The results obtained underscore a differential regulation of p53 activity by PKC isoforms with the identification of kinases responsible for both a positive and negative regulation of p53.

2.3. MATERIAL AND METHODS

2.3.1. Plasmids

Constructed yeast expression plasmids YEplac181-LEU2 encoding bovine PKC–α, rat PKC–δ, mouse PKC–ε or PKC–ζ and the empty vector, and pLS89-TRP1 encoding human WT p53 and the empty vector, were kindly provided by Dr. Nigel Goode (The Royal Veterinary College, UK) and Dr. Richard Iggo (Swiss Institute for Experimental Cancer Research, Switzerland), respectively. Plasmids used have a galactose-inducible *GAL1-10* promoter.

2.3.2. Yeast strain, transformation and growth conditions

S. cerevisiae CG379 was co-transformed by the lithium acetate method, as reported (9). For selection of co-transformed yeast, cells were routinely grown in a minimal selective medium, with 2% glucose, 0.67% yeast nitrogen base without amino acids and all the amino acids required for yeast growth (50 μg/ml) except leucine and tryptophan, to approximately 1 optical density $(OD)_{600}$. To induce expression of mammalian proteins, yeast were diluted to 0.05 OD_{600} in selective medium with 2% galactose and raffinose instead of glucose and incubated at 30 ºC with shake (200 r.p.m.) for up to 60 hours in growth curves experiments, or for approximately 45 hours (time required by control yeast, co-transformed with the empty vectors pLS89 and YEplac181, to achieve 0.5 OD_{600}) in all other experiments. Yeast growth was analysed by counting the number of colony-forming units per ml (CFU/ml) after 2 days incubation at 30 ºC on Sabouraud Dextrose Agar plates.

2.3.3. Effect of the selective PKC inhibitor Ro 32-0432 on yeast growth

To analyse the effect of Bisindolylmaleimide XI hydrochloride (Ro 32-0432; ALX-270-058, Alexis Biochemicals) on yeast growth, co-transformed cells were incubated in galactose selective medium with 1 μM Ro 32-0432 or solvent only (0.1% DMSO) for approximately 45 h, at 30 ºC with shake (200 r.p.m.). Yeast growth was analysed as described above.

2.3.4. Cell death markers

Plasma membrane integrity and DNA fragmentation were analysed by fluorescence microscopy using propidium iodide (PI) and *In Situ Cell Death Detection Kit*, Fluorescein, respectively, whereas yeast metacaspase (Yca1p) activation and reactive oxygen species (ROS) accumulation were analysed by flow cytometry using FITC-VAD-fmk and dihydroethidium (DHE), respectively, as described (8).

2.3.5. Cell cycle

Flow cytometric analysis of DNA content was performed using Sytox Green Nucleic Acid, as described (8). Yeast cell cycle phases were identified and quantified using ModFit LT software (Verity Software House Inc., Topsham, USA).

2.3.6. Western blot

For human WT p53 and mammalian PKC isoforms detection, anti-p53 (DO-1) and anti-PKC$-\alpha$/PKC$-\delta$/PKC$-\varepsilon$/PKC$-\zeta$ mouse monoclonal antibodies (Santa Cruz Biotechnology) were used. p53 phosphorylation was analysed using phospho-p53(Ser15) mouse monoclonal antibody, phospho-p53(Ser20) and phosphop53(Ser46) rabbit polyclonal antibodies (Cell Signalling Technology), and PAb421 mouse monoclonal antibody (Calbiochem). The etoposite-treated MCF7 cell lysate (sc-2281; Santa Cruz Biotechnology) was used as positive control. Immunoblots were developed by enhanced chemiluminescence, as described (9). Band intensities were quantified using Bio-Profil Bio-1D++ software.

2.3.7. Statistical analysis

Data were analysed statistically using *SigmaStat 3.5 programme*. Differences between means were tested for significance using the unpaired Student's *t*-test ($P < 0.05$).

2.4. RESULTS

2.4.1 Differential regulation of WT p53-induced yeast growth inhibition by PKC isoforms

In accordance with other authors (11,12), we verified that expression of human WT p53 in *S. cerevisiae* inhibited cell growth (Fig. 2.1A,B). Instead, and as previously reported by us (8,9), in our experimental conditions expression of a mammalian PKC isoform did not significantly interfere with yeast growth (Fig. 2.1A,B). However, when PKC$-\alpha$, $-\delta$, $-\varepsilon$ or $-\zeta$ was co-expressed with p53, a differential regulation of p53-induced growth inhibition by PKC isoforms was obtained (Fig. 2.1A,B). This was particularly evident for 45 hours incubation (Fig. 2.1B). For this time, whereas PKC$-\alpha$ significantly reduced the p53-induced growth inhibition, PKC$-\delta$ and $-\varepsilon$ significantly increased the p53 growth-inhibitory effect and PKC$-\zeta$ did not interfere with the p53 effect.

The direct effect of PKC isoforms on p53 was evidenced using the selective PKC inhibitor Ro 32-0432. Though 1 μM Ro 32-0432 did not significantly interfere with the growth of yeast expressing p53 or a PKC isoform only, it significantly reduced the effects exhibited by PKC$-\alpha$, $-\delta$ and $-\varepsilon$ on p53 activity (Fig. 2.1C).

As confirmed by Western blot analysis, this distinct influence of each PKC isoform on p53 effect was not a consequence of significant changes in the expression levels of p53 or a PKC isoform in yeast co-expressing both proteins (Fig. 2.2).

2.4.2. WT p53 yeast growth-inhibitory effect and its stimulation by PKC$-\delta$ and $-\varepsilon$ are not associated with cell death

A recent work associated the human WT p53 growth-inhibitory effect to the induction of an apoptotic cell death in *S. cerevisiae* (11). Hence, typical necrotic and apoptotic markers, as loss of plasma membrane integrity, DNA fragmentation, ROS accumulation and Yca1p activation, were investigated.

However, in our experimental conditions, expression of human WT p53 in yeast did not significantly increase the PI and TUNEL positive cells (Fig. 2.3A,B), Yca1p activation and ROS levels (Fig. 2.3C,D). Even in the presence of a PKC isoform, particularly PKC$-\delta$ or $-\varepsilon$, characteristic features of cell death were not detected (Fig. 2.3B–D). Indeed, though yeast co-expressing p53 and PKC$-\varepsilon$ presented a significant increase in ROS levels, this was not accompanied by an increase of PI and TUNEL positive cells and Yca1p activation (Fig. 2.3B–D).

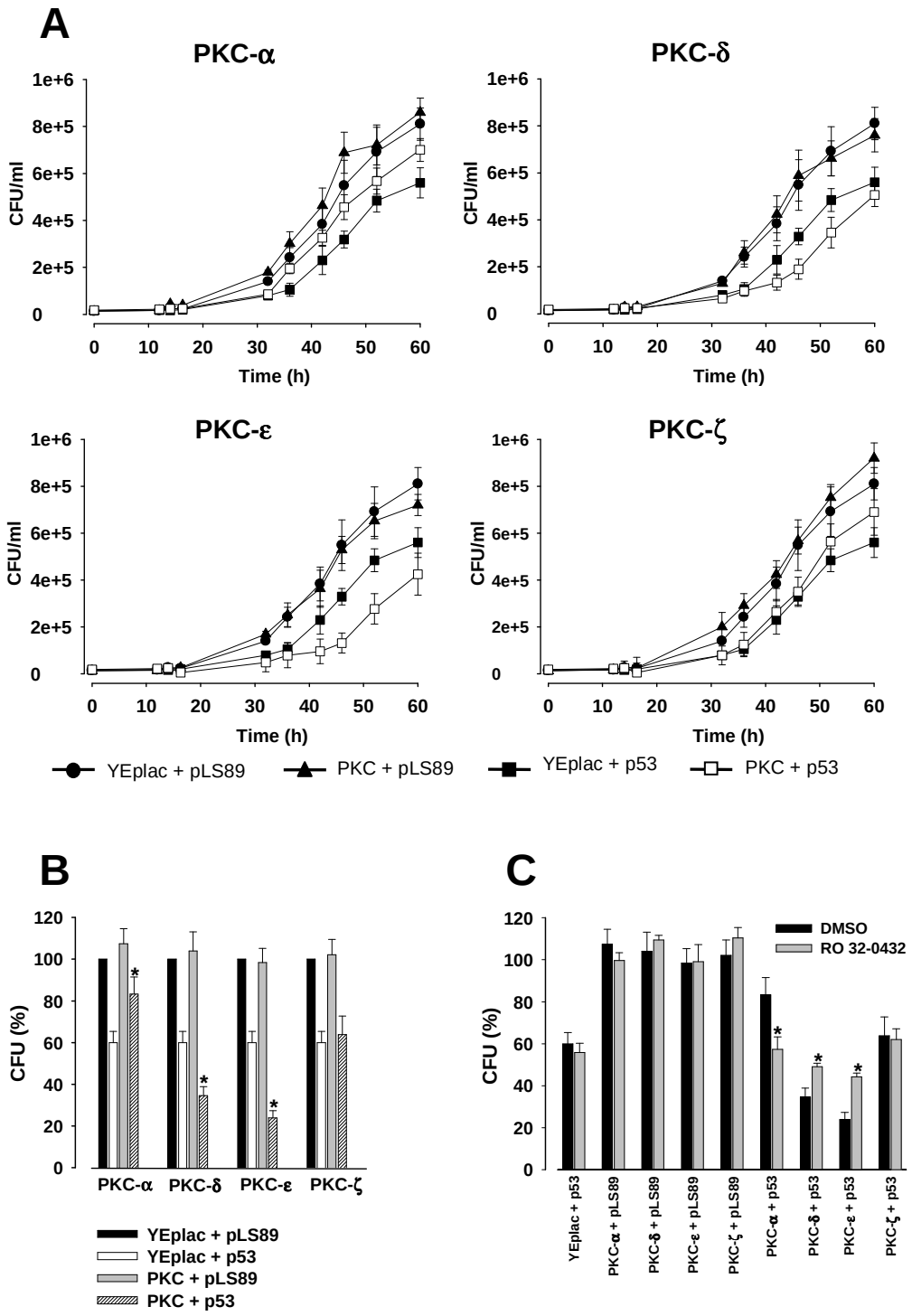

Fig. 2.1. WT p53-induced yeast growth inhibition is differently regulated by PKC isoforms. (**A**) Growth curves were obtained by CFU counts. (**B**) Percentage of growth obtained for 45 hours incubation; value of yeast co-expressing p53 and a PKC isoform significantly different from that of yeast expressing p53 only: **P*<0.05. (**C**) Effect of 1 μM RO 32-0432 on the growth of co-transformed cells for 45 hours incubation; value obtained with RO 32-0432 significantly different from that obtained with DMSO only: **P*<0.05. In **B** and **C**, values were obtained considering the growth achieved with control yeast (YEplac181+pLS89) as 100%. Data represent mean ± S.E.M. (n=4).

According to Amor et al. (11), a complete abolishment of yeast growth associated with cell death can be achieved using a multicopy vector for p53 expression and minimal medium culture conditions that rendered yeast more responsive to target gene regulation by p53. In fact, similarly to that obtained by us, Nigro et al. (12) only observed a modest yeast growth inhibition using a centromeric low-copy-number vector for p53 expression. However, considerable levels of p53 expression were also achieved in our work using a pLS89 centromeric vector (Fig. 2.2A,B). Additionally, a minimal medium similar to that described by Amor et al. (11) was used in our study. In order to understand the different results obtained, co-transformed yeast expressing WT p53 only (YEplac181 + p53) were treated with different concentrations of hydrogen peroxide (a known yeast apoptotic inducer, see (13)). In accordance with the high-level resistance to cell death detected in a previous work performed by us with co-transformed yeast (9), a significant percentage of TUNEL positive cells (21.3 ± 5.4%; n = 3), with a low percentage of IP positive cells (9.2 ± 4.1%; n = 3), was only achieved at high concentrations of hydrogen peroxide (10 mM H_2O_2, 1 hour treatment). Thus, the use of co-transformed yeast strains with high-level resistance to cell death may be a possible explanation for the results obtained by us.

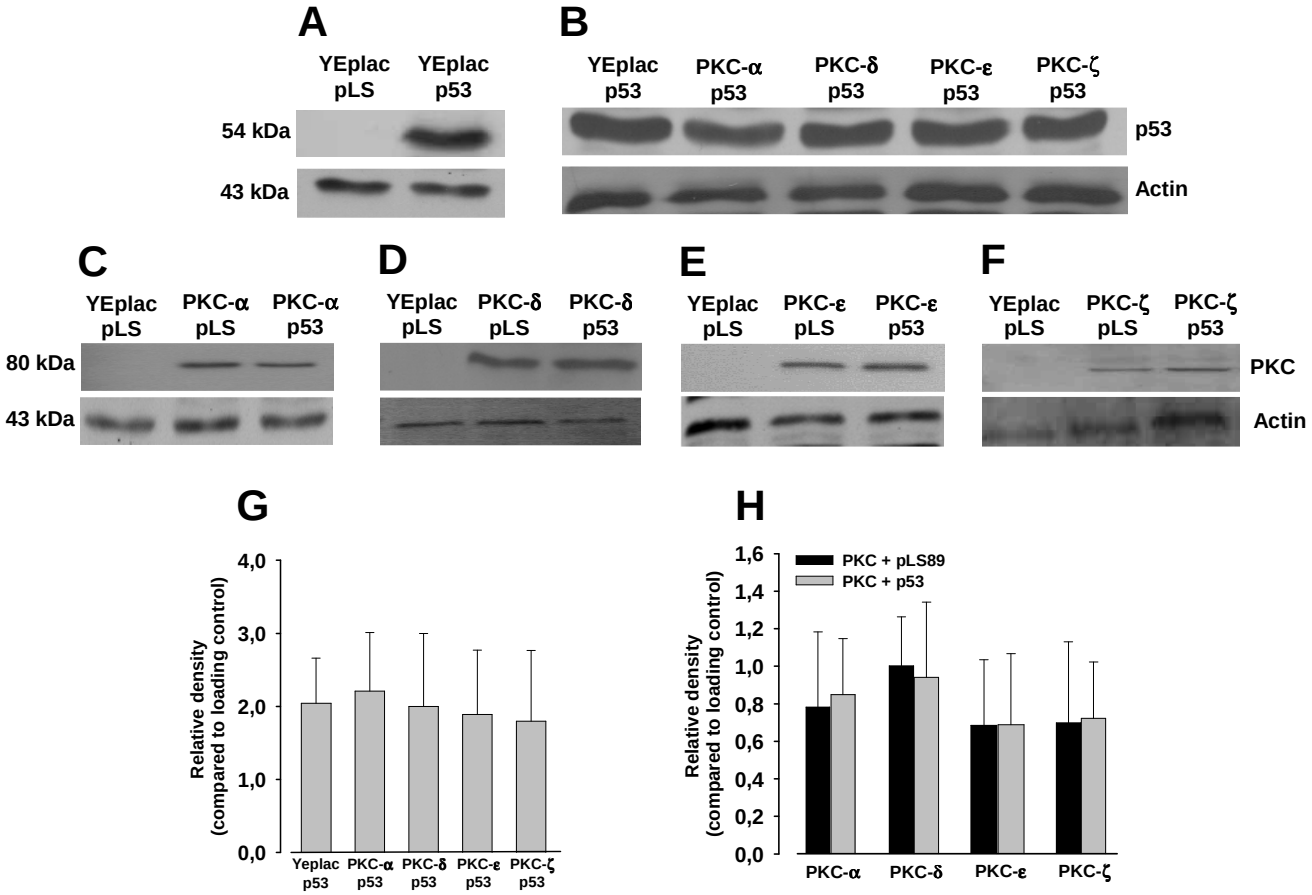

Fig. 2.2. Expression of human WT p53 or a mammalian PKC isoform is not affected by co-expression of both proteins in yeast. Western blot analysis of (**A**, **B**) p53, (**C**) PKC-α (**D**) PKC-δ (**E**) PKC-ε and (**F**) PKC-ζ

represent 1 of duplicate experiments; β-actin was used as loading control. (**G**, **H**) Quantification of band intensities of Western blots obtained for (**G**) p53 and (**H**) PKC isoforms; data represent mean ± S.E.M. (n=2).

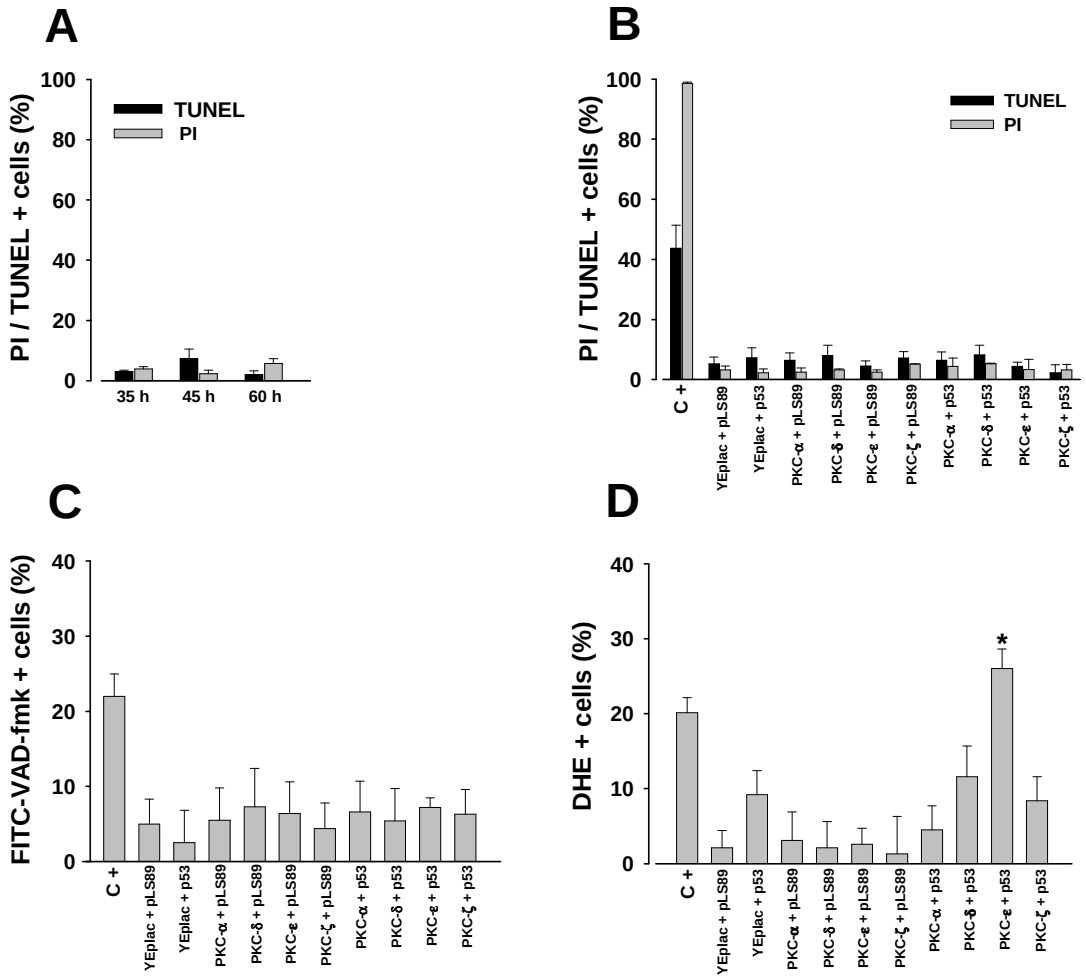

Fig. 2.3. Effects of WT p53 and/or a PKC isoform on yeast growth are not associated with cell death. (**A**) Cells with DNA fragmentation (TUNEL +) and necrotic cells (PI +) obtained from yeast expressing WT p53 for 35, 45 and 60 hours incubation; (**B**) TUNEL and PI positive cells, (**C**) Yca1p activation and (**D**) ROS accumulation obtained from yeast expressing WT p53 and/or a PKC isoforms for 45 hours incubation. In **B**, **C** and **D**, yeast expressing PKC-ε treated with 1 μM coleon U (see (8)) were used as positive control (C+). Data represent mean ± S.E.M. (n=2); values significantly different from control yeast (YEplac181+pLS89): * P<0.05.

2.4.3. WT p53 yeast growth-inhibitory effect is associated with S-phase cell cycle arrest that is differently regulated by PKC isoforms

As in mammalian cells, where p53 controls cell cycle mainly through the G1/S checkpoint (2), we detected that p53-induced yeast growth inhibition was associated with S-phase cell cycle arrest (Fig. 2.4). Instead, and in agreement with the absence of effect on yeast growth, PKC isoforms only slightly interfered with the yeast cell cycle progression (Fig. 2.4). However, when PKC–α, –δ, –ε or –ζ was co-expressed with p53, a specific PKC isoform-dependent modulation of cell cycle through p53 was obtained (Fig. 2.4).

Whereas PKC–α decreased the percentage of cells in S-phase and increased the percentage of cells in G2/M, PKC–δ and –ε markedly increased the percentage of cells arrested in S-phase. Consistently, PKC–ζ that did not affect the p53-induced growth inhibition also did not interfere with the p53-induced S-phase arrest.

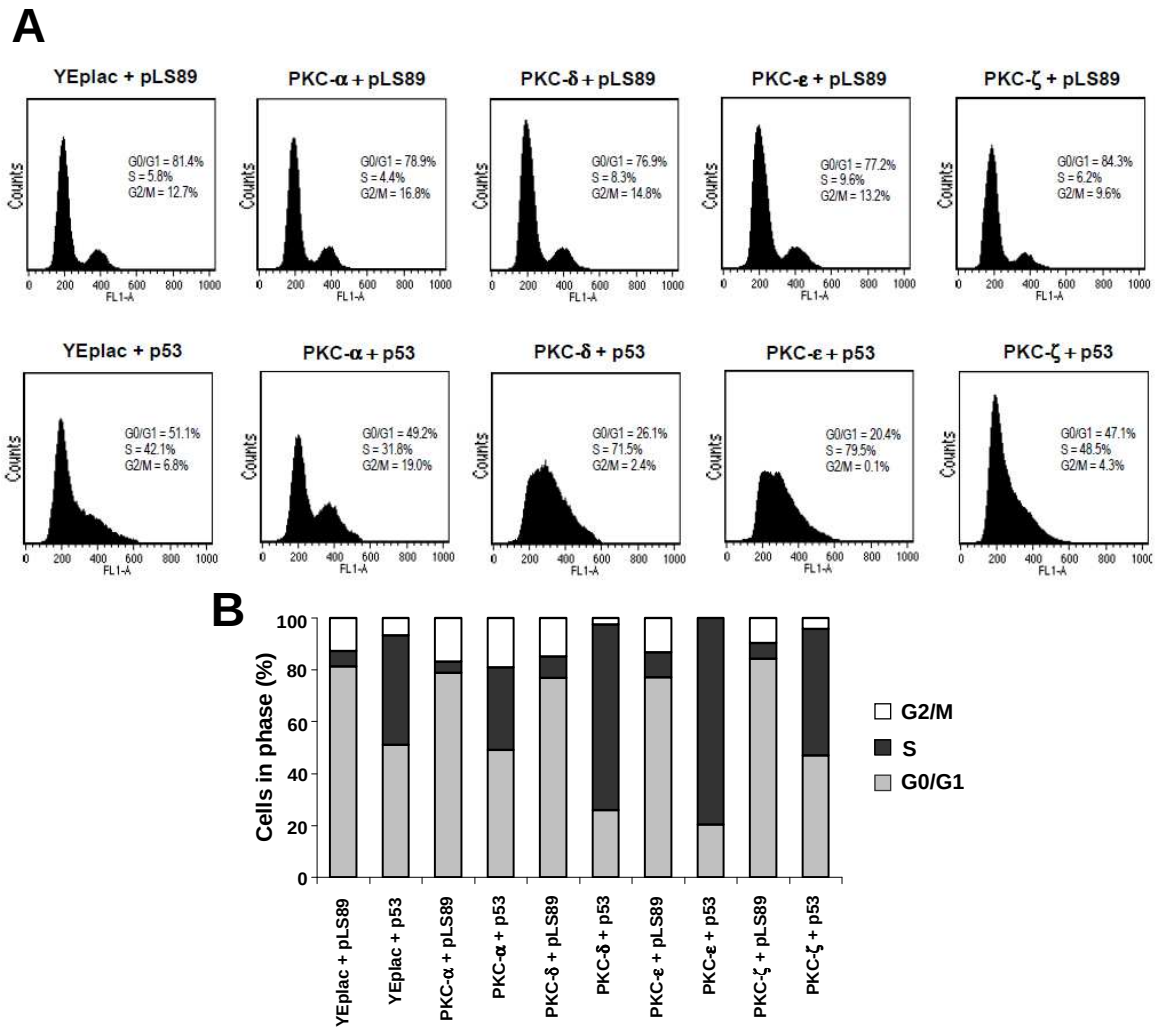

Fig. 2.4. WT p53-induced S-phase cell cycle arrest is differently regulated by PKC isoforms. (**A**) Histograms represent 1 of triplicate experiments. (**B**) Quantification of yeast cell cycle phases; data represent mean values (n=3).

2.4.4. Differential regulation of p53 effects by PKC isoforms is associated with distinct patterns of p53 phosphorylation in yeast

Phosphorylation of WT p53 at commonly reported p53 phosphorylation sites, Ser15, Ser20, Ser46 and Ser376-378 (2,3,14), was checked by Western blot analysis. p53 phosphorylation at Ser15, Ser20 and Ser46, analysed using a phospho-p53 antibody specific for each serine, was not detected neither in yeast expressing p53 only nor in yeast co-expressing p53 and a PKC isoform (Fig. 2.5A–C).

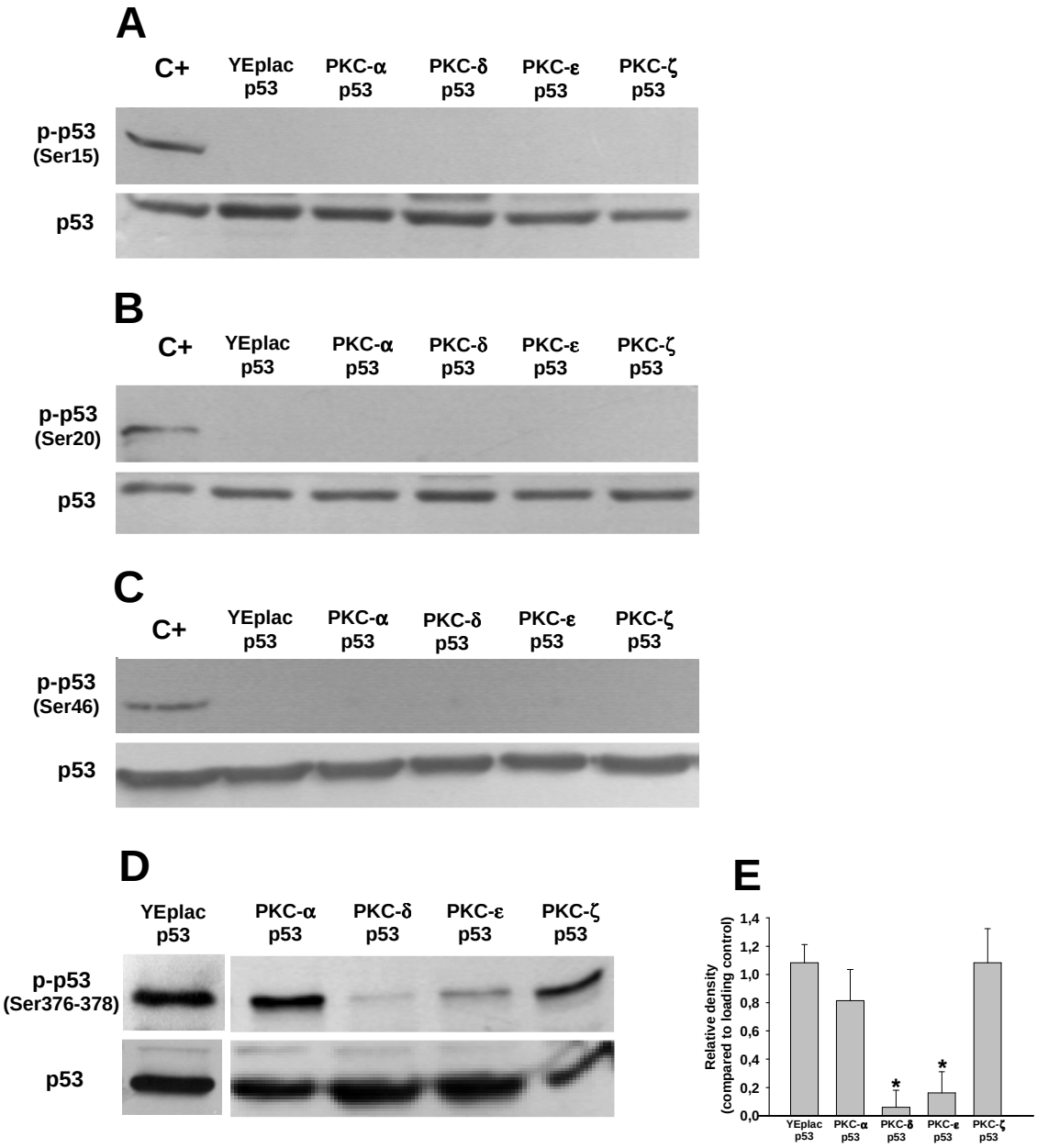

Fig. 2.5. PKC isoforms cause distinct patterns of p53 phosphorylation in yeast. p53 phosphorylation was analysed using (**A**) phospo-p53(Ser15), (**B**) phospo-p53(Ser20), (**C**) phospo-p53(Ser46) and (**D**) PAb421 (Ser376-378) antibodies. Detection of p53 with DO-1 antibody was used as a loading control. In **A**, **B** and **C**, the etoposite-treated MCF7 cell lysate was used as positive control (C+); in **D**, the positive control corresponds to p53 from yeast cells expressing p53 only (YEplac181+p53), which expression was confirmed using the anti-p53 DO-1 antibody. Immunoblots represent 1 of duplicate experiments. In **D**, YEplac181/p53 lane was located in a different part of the same gel. (**E**) Quantification of band intensities of **D**; data represent mean ± S.E.M. (n=2); values significantly different from control band (YEplac181+p53): **P*<0.05.

p53 phosphorylation at Ser376-378 was analysed using the PAb421 antibody which preferentially recognises unphosphorylated p53 residues at Ser376-378. Indeed, it was demonstrated that when p53 was phosphorylated at Ser376-378 by PKC the PAb421 band almost disappeared (3). In our case, a high intensity PAb421 band was obtained for

yeast expressing p53 only (Fig. 2.5D,E), indicating the absence of p53 phosphorylation at Ser376–378 by endogenous yeast kinases. This band was therefore used as control. Interesting, when compared to control band, whereas PKC–α slightly decreased and PKC–ζ did not interfere with the PAb421 band intensity, PKC–δ and –ε markedly decreased the intensity of this band (Fig. 2.5D,E). This indicated that, while PKC–α and –ζ did not significantly interfere with the degree of p53 phosphorylation, PKC–δ and –ε markedly increased p53 phosphorylation.

Overall, despite phosphorylation of human WT p53 by endogenous kinases was already reported for *S. cerevisiae* (12), to our knowledge the sites of p53 phosphorylation were still unclear. The results obtained indicated that p53 is not phosphorylated at Ser15, Ser20, Ser46 and Ser376-378 by yeast kinases. Importantly, though p53 phosphorylation by PKC has been described at distinct serine residues (2,3,14), in yeast we only detected phosphorylation at Ser376–378, which is the most commonly reported PKC phosphorylation site in mammalian cells (2,3). For this p53 residue, a PKC isoform-dependent phosphorylation pattern was identified.

2.5. DISCUSSION

The involvement of PKC family in the regulation of cell proliferation was early recognised. While PKC–α and –ζ are frequently associated to the proliferation of human cancers and PKC–δ is often linked to an anti-proliferative effect (4,5), PKC–ε has been associated both to proliferative (4,5) and anti-proliferative (15) effects. In spite of this, the molecular mechanism of induction or suppression of cell proliferation by PKC isoforms remains unclear. Another open issue is the role of PKC isoforms in the regulation of p53, a key player in cell proliferation and death. The PKC-mediated effects are in fact largely tissue and cell-type-specific, thus it has been difficult and controversial to extrapolate conclusions from one mammalian cell type to another. This study represents the first attempt to reconstitute in yeast key parts of the intricate mammalian p53-PKC network. With this yeast approach, it was possible to ascertain the effect of individual PKC isoforms, representative of the classical (PKC–α), novel (PKC–δ and –ε) and atypical (PKC–ζ) PKC subfamilies and considered major isoforms in carcinogenesis, cell proliferation and human WT p53 activity. The results obtained revealed that even though the PKC isoforms per se had no effect on yeast growth and cell cycle, they differentially interfered with the p53-induced yeast growth inhibition and cell cycle arrest and p53 phosphorylation. They underscored an anti-proliferative effect of PKC–δ and –ε through phosphorylation and consequent activation of p53. In opposition, they evidenced a proliferative effect of PKC–α through inhibition of p53 function. Further studies are underway to better elucidate the molecular mechanism of this PKC–α-inhibitory effect. Finally, the absence of effect of PKC–ζ on the activity and phosphorylation state of p53 indicated that p53 was not a target of this kinase. As a whole, this study established a unifying mechanism between the modulation of cell proliferation by PKC–α, –δ and –ε and the regulation of p53. For PKC–ζ, a p53-independent effect on cell proliferation was suggested. Additionally, it underscored a differential regulation of p53 activity and phosphorylation state by PKC isoforms, with the identification of kinases responsible for both a positive and negative regulation of p53. These kinases represent therefore promising molecular and pharmacological targets in anti-cancer therapy. As reported by others, the distinct roles of individual PKC isoforms in cancer progression suggests that either PKC activators or inhibitors may serve as anti-tumour agents if target specific members of the PKC family (5). Accordingly, our data support that selective PKC–α inhibitors as well as selective PKC–δ and –ε activators may be beneficial in the therapy of several cancers by inhibiting the proliferation particularly of those tumours where PKC–α

is up-regulated and PKC$-\delta$ or $-\varepsilon$ are down-regulated. This study will certainly contribute to the elucidation of the effect of PKC activators and inhibitors on tumour cells proliferation, providing new perspectives for PKC modulators as anti-cancer agents.

2.6. ACKNOWLEDGEMENTS

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2.7. REFERENCES

(1) Lu C, El-deiry WS. Targeting p53 for enhanced radio- and chemosensitivity. Apoptosis 2009 Apr; 14 (4): 597-606.

(2) Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ 2006 Jun; 13 (6): 941-50.

(3) Pospísilová S, Brázda V, Kucharíková K, Luciani MG, Hupp TR, Skládal P et al. Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. Biochem J 2004 Mar; 378 (3): 939-47.

(4) Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. Cancer Lett 2006 Apr; 235 (1): 1-10.

(5) Caino MC, Meshki J, Kazanietz MG. Hallmarks for senescence in carcinogenesis: novel signalling players. Apoptosis 2009 Apr; 14 (4): 392-408.

(6) Petranovic D, Nielsen J. Can yeast systems biology contribute to the understanding of human disease? Trends Biotechnol 2008 Nov; 26 (11):584-90.

(7) Parissenti AM, Riedel H. Yeast as a host to screen for modulators and regulatory regions of mammalian protein kinase C isoforms. Meth Mol Biol 2003; 233: 491-516.

(8) Coutinho I, Pereira G, Simões MF, Côrte-Real M, Gonçalves J, Saraiva L. Selective activation of protein kinase C-d and -e by 6, 11, 12, 14- tetrahydroxy-abieta-5, 8, 11, 13-tetraene-7-one (coleon U). Biochem Pharmacol 2009 Sep; 78 (5): 449-59.

(9) Saraiva L, Silva RD, Pereira G, Gonçalves J, Côrte-Real M. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci 2006 Aug; 119 (15): 3171-81.

(10) Smardová J, Smarda J, Koptíková J. Functional analysis of p53 tumor suppressor in yeast. Differentiation 2005 Jul; 73 (6): 261-77.

(11) Amor IY-H, Smaoui K, Chaabène I, Mabrouk I, Djemal L, Elleuch H, et al. Human p53 induces cell death and downregulates thioredoxin expression in Saccharomyces cerevisiae. FEMS Yeast Res 2008 Dec; 8 (8): 1254-62.

(12) Nigro JM, Sikorski R, Reed SI, Vogelstein B. Human p53 and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae. Mol Cell Biol 1992 Mar; 12 (3): 1357-65.

(13) Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH et al. Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 1999 May; 145 (4): 757-67.

(14) Yoshida K, Liu H, Miki Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. J Biol Chem 2006 Mar 3; 281 (9): 5734-40.

(15) Zhang Y, Venugopal SK, He S, Liu P, Wu J, Zern MA. Ethanol induces apoptosis in hepatocytes by a pathway involving novel protein kinase C isoforms. Cell Signal 2007 Nov; 19 (11): 2339-50.

CHAPTER 3

Distinct regulation of p53-mediated apoptosis by protein kinase Cα, δ, ε and ζ: evidence in yeast for transcription-dependent and -independent p53 apoptotic mechanisms

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DISTINCT REGULATION OF P53-MEDIATED APOPTOSIS BY PROTEIN KINASE Cα, δ, ε AND ζ: EVIDENCE IN YEAST FOR TRANSCRIPTION-DEPENDENT AND -INDEPENDENT P53 APOPTOTIC MECHANISMS

3.1. ABSTRACT

The role of individual protein kinase C (PKC) isoforms in the regulation of p53-mediated apoptosis is still uncertain. Using yeast cells co-expressing the human wild-type p53 and a single mammalian PKCα, δ, ε or ζ, we showed a differential regulation of p53-mediated apoptosis by these PKC isoforms. Whereas PKCα and ζ had no effect on p53 activity, PKCδ and ε stimulated a p53-mediated mitochondria-dependent apoptosis. Moreover, using pifithrin-α and -μ, selective inhibitors of p53 transcriptional activity and mitochondrial p53 translocation, respectively, we showed the activation of a transcription-dependent and -independent p53-mediated apoptosis by PKCδ and ε. The activation of mitochondrial p53 translocation by PKCδ and ε was further confirmed by immunofluorescence and Western blot analysis.

Together, this work reveals the conservation in yeast of functional transcription-dependent and -independent p53 apoptotic mechanisms. Furthermore, it gives mechanistic insights about the regulation of p53-mediated apoptosis by PKCδ and ε through modulation of p53 transcriptional activity and of its translocation to mitochondria. Finally, it underscores a major role of PKCδ and ε as positive regulators of p53-mediated apoptosis, and therefore as promising therapeutic targets in cancer.

Keywords: p53; PKC isoforms; Apoptosis; Transcription; Mitochondria; Yeast

3.2. INTRODUCTION

The major tumour suppressor protein, p53, is a sequence-specific transcription factor that determines the fate of a cell. p53 regulates the expression of an assortment of genes involved in cell cycle regulation, apoptosis and numerous other processes. In large part, the potent tumour suppressing function of p53 has been attributed to its ability to induce apoptosis. Despite the prominence of p53 nuclear transcriptional activity in the induction of apoptosis, a transcription-independent mechanism involving mitochondrial p53 translocation, is receiving increasing attention. Indeed, recent findings provide encouragement to further explore the potential of mitochondrial p53-based cancer therapeutics (1-3).

Inactivating mutations of the p53 gene are found in approximately half of all human cancers. In most of the remaining cancers that retain a wild-type (WT) p53, the p53 pathway is deactivated by an increase in its inhibitors, a reduction in its activators or by inactivation of downstream targets (1). In these cases, restoring WT p53 apoptotic function has been recognised as a promising strategy for cancer therapy. Hence, a detailed understanding of the mechanisms of regulation of p53-mediated apoptosis has been an important research objective with significant clinical impact (3).

One of the key enzymes involved in the regulation of WT p53 function is the protein kinase C (PKC). PKC is a family of serine/threonine kinases with at least 10 isoforms grouped into three major subfamilies according to their primary structure and cofactors required for activation: classical (α, βI, βII and γ), novel (δ, ε, η and θ) and atypical (ζ and λ\ι). PKC isoforms are important regulators of several cellular processes, such as cell proliferation and death, and a striking feature is that individual isoforms can exert either similar or opposite effects in these processes (4). In fact, several studies underscore a major role of PKC, particularly of PKCδ, in the regulation of p53 apoptotic activity. It was demonstrated that PKCδ leads to p53 accumulation and phosphorylation with consequent activation of a p53-mediated apoptosis (5-10).

However, due to the high complexity of the mammalian p53 pathway and PKC family, namely the coexistence of several PKC isoforms in the same cell, the discrimination of the role of PKC isoforms in the regulation of p53 apoptotic mechanisms, particularly of its transcription-dependent and -independent activity, is still uncertain. To address this issue, yeast cells co-expressing the human WT p53 and an individual mammalian PKC isoform of the classical (PKCα), novel (PKCδ and ε) and atypical (PKCζ)

PKC subfamilies, and considered the isoforms most commonly involved in carcinogenesis, were used. In fact, using this yeast co-expression system, a differential modulation of p53-induced yeast growth inhibition and cell cycle arrest by distinct PKC isoforms was shown in our previous work (11). Moreover, other authors have confirmed the versatility of the yeast cell model to study different aspects of p53 function (12). For instance, a recent work showed that p53 could also function as a sequence-specific transcription factor in yeast. This study revealed several remarkable similarities between the transcription-dependent p53 activity in yeast and mammalian cells (13).

With the yeast approach used, it was possible to ascertain the role of PKCα, δ, ε and ζ in the regulation of p53-mediated apoptosis. In contrast to PKCα and ζ, PKCδ and ε activated transcription-dependent and -independent p53 mechanisms that cooperated to ultimately cause an apoptotic cell death. This study therefore identified PKCδ and ε as major positive regulators of p53-mediated apoptosis, and consequently as promising therapeutic targets for cancer treatment. Another relevant point arising from this study was to provide the first evidence for the conservation in yeast of a transcription-dependent and -independent p53-mediated apoptosis, with the validation of the yeast cell model to further understand the regulation of these p53 apoptotic mechanisms.

3.3. MATERIAL AND METHODS

3.3.1. Plasmids

The following yeast expression plasmids were used: pLS89-*TRP1* encoding human WT p53 and the respective empty vector (kindly provided by Dr. Richard Iggo, Swiss Institute for Experimental Cancer Research, Switzerland); YEplac181-*LEU2* encoding bovine PKCα, rat PKCδ, mouse PKCε or PKCζ and the respective empty vector (kindly provided by Dr. Nigel Goode, The Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK). All plasmids have a galactose-inducible *GAL1-10* promoter.

3.3.2. Yeast strain, transformation and growth conditions

Co-transformants of *Saccharomyces cerevisiae* strain CG379 were prepared in previous work (11). To ensure selection of co-transformed yeast, cells were routinely grown in a minimal selective medium with 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids, and all the amino acids required for yeast growth (50 μg/ml) except leucine and tryptophan, at 30ºC with mechanical shaking (200 r.p.m.) to approximately 1 optical density measured at 600 nm (OD_{600}; Jenway 6310 Spectrophotometer, Jenway). To induce expression of mammalian proteins, yeast cultures were diluted to 0.05 OD_{600} in selective medium with 2% (w/v) galactose and 1% (w/v) raffinose, instead of glucose, and incubated at 30ºC with mechanical shaking (200 r.p.m.) to 0.45 OD_{600}. Expression of human WT p53 and/or a mammalian PKC isoform (α, δ, ε or ζ in *S. cerevisiae* was confirmed by Western blot analysis in previous work (11).

3.3.3. Cell death assays

Co-transformed yeast cells, previously grown in galactose selective medium to 0.45 OD_{600}, were treated with 5 mM H_2O_2 for 1 hour at 30ºC with mechanical shaking (200 r.p.m.). Cell death was assessed by counting the number of colony-forming units (CFU) after 2 days incubation at 30ºC on Sabouraud Dextrose Agar plates. For each co-transformant, the percentage of dead cells was estimated considering 100% survival (0% death) as the number of CFU obtained with cells incubated in the same conditions but without H_2O_2.

3.3.4. Propidium iodide (PI) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining

PI and TUNEL staining, to monitor plasma membrane integrity and DNA fragmentation, respectively, were analysed by fluorescence microscopy, as described (14,15). For PI staining, cells were incubated with 5 μg/ml PI (Molecular Probes) for 10 minutes at room temperature. TUNEL assay was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science).

3.3.5. Reactive oxygen species (ROS) accumulation and modification of mitochondrial transmembrane potential ($\Delta\psi m$)

Mitochondrial ROS accumulation and modification of $\Delta\psi m$ were monitored by flow cytometry, as described (15). For analysis of ROS accumulation and modification of $\Delta\psi m$, about 106 untreated and 5 mM H_2O_2-treated cells were incubated with 5 μg/ml dihydroethidium (DHE; Sigma-Aldrich) and 1 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Molecular Probes), respectively, for 30 minutes at 30ºC. For analysis of modification of $\Delta\psi m$, 2 μg/ml PI was added to exclude necrotic cells; yeast cells treated with 0.4 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich) for 15 minutes at 30ºC, instead of H_2O_2, were used as positive control of depolarised cells.

3.3.6. Mitochondrial network fragmentation

Fragmentation of mitochondrial network was analysed using cells transformed with the pCLbGFP-*URA3* plasmid encoding green fluorescent protein (GFP) fused to the mitochondrial presequence of citrate synthase (mt-GFP) under the control of the *GAL1/10* promoter, as described (15). In this set of experiments, uracil was also omitted from the selective medium. Untreated and 5 mM H_2O_2-treated cells were observed under a fluorescence microscopy.

3.3.7. Generation of rho^0 mutants from WT co-transformed yeast cells, growth conditions and cell death assays

For abrogation of mtDNA (rho^0), WT co-transformed yeast cells were treated basically as described (16). Briefly, cells were grown in minimal selective medium containing 10 μg/ml ethidium bromide (Bio-rad) for 3 days. Treatment with ethidium bromide was repeated twice. The resulting respiratory deficiency was confirmed by

complete lack of growth on obligatory respiratory selective medium, containing 3% (v/v) glycerol instead of glucose. Depletion of mtDNA was also confirmed by fluorescence microscopy using 4 μg/ml 4`,6-diamidino-2-phenylindole (DAPI; Molecular Probes). To induce expression of mammalian proteins, rho^0 co-transformed yeast cells were grown in the galactose selective medium described in 2.2., supplemented with 0.2% (w/v) glucose, as reported (16). Effect of 5 mM H_2O_2 on cell death of WT and rho^0 co-transformed yeast cells was assessed by CFU counts as described in 3.3.3.

3.3.8. Ro 32-0432, pifithrin-α and pifithrin-μ assays

To analyse the effect of bisindolylmaleimide XI hydrochloride (Ro 32-0432; Alexis Biochemicals) and pifithrin-α (PFT-α; Sigma-Aldrich) on cell death, co-transformed yeast cells were grown in galactose selective medium with 1 μM Ro 32-0432, 10 μM PFT-α or solvent (0.1% DMSO) only at 30ºC with mechanical shaking (200 r.p.m.) to 0.45 OD_{600} before treatment with 5 mM H_2O_2. For pifithrin-μ (PFT-μ, Calbiochem), co-transformed yeast cells were grown in galactose selective medium at 30ºC with mechanical shaking to 0.4 OD_{600}, and then incubated with 75 μM PFT-μ or 0.1% DMSO only for 4 hours before treatment with 5 mM H_2O_2; effect of PFT-μ on $\Delta\psi m$ was monitored by flow cytometry as described in 3.3.5. Effects of Ro 32-0432, PFT-α and PFT-μ on yeast cell death were assessed by CFU counts as described in 3.3.3. To analyse the effect of PFT-α on yeast cell growth, co-transformed yeast cells were incubated in galactose selective medium with 10 μM PFT-α or 0.1% DMSO only at 30ºC with mechanical shaking for approximately 45 hours (time required by control yeast, co-transformed with the empty vectors YEplac181 and pLS89, to achieve 0.5 OD_{600}); yeast cell growth was assessed by CFU counts considering 100% growth (0% growth inhibition) as the number of CFU obtained with control yeast.

3.3.9. Immunofluorescence assays

Immunofluorecence assays were performed basically as described (15). About 10^7 untreated and 5 mM H_2O_2-treated yeast cells were incubated with 0.4 μg/ml MitoTrackerRed CM-H_2XROS (Molecular Probes) for 20 minutes at room temperature for mitochondrial labelling. After fixation, spheroplasts formation and permeabilization, spheroplasts were incubated with the anti-mouse p53 monoclonal antibody DO-1 (1:500; Santa Cruz Biotechnology) for 2 hours at room temperature, followed by the anti-mouse Alexa Fluor 488 secondary antibody (1:200; Molecular Probes) for 2 hours at room

temperature. Mounting medium containing 1.5 μg/ml DAPI (Vector Laboratories) was used to visualize nuclear DNA. Samples were observed under a fluorescence microscope.

3.3.10. Preparation of whole cell extracts and mitochondrial fractions

Whole cell extracts (WCE) and mitochondrial fraction were prepared basically as described (17). Briefly, for spheroplasts formation, untreated and 5 mM H_2O_2-treated yeast cells were incubated in dithiothreitol (DTT) buffer (100 mM Tris–H2SO4, pH 9.4, 10 mM DTT) for 30 minutes at 30℃ with mechanical shaking (80 r.p.m.), and then in zymolyase buffer (1.2 M sorbitol, 1 mM EDTA, 60 mM K2PO4, pH 7.5) containing 2.5 mg/g of cells (wet weight) of zymolyase-20T (MP Biomedicals Solon) for approximately 45 minutes at 30℃ with mechanical shaking (80 r.p.m.). For cell homogenization, spheroplasts were resuspended in ice-cold homogenization buffer (0.5 M sorbitol, 20 mM Tris-base, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride). The resulting homogenate (WCE) was centrifuged twice (at 1500 g and 3000 g) for 5 minutes at 4℃ to pellet cell debris and nuclei. Mitochondrial fraction was pellet by centrifugation at 12,000 g for 15 minutes at 4℃. Proteins were precipitated with 3 M trichloroacetic acid.

3.3.11. Western blot analysis

Western blot was performed basically as described (11). Protein content was determined using Coomassie Protein Assay Reagent Kit (Pierce). p53 phosphorylation in 5 mM H_2O_2-treated yeast cells was analysed as described (11) and using the primary rabbit polyclonal antibodies phospho-p53(Ser15) (1:4000; Calbiochem), phospho-p53(Ser20) (1:500; Calbiochem) and phospho-p53(Ser46) (1:500; Calbiochem) and the mouse monoclonal antibody PAb421 (1:100; Calbiochem), followed by incubation with the respective horseradish peroxidise (HRP)-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology). The etoposide-treated MCF7 cell lysate (Santa Cruz Biotechnology) was used as positive control. For p53 subcellular localization, 10 μg of WCE and mitochondrial fraction were analysed using the primary mouse monoclonal antibodies against p53 (DO-1) (1:500; Santa Cruz Biotechnology), yeast porin (Por1p) (1:5000; Molecular Probes) and yeast phosphoglycerate kinase (Pgk1p) (1:6000; Molecular Probes), followed by the anti-mouse HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Immunoblots were developed by chemiluminescence. Band intensities were quantified using Bio-Profil Bio-1D++ software (Vilber-Lourmat).

3.3.12. Flow cytometric data acquisition and analysis

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) and the CellQuest software (BD Biosciences).

3.3.13. Fluorescence microscopy

For fluorescent microscopic examination, samples were observed under an Eclipse E400 fluorescence microscope (Nikon) equipped with a 100 W mercury lamp and appropriate filter setting. Yeast cells were observed with an oil immersion lens (Plan Fluor 100/1.30) and images were captured by a Digital Sight Camera System (Nikon DS-5Mc) carrying built-in software for image acquisition (Nikon ACT-2U).

3.3.14. Statistical analysis

Data were analysed statistically using the *SigmaStat 3.5 software*. Differences between means were tested for significance using the unpaired Student´s *t* test ($P < 0.05$).

3.4. RESULTS

3.4.1 Differential regulation of p53 effects by PKCα, δ, ε and ζ in H_2O_2-treated yeast cells

To ascertain the role of individual mammalian PKC isoforms in the regulation of human WT p53-mediated apoptosis, co-transformed yeast cells were treated with H_2O_2. The H_2O_2 is an inducer of yeast apoptosis (18) and of a p53-mediated apoptotic pathway in mammalian cells (5,19). Moreover, the involvement of PKCδ in the activation of a p53-mediated apoptosis was previously demonstrated in H_2O_2-treated mammalian cells (5,7). To estimate the percentage of dead cells, for each co-transformant the number of CFU obtained with yeast cells incubated for 1 hour without H_2O_2 was considered as 100% survival (0% death). With this procedure, the previously reported growth inhibition induced by expression of human WT p53 in yeast (11) was not taken into account.

In accordance with the previously reported high-level resistance of co-transformed yeast cells to cell death (11,14), a pronounced resistance to H_2O_2-induced yeast apoptosis was also observed with the experimental conditions and co-transformed yeast cells used in this study. Indeed, upon 1 hour treatment with 5 mM H_2O_2, control yeast (co-transformed with the empty vectors, YEplac181 and pLS89) exhibited no more than 37% of dead cells (Fig. 3.1A) and a non-significant percentage of TUNEL-positive (DNA fragmentation; Fig. 3.1B) and PI-positive (loss of plasma membrane integrity; Fig. 3.1C) cells.

We firstly analysed the effect of single expression of human WT p53 and a mammalian PKCα, δ, ε or ζ on 5 mM H_2O_2-induced yeast cell death. Surprisingly, with the exception of PKCε, expression of these proteins did not significantly increase the percentage of dead cells, TUNEL- and PI-positive cells, when compared to control yeast (Fig. 3.1A-C).

However, when PKCα, δ, ε and ζ were individually co-expressed with p53, a differential regulation of p53 effects was obtained in H_2O_2-treated yeast cells. In fact, when compared to yeast cells expressing only p53, contrarily to PKCα and ζ, co-expression of PKCδ\ε with p53 significantly increased the p53 effects. Indeed, co-expression of PKCδ/ε with p53 significantly increased the percentage of dead cells and cells with DNA fragmentation obtained with the single expression of p53, without interfering with the plasma membrane integrity (Fig. 3.1A-C). Although expression of PKCε significantly increased per se H_2O_2-induced cell death, when co-expressed with p53 a 1.3-fold increase in the percentage of dead cells was obtained. The stimulation of p53-mediated

cell death by PKCδ and ε was further supported using the selective PKC inhibitor Ro 32-0432. Indeed, though 1 μM Ro 32-0432 did not interfere with the percentage of H_2O_2-induced dead cells obtained with control yeast and yeast expressing only p53 or PKC isoform, it reduced the increase in the percentage of H_2O_2-induced dead cells obtained when PKCδ/ε were co-expressed with p53 (Fig. 3.1D).

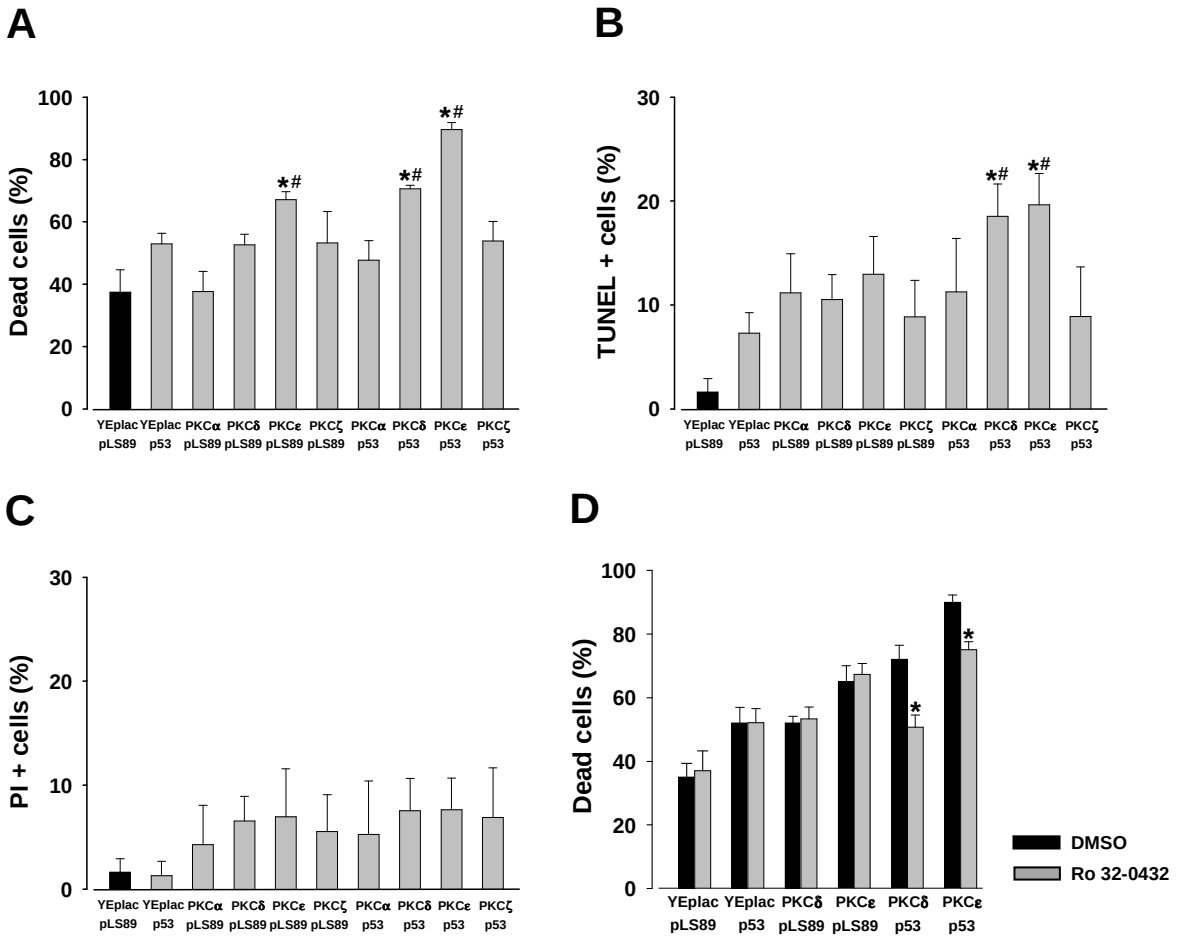

Fig. 3.1. PKCδ and ε increase p53 effects on cell death and DNA fragmentation without interfering with the plasma membrane integrity of 5 mM H_2O_2-treated yeast cells. Control yeast (YEplac/pLS89) and yeast expressing p53 and/or a PKC isoform (α, δ, ε or ζ) were incubated in galactose selective medium to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. Cell death, DNA fragmentation and plasma membrane integrity were analysed by CFU counts (for each co-transformant 100% survival, 0% death, was considered the number of CFU obtained with H_2O_2-untreated cells), TUNEL and PI staining, respectively. Percentage of (**A**) dead cells, (**B**) cells with DNA fragmentation and (**C**) cells with loss of plasma membrane integrity obtained with control yeast and yeast expressing p53 and/or a PKC isoform after treatment with 5 mM H_2O_2. Data represent means ± s.e.m. of 4 independent experiments; values significantly different from control yeast (*P < 0.05) and yeast expressing only p53 ($^{\#}P$ < 0.05). (**D**) Effect of the selective PKC inhibitor Ro 32-0432 on the percentage of dead cells obtained with 5 mM H_2O_2-treated control yeast and yeast expressing p53 and/or PKCδ/ε. Before treatment with 5 mM H_2O_2, yeast cells were incubated in galactose selective medium with 1 μM Ro 32-0432 or DMSO only to 0.45 OD_{600}. Data represent means ± s.e.m. of 4 - 5 independent experiments; values significantly different from DMSO only, *P < 0.05.

Additionally, co-expression of PKCδ/ε with p53 significantly increased the percentage of cells with mitochondrial ROS accumulation (Fig. 3.2A,B) and Δψm loss (Fig. 3.3A,B) obtained with H_2O_2-treated yeast cells expressing only p53. Curiously, as previously reported, even in the absence of a stress stimulus, a marked mitochondrial ROS accumulation was observed in yeast cells co-expressing p53 and PKCε (11). Moreover, though the single expression of p53 or PKC isoform did not increase the percentage of H_2O_2-induced mitochondrial network fragmentation obtained with the control yeast (56.2 ± 8.3%, n=4), this percentage was significantly increased when PKCδ/ε were co-expressed with p53 (Fig. 3.3C). In ROS accumulation, Δψm modification and mitochondrial network fragmentation experiments, to estimate the increase of p53 effects by a PKC isoform, the effect obtained with yeast expressing only p53 was subtracted to that obtained with yeast co-expressing p53 and PKC isoform. The requirement of respiring mitochondria for the PKCδ and ε stimulatory effect was confirmed using co-transformed yeast cells devoid of mtDNA (rho^0). Surprisingly, although respiratory deficient yeast cells have been reported as more sensitive to H_2O_2 (20), this was not detected with our rho^0 co-transformed yeast cells (Fig. 3.3D, control yeast) In spite of this, and even though similar expression levels of p53 and PKCδ/ε were detected in WT and rho^0 co-transformed yeast cells (data not shown), PKCδ and ε failed to stimulate p53-mediated cell death in H_2O_2-treated rho^0 cells (Fig. 3.3D).

Together, these results indicated that PKCδ and ε, contrarily to PKCα and ζ were activators of a p53-mediated mitochondria-dependent apoptosis.

3.4.2. Phosphorylation of p53 at Ser376-378 was detected in H_2O_2-treated yeast cells co-expressing p53 and PKCδ/ε

As previously performed for untreated yeast cells (11), phosphorylation of p53 by PKCδ and ε at commonly reported PKC phosphorylation sites, namely Ser15 (5,9), Ser20 (5), Ser46 (7,10) and Ser376-378 (6,8), was also checked in H_2O_2-treated yeast cells by Western blot analysis.

In 5 mM H_2O_2-treated yeast cells, phosphorylation of p53 at Ser15, Ser20 and Ser46 was not detected neither in yeast cells expressing only p53 nor in yeast cells co-expressing p53 and PKCδ/ε (Fig. 3.4A-C). However, phosphorylation of p53 at Ser376-378 was detected in yeast cells co-expressing p53 and PKCδ/ε. In fact, using the PAb421 antibody, which preferentially recognises unphosphorylated p53 residues at Ser376-378 (6), a high intensity PAb421 band was obtained with yeast cells expressing only p53.

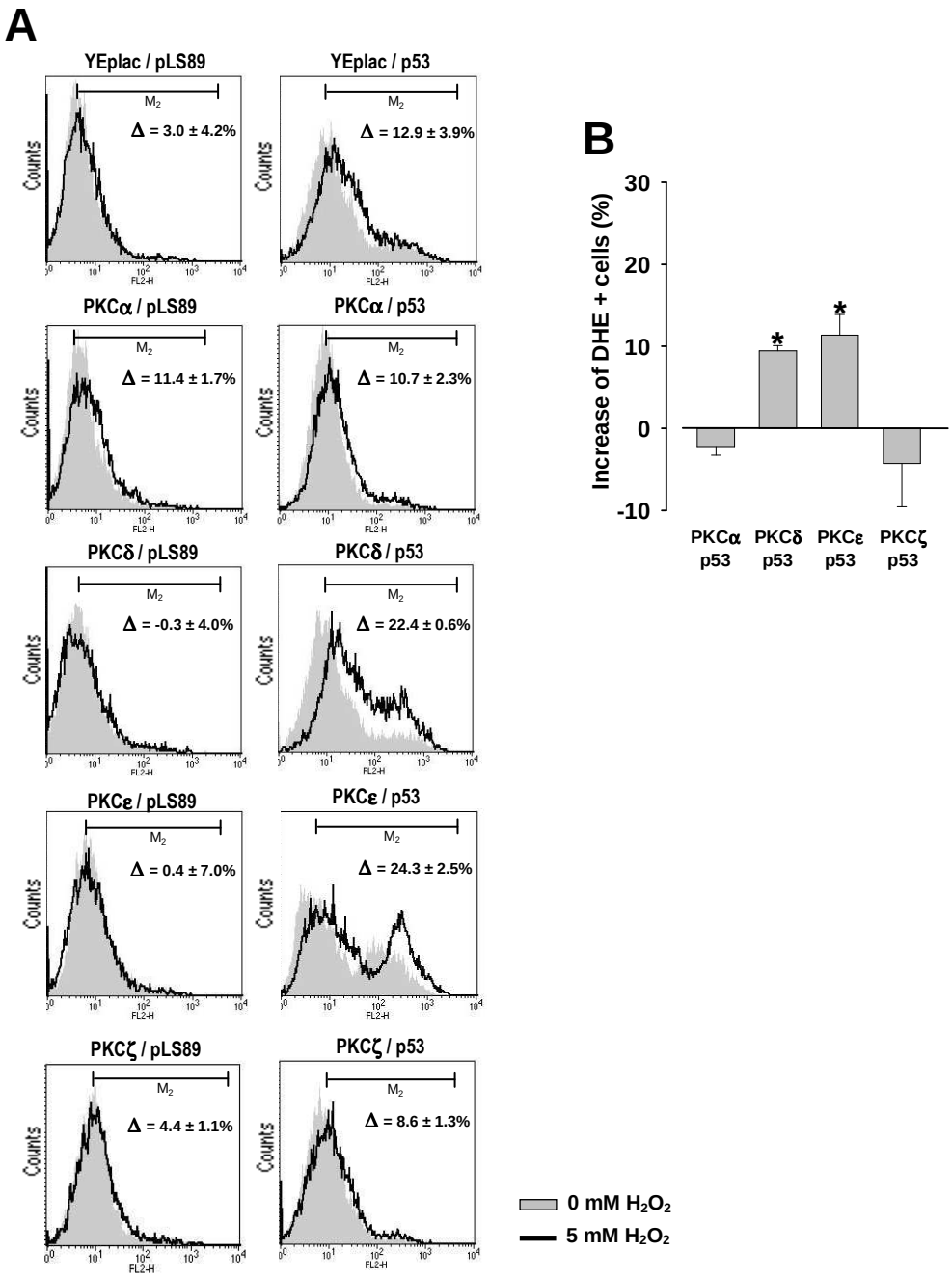

Fig. 3.2. PKCδ and ε increase the p53 effect on mitochondrial ROS accumulation. Control yeast (YEplac/pLS89) and yeast expressing p53 and/or a PKC isoform (α, δ, ε or ζ) were incubated in galactose selective medium to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. ROS accumulation was analysed by flow cytometry using DHE. (**A**) Overlays of red fluorescence histograms were obtained with untreated (0 mM) and 5 mM H_2O_2-treated cells; histograms represent 1 of 2 independent experiments. M_2 cursor indicates the sub-populations analysed; Δ values correspond to the increase in the percentage of DHE positive cells obtained when cells were treated with 5 mM H_2O_2, and represent means ± s.e.m. of 2 independent experiments. (**B**) Increase in the percentage of DHE positive cells obtained when PKCα, δ, ε or ζ were co-expressed with p53; values correspond to differences between the effect obtained with yeast co-expressing p53 and PKC isoform and that obtained with yeast expressing only p53. Data represent means ± s.e.m. of 2 independent experiments; value of yeast co-expressing p53 and PKC isoform significantly higher than that obtained with yeast expressing only p53, **P* < 0.05.

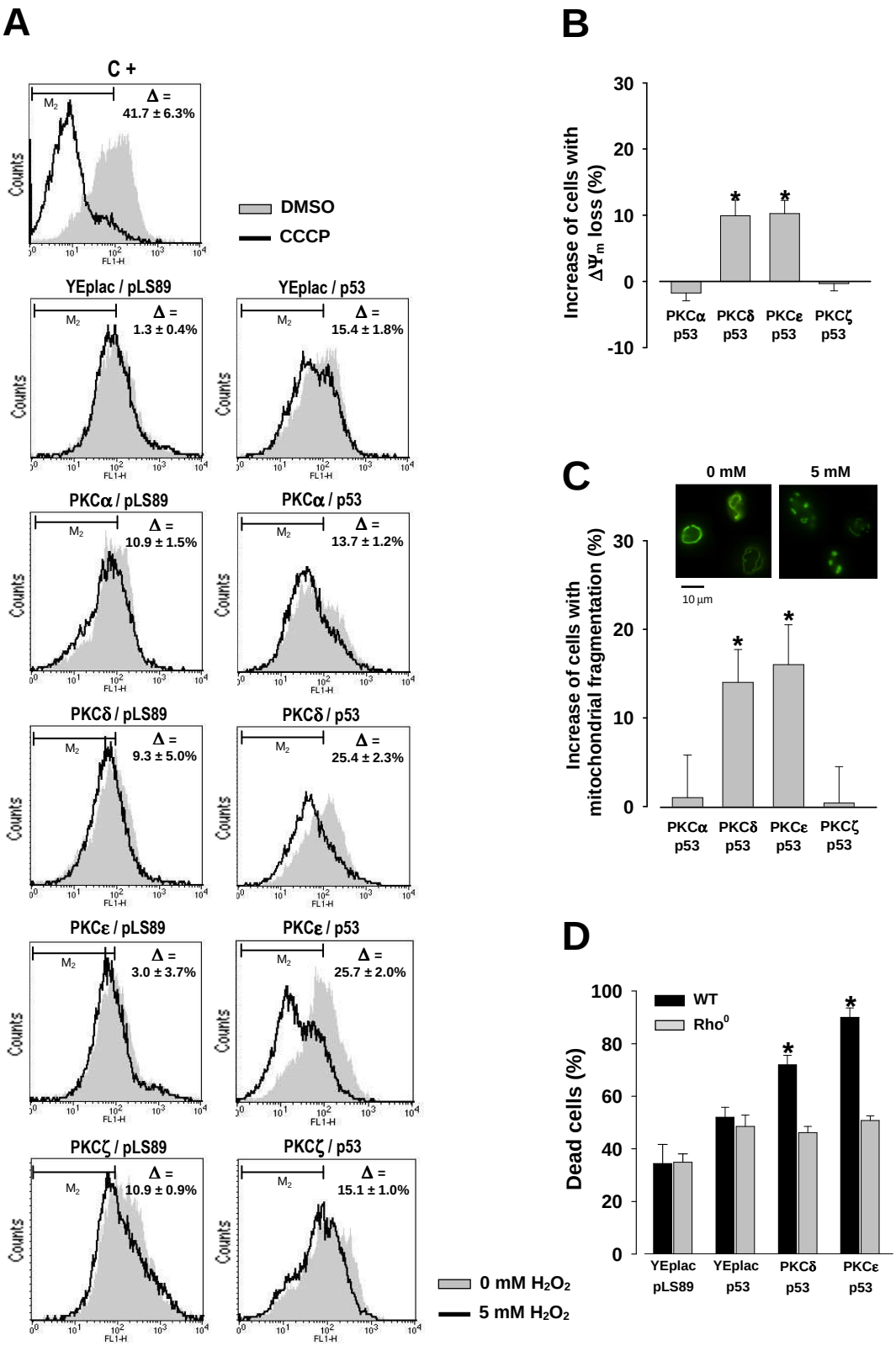

Fig. 3.3. PKCδ and ε increase the p53 effect on $\Delta\psi_m$ loss and mitochondrial network fragmentation, and fail to stimulate p53-mediated cell death in rho^0 co-transformed yeast cells. Control yeast (YEplac/pLS89) and yeast expressing p53 and/or a PKC isoform (α, δ, ε or ζ) were incubated in galactose selective medium to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. (**A**, **B**) Δψm was monitored by flow cytometry using $DiOC_6(3)$. (**A**) Overlays of green fluorescence histograms were obtained with untreated (0 mM) and 5 mM

H_2O_2-treated cells (or CCCP for positive control; C+); histograms represent 1 of 2 - 4 independent experiments. M_2 cursor indicates the sub-populations analysed; Δ values correspond to the increase in the percentage of cells with Δψm loss obtained when cells were treated with 5 mM H_2O_2 (CCCP for C+), and represent means ± s.e.m. of 2 - 4 independent experiments. Increase in the percentage of cells with (**B**) Δψm loss and (**C**) mitochondrial network fragmentation obtained when PKCα, δ, ε or ζ were co-expressed with p53; values correspond to differences between the effect obtained with yeast co-expressing p53 and PKC isoform and that obtained with yeast expressing only p53. In **C**, normal tubular mitochondria observed in untreated cells (0 mM) and fragmentation of the mitochondrial network observed in 5 mM H_2O_2-treated cells (5 mM) were evaluated using cells expressing mt-GFP, p53 and PKC isoform. Data represent means ± s.e.m. of 2 - 4 independent experiments; value of yeast co-expressing p53 and PKC isoform significantly higher than that obtained with yeast expressing only p53, **P* < 0.05. (**D**) Effect of mtDNA deletion on the stimulation of p53-mediated cell death by PKCδ and ε. The percentage of dead cells obtained with 5 mM H_2O_2-treated yeast cells with (WT) and without (Rho^0) mtDNA was assessed by CFU counts, considering 100% survival (0% death) as the number of CFU obtained with H_2O_2-untreated cells. Data represent means ± s.e.m. of 4 independent experiments; values of yeast co-expressing p53 and PKCδ/ε significantly higher than that obtained with yeast expressing only p53, **P* < 0.05.

This indicated a low level of p53 phosphorylation at Ser376-378 by endogenous yeast kinases. On the other hand, a pronounced decrease in the intensity of PAb421 band, indicative of a high level of p53 phosphorylation at Ser376-378, was obtained with yeast cells co-expressing p53 and PKCδ/ε (Fig. 3.4D,E).

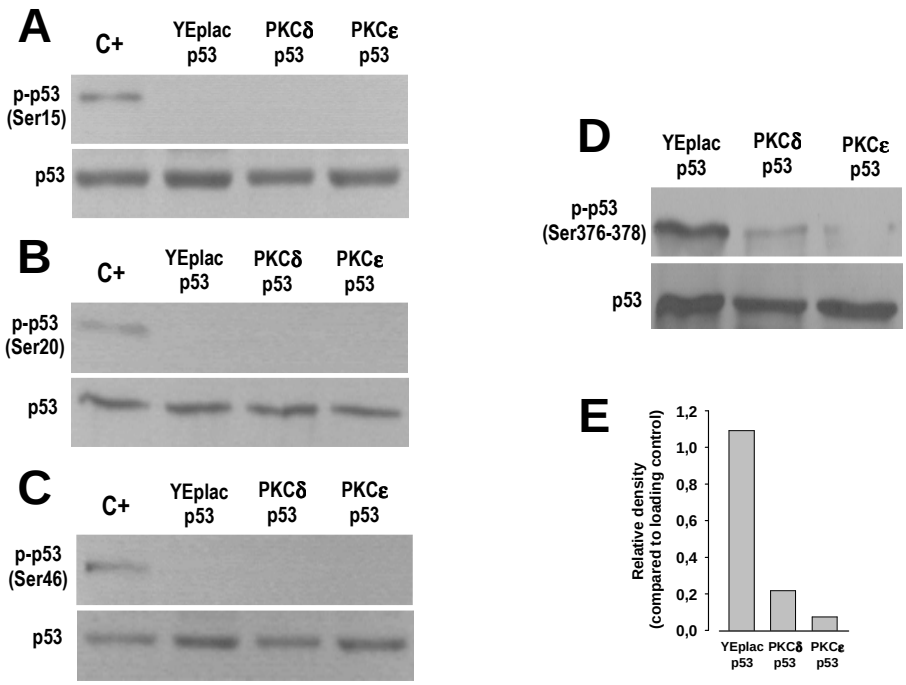

Fig. 3.4. PKCδ and ε phosphorylate p53 at Ser376-378 in H_2O_2-treated yeast cells. Yeast cells expressing only p53 and co-expressing p53 and PKCδ/ε were incubated in galactose selective medium to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. p53 phosphorylation was analysed using (**A**) phospho-

p53(Ser15), (**B**) phospho-p53(Ser20), (**C**) phospho-p53(Ser46) and (**D**) PAb421 (Ser376-378) antibodies. Detection of p53 with DO-1 antibody was used as loading control. In **A** - **C**, the etoposite-treated MCF7 cell lysate was used as positive control (C+); in **D**, p53 from yeast expressing only p53 (YEplac/p53), which expression was previously confirmed with the anti-p53 DO-1 antibody, was used as positive control. (**E**) Quantification of band intensities obtained in **D**.

In fact, similar results were obtained with untreated yeast cells co-expressing p53 and PKCδ/ε (11). This indicated that stimulation of p53-mediated apoptosis, likewise stimulation of p53-induced growth inhibition (11), by PKCδ and ε was accompanied by p53 phosphorylation at Ser376-378.

3.4.3. Stimulation of p53 translocation to mitochondria by PKCδ and ε in H_2O_2-treated yeast cells

As previously reported (21), immunofluorescence studies performed with untreated yeast cells expressing only p53 confirmed that human WT p53 was predominantly localized in the nucleus of yeast cells (Fig. 3.5A,B). Additionally, we verified that p53 nuclear localization was not significantly modified in yeast by co-expression with PKCα, δ, ε or ζ (Fig. 3.5B).

Under stress conditions, an increase in the percentage of cells exhibiting p53 nuclear staining with partial mitochondrial co-localization was observed in all co-transformed yeast cells tested (Fig. 3.5A,C). However, a significant increase in the percentage of these cells was only achieved with yeast cells co-expressing p53 and PKCδ/ε. Indeed, when compared to yeast expressing only p53, about a 3-fold increase in the percentage of cells exhibiting nuclear and mitochondrial p53 co-localization was obtained with yeast co-expressing p53 and PKCδ/ε (Fig. 3.5C). Additionally, Western blot analysis of mitochondrial fractions of untreated and H_2O_2-treated yeast cells expressing only p53 and co-expressing p53 and PKCδ/ε revealed a marked increase in the level of mitochondrial p53 in H_2O_2-treated yeast cells co-expressing p53 and PKCδ/ε. In fact, when compared to yeast expressing only p53, a 13- and 9-fold increase in the amount of mitochondrial p53 were obtained in the presence of PKCδ and ε, respectively (Fig. 3.5D-F). Together, these results showed that, under stress conditions, PKCδ and ε stimulated mitochondrial p53 translocation. A correlation between stimulation of p53-mediated apoptosis and enhancement of mitochondrial p53 localization by PKCδ and ε was therefore established.

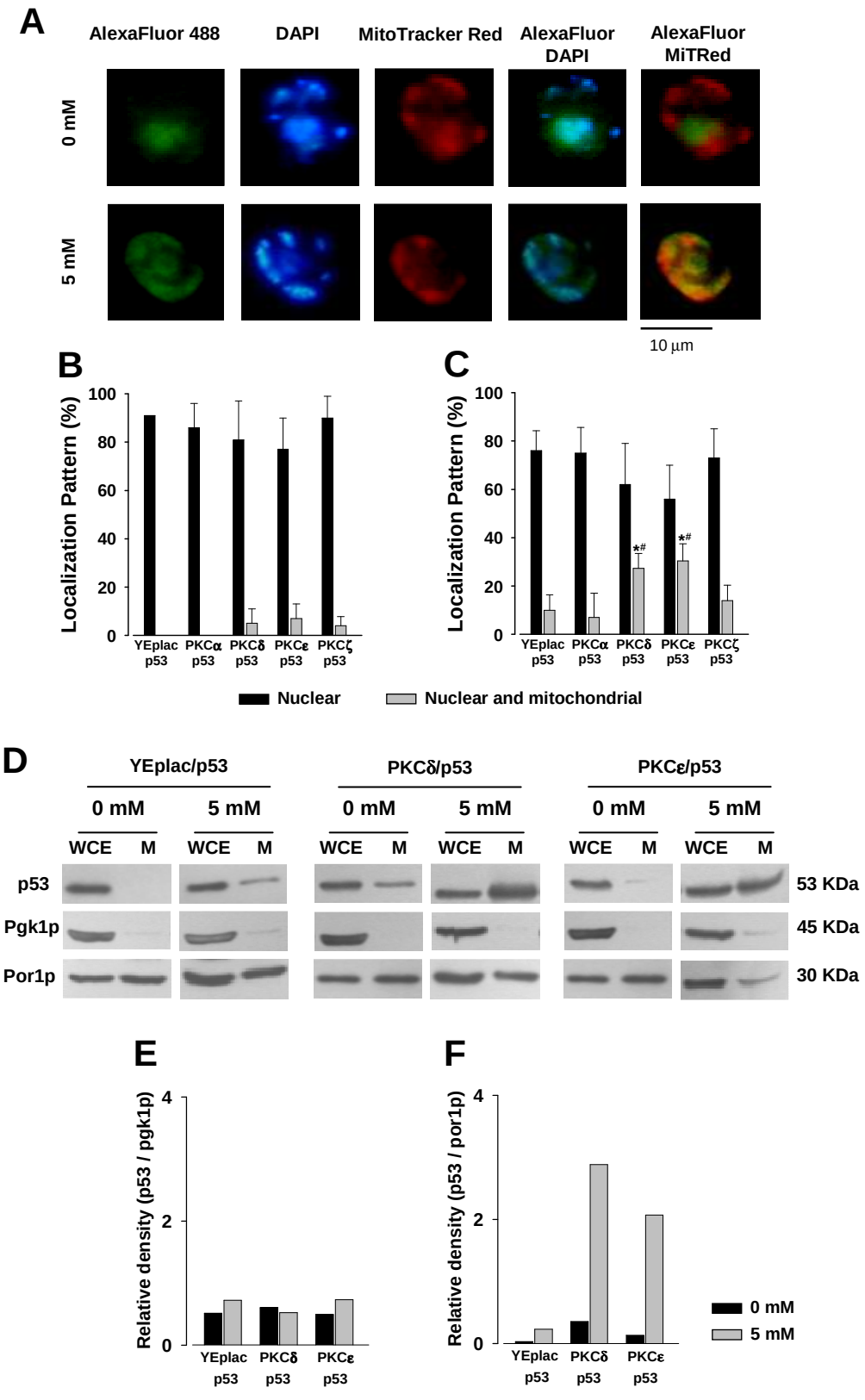

Fig. 3.5. PKCδ and ε activate mitochondrial p53 translocation in H_2O_2-treated yeast cells. Control yeast (YEplac/pLS89) and yeast expressing p53 and/or a PKC isoform (α, δ, ε or ζ) were incubated in galactose selective medium to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. (**A** - **C**) Subcellular localization of p53 in yeast assessed by immunofluorescence microscopy using the anti-p53 DO-1 antibody followed by Alexa Fluor 488 secondary antibody; nuclear and mitochondrial DNA were visualized using DAPI;

mitochondrial labelling was also performed using MitoTrackerRed. (**A**) Nuclear p53 localization observed in H_2O_2-untreated cells (0 mM); nuclear and mitochondrial p53 localization observed in 5 mM H_2O_2-treated yeast cells co-expressing p53 and PKCδ (as representative of the two nPKCs) (5 mM). (**B**, **C**) Quantification of p53 subcellular localization in (**B**) untreated and (**C**) 5 mM H_2O_2-treated yeast cells; data represent means ± s.e.m. of 4 independent experiments; values significantly different from that obtained with yeast expressing only p53, $^{*}P < 0.05$; values obtained in **C** significantly different from those obtained with the same co-transformed yeast in B, $^{\#}P < 0.05$. (**D** - **F**) Mitochondrial localization of p53 in yeast assessed by Western blot analysis of whole cell extract (WCE) and mitochondrial fraction (M) of untreated (0 mM) and 5 mM H_2O_2-treated yeast cells expressing only p53 and co-expressing p53 and PKCδ/ε. Pgk1p and por1p were used as loading controls for WCE and mitochondrial fraction, respectively. Quantification of p53 levels in (**E**) whole cell extract and (**F**) mitochondrial fraction. Data represent 1 of 2 independent experiments.

3.4.4. Stimulation of transcription-dependent and -independent p53 activities by PKCδ and ε in yeast

Our previous work showed that expression of human WT p53 in yeast induced a marked growth inhibition (11). Yeast cell growth was assessed by CFU counts, but considering 100% growth (0% growth inhibition) the number of CFU obtained with control yeast. In the present study, we verified that the treatment of yeast cells expressing only p53 with 10 μM PFT-α, a selective inhibitor of p53 transcriptional activity (22,23), practically abolished the p53-induced yeast growth inhibition (Fig. 3.6A). These results corroborated the preservation in yeast of a p53 transcriptional activity previously reported by others (13). Additionally, they established a correlation between p53 transcriptional activity and p53 growth-inhibitory effect in yeast.

The effect of 10 μM PFT-α on p53-mediated cell death was also analysed. Unexpectedly, a significant reduction in the percentage of dead cells was achieved when 5 mM H_2O_2-treated yeast cells expressing only p53 were pretreated with 10 μM PFT-α (Fig. 3.6B). In fact, the single expression of p53 in yeast only slightly increased 5 mM H_2O_2-induced cell death. This seemed therefore to indicate the activation of a transcription-dependent p53 apoptotic activity, which was not strong enough to significantly stimulate H_2O_2-induced cell death in yeast expressing only p53. However, a pronounced reduction in the percentage of dead (Fig. 3.6B) and TUNEL-positive (Fig. 3.6C) cells was achieved when yeast cells co-expressing p53 and PKCδ/ε were pretreated with 10 μM PFT-α. Together, these results identified PKCδ and ε as activators of a transcription-dependent p53 apoptotic activity.

Accumulating evidence for a transcription-independent p53 apoptosis in mammalian cells, characterized by translocation of a fraction of p53 to mitochondria in response to stress stimuli (1-3), together with the observation of mitochondrial p53

translocation in H_2O_2-treated yeast cells co-expressing p53 and PKCδ/ε, led us to also investigate the existence in yeast of a transcription-independent p53 apoptotic mechanism. With this goal, before treatment with H_2O_2, yeast cells were treated with 75 μM PFT-μ, an inhibitor of the transcription-independent p53 apoptosis that selectively inhibits p53 translocation to mitochondria without interfering with its transcriptional activity (2,3,24). Although PFT-μ did not interfere with the percentage of dead cells of yeast expressing only p53 or PKCδ/ε, it significantly reduced the percentage of dead cells (Fig. 3.6D), TUNEL-positive cells (Fig. 3.6E) and cells exhibiting Δψm loss (Fig. 3.6F) of yeast co-expressing p53 and PKCδ/ε. Together, these results showed, for the first time, the preservation in yeast of a transcription-independent p53 apoptotic mechanism, which was activated by PKCδ and ε.

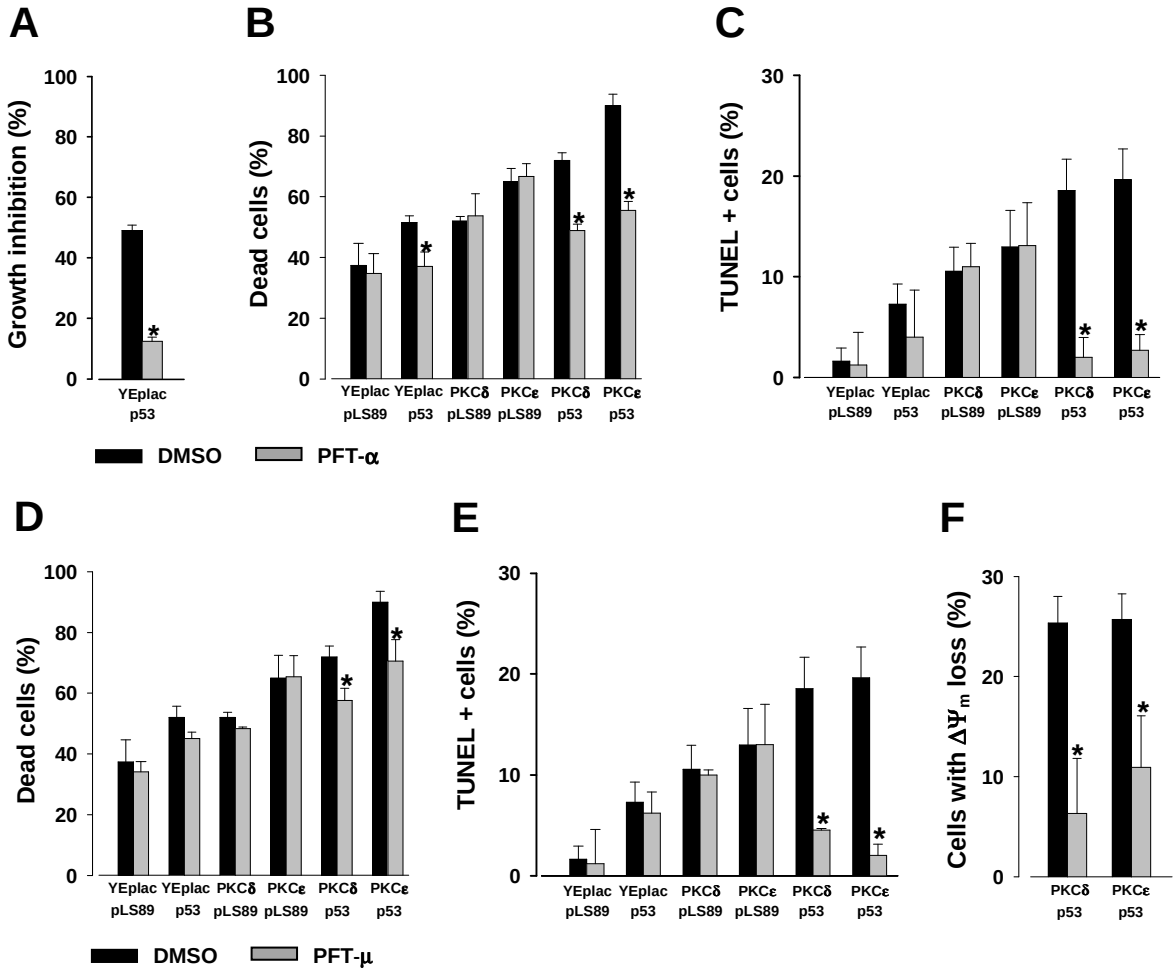

Fig. 3.6. PKCδ and ε activate transcription-dependent and -independent p53 mechanisms in yeast. (**A**) Effect of PFT-α, a selective inhibitor of p53 transcriptional activity, on the growth of yeast cells expressing only p53 Yeast cells were incubated in galactose selective medium with 10 μM PFT-α or DMSO only at 30ºC for approximately 45 hours (time required by control yeast to achieve 0.5 OD_{600}). Effect of PFT-α on yeast cell

growth was assessed by CFU counts, considering 100% growth (0% growth inhibition) as the number of CFU obtained with control yeast. Effect of PFT-α on (**B**) cell death and (**C**) DNA fragmentation of 5 mM H_2O_2-treated yeast cells. Co-transformed cells were incubated in galactose selective medium with 10 μM PFT-α or DMSO only to 0.45 OD_{600} before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. Effect of PFT-α on yeast cell death was assessed by CFU counts, considering for each co-transformant 100% survival (0% death) as the number of CFU obtained with H_2O_2-untreated cells. Effects of PFT-μ, a selective inhibitor of p53 translocation to mitochondria, on (**D**) cell death, (**E**) DNA fragmentation, and (**F**) $\Delta\psi m$ loss of 5 mM H_2O_2-treated yeast cells. Co-transformed cells were incubated in galactose selective medium to 0.4 OD_{600}, and then pretreated with 75 μM PFT-μ or DMSO only for 4 hours before treatment with 5 mM H_2O_2 for 1 hour at 30ºC. Effect of PFT-μ on cell death was assessed by CFU counts as in B. Effect of PFT-μ on $\Delta\psi m$ was monitored by flow cytometry using $DiOC_6(3)$; values correspond to the increase in the percentage of cells with $\Delta\psi m$ loss obtained upon treatment with 5 mM H_2O_2. In **A** - **F**, data represent means ± s.e.m. of 4 - 5 independent experiments; values significantly different from DMSO only, $^*P < 0.05$.

3.5. DISCUSSION

Contrarily to what was reported in mammalian cells (5,19), in the present work we observed that human WT p53 is unable to stimulate per se H_2O_2-induced yeast cell death. The absence in yeast of orthologues of mammalian positive regulators of p53 activity might explain this result. Since PKC family isoforms, particularly PKCδ, have been described as regulators of p53-mediated apoptosis (5-10), we addressed the question whether PKC isoforms could regulate the p53 apoptotic activity in yeast. Additionally, though several works have already reported the p53 phosphorylation and the activation of a p53-mediated apoptosis by PKCδ, the involvement of this PKC isoform in crucial aspects of the p53 apoptotic activity, namely in its transcription-dependent and -independent mechanisms, is still unknown. Besides, the discrimination of the role of other PKC isoforms commonly involved in carcinogenesis in the regulation of p53-mediated apoptosis is also unclear. To address these issues, yeast cells co-expressing the human WT p53 and an individual mammalian PKCα, δ, ε or ζ were used.

The present study reveals a distinct regulation of p53-mediated apoptosis by PKCα, δ, ε and ζ and identifies PKCδ and ε as positive regulators of p53 activity. Whereas the classical PKCα and the atypical PKCζ had no effect on p53 activity, the novel PKCδ and ε stimulated a p53-mediated mitochondria-dependent apoptosis in H_2O_2-treated yeast cells. In fact, PKCδ and ε increased the p53 effects on cell death, DNA fragmentation, mitochondrial ROS accumulation, $\Delta\psi m$ loss and mitochondrial network fragmentation, without interfering with the plasma membrane integrity. The strict requirement of respiring mitochondria in the stimulation of p53-mediated apoptosis by PKCδ and ε was confirmed using rho^0 co-transformed yeast cells, where PKCδ and ε failed to stimulate p53-mediated cell death. The enhancement of p53-mediated apoptosis by PKCδ and ε was further supported using the selective PKC inhibitor, Ro 32-0432, which markedly reduced the stimulatory effect of these two kinases. The absence of effect of Ro 32-0432 on the isolated PKCε-mediated cell death may be probably due to the mechanism of action of this PKC inhibitor. Ro 32-0432 interacts with the PKC catalytic region (specifically with the ATP binding site), inhibiting the protein kinase activity, and therefore the PKC capability to phosphorylate its substrates (25,26). Based on this, the effect of this inhibitor on yeast cells co-expressing PKCε and p53 supports the regulation of p53 activity by PKCε through a mechanism that involves the PKCε kinase activity. The absence of effect of Ro 32-0432 on the isolated PKCε-mediated cell death may suggest that, in this case, a kinase-

independent mechanism is involved in the PKCε activity. In fact, nontraditional PKC activation mechanisms, such as kinase-independent actions of freed regulatory domains, are frequently reported for PKCε (27). For example, PKCε, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells (28).

In the present work, phosphorylation of p53 by PKCδ and ε at commonly reported PKC phosphorylation sites, namely Ser15, Ser20, Ser46 and Ser376-378, was also analysed. The results obtained showed that under stress conditions p53 is not phosphorylated by PKCδ and ε at Ser15, Ser20 and Ser46. Although it was shown that stimulation of p53-mediated apoptosis by PKCδ and ε was accompanied by p53 phosphorylation at Ser376-378, similar results were also obtained with stimulation of p53-induced growth inhibition by PKCδ and ε in unstressed yeast cells (11). Therefore, it is still unclear whether phosphorylation of p53 by PKCδ and ε in yeast is a relevant factor for the stimulation of p53-mediated apoptosis by PKCδ and ε. Further work must be carried out in order to clarify this issue.

This work confirms the already reported nuclear localization of human WT p53 expressed in yeast (21). Though the conservation in yeast of a transcription-dependent p53 function was already demonstrated (13), as well as the p53-induced yeast growth inhibition (29), the regulation of yeast cell growth by p53 through a transcription-dependent mechanism was pointed out in the present work. Additionally, it reveals that the transcription-dependent p53 apoptotic mechanism in yeast is activated by PKCδ and ε.

Moreover, we demonstrate that the stimulation of p53-mediated apoptosis by PKCδ and ε entailed translocation of a fraction of p53 to mitochondria and was reduced by PFT-μ, a selective inhibitor of mitochondrial p53 translocation (2,3,24). Hence, another interesting outcome of the present work is to provide the first evidence for the conservation in yeast of a functional transcription-independent p53-mediated apoptosis, which is activated under cell death conditions and in the presence of PKCδ or ε. These data therefore support the possibility raised by others that mitochondrial localization of p53 is a subtle deciding factor that dictates whether cells die or arrest growth (2,3). Additionally, they provide new insights about an unclear issue concerning the regulation of p53 translocation to mitochondria.

As a whole, this study underscores a major role of PKCδ and ε as key positive regulators of transcription-dependent and -independent p53 activities that cooperate to

ultimately cause an apoptotic cell death. In fact, supporting our data, previous works reported that the treatment of mouse skin epidermal JB6 cells and skin tissues with phorbol 12-myristate 13-acetate, a potent selective activator of classical and novel PKC isoforms, increased not only the p53 transcriptional activity, but also the translocation of a fraction of p53 to mitochondria (30,31).

However, why only nPKCδ and ε can affect p53-mediated apoptosis? It is well known that individual PKC isoforms can exert either similar or opposite effects in distinct cellular processes, such as cell proliferation and death (4). One of the major regulatory mechanisms implicated in these PKC isoform-specific activities is the phosphorylation of distinct target substrates. For example, it was reported that PKCα is a potent kinase for histones, myelin basic protein and protamine, whereas PKCδ and ε do not exhibit this activity (32). Crucial for substrate recognition is the relief of the inhibitory pseudosubstrate region within the regulatory domain. However, other mechanisms must exist to direct these PKC isoforms to distinct signalling pathways. These include isoforms-specific subcellular compartmentalization patterns, protein-protein interactions, and posttranslational modifications that influence catalytic function (27,32). The present work gives an additional information regarding the specific actions (and cellular substrate) of individual PKC isoforms in the apoptotic cell death. The identification of p53 as a cellular target for some PKC isoforms may represent a challenge and opportunity for future research in the p53 field.

It has been proposed that direct participation of p53 in the intrinsic mitochondria-mediated apoptotic pathway involves the interaction with the multidomain members of the Bcl-2 family, and particularly the activation of one of its pro-apoptotic members Bax or Bak, to induce MOMP. However, considering the large number of proteins that control MOMP, it has also been suggested that the regulation of p53 transcription-independent apoptosis might involve many more factors than the original concept of a direct interaction with Bcl-2 proteins implies (3). Since there are no orthologues of the Bcl-2 family members in yeast (12), the present work corroborates this hypothesis. Further studies are underway to ascertain whether p53 interacts with the already identified yeast orthologues involved in MOMP (33).

3.6. CONCLUSION

The present work represents the first attempt to reconstitute in yeast a mammalian p53-PKC isoform apoptotic network. Additionally, it provides the first evidence for the conservation in yeast of a transcription-dependent and -independent p53-mediated apoptosis and further validates the yeast cell model to elucidate the mechanisms underlying the regulation of p53-mediated apoptosis. Moreover, it gives a mechanistic insight on apoptosis regulation by PKCδ and ε through regulation of p53 transcriptional activity and p53 translocation to mitochondria. Since compelling evidence indicates that the transcription-independent pathway driven by p53 contributes significantly to the effect and outcome of cancer therapy, this work underscores that PKCδ and ε are promising therapeutic targets in cancer and that selective activators of these isoforms are potential anti-cancer agents.

3.7. ACKNOWLEDGMENTS

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3.8. REFERENCES

(1) Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature 2009 Apr; 458 (7242): 1127-30.
(2) Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochim Biophys Acta 2009 May; 1787 (5): 414-20.
(3) Speidel D, Transcription-independent p53 apoptosis: an alternative route to death. Trends Cell Biol 2010 Jan; 20 (1): 14-24.
(4) Reyland ME, Protein kinase C isoforms: Multi-functional regulators of cell life and death, Frontiers in Bioscience 2009 Jan; 14: 2386-99.
(5) Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Karino T et al. Roles of protein kinase C delta in the accumulation of p53 and the induction of apoptosis in H2O2-treated bovine endothelial cells. Free Radic Res 2002 Nov; 36 (11): 1147-53.
(6) Pospísilová S, Brázda V, Kucharíková K, Luciani MG, Hupp TR, Skládal P et al. Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. Biochem J 2004 Mar; 378 (3): 939-47.
(7) Ryer EJ, Sakakibara K, Wang C, Sarkar D, Fisher PB, Faries PL et al. Protein kinase C delta induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. J Biol Chem 2005 Oct; 280 (42): 35310-7.
(8) Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ 2006 Jun; 13 (6): 941-50.
(9) Lee SJ, Kim DC, Choi BH, Ha H, Kim KT. Regulation of p53 by activated protein kinase C-delta during nitric oxide-induced dopaminergic cell death. J Biol Chem 2006 Jan; 281 (4): 2215-24.
(10) Yoshida K, Liu H, Miki Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. J Biol Chem 2006 Mar; 281 (9): 5734-40.
(11) Coutinho I, Pereira G, Leão M, Gonçalves J, Côrte-Real M, Saraiva L. Differential regulation of p53 function by protein kinase C isoforms revealed by a yeast cell system. FEBS Lett 2009 Nov; 583 (22): 3582-8.
(12) Greenwood MT, Ludovico P. Expressing and functional analysis of mammalian apoptotic regulators in yeast. Cell Death Differ 2010 May; 17 (5): 737-45.
(13) Yousef AF, Xu GW, Mendez M, Brandl CJ, Mymryk JS. Coactivator requirements for p53-dependent transcription in the yeast Saccharomyces cerevisiae. Int J Cancer 2008 Feb; 122 (4): 942-6.
(14) Saraiva L, Silva R, Pereira G, Gonçalves J, Côrte-Real M, Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Science 2006 Aug; 119 (15): 3171-81.
(15) Coutinho I, Pereira G, Simões MF, Côrte-Real ., Gonçalves J, Saraiva L. Selective activation of protein kinase C-delta and -epsilon by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U). Biochem Pharmacol 2009 Sep;78 (5): 449-59.
(16) Büttner S, Bitto A, Ring J, Augsten M, Zabrocki P, Eisenberg T et al., Functional mitochondria are required for alpha-synuclein toxicity in aging yeast. J Biol Chem 2008 Mar;283 (12): 7554-60.
(17) Meisinger C, Pfanner N, Truscott K.N. Isolation of Yeast Mitochondria. Methods in Molecular Biology 2006; 313: 33-9.
(18) Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ. Wolf D.H et al. Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 1999 May; 145 (4): 757-67.
(19) Yamamoto H, Ozaki T, Nakanishi M, Kikuchi H, Yoshida K, Horie H et al. Oxidative stress induces p53-dependent apoptosis in hepatoblastoma cell through its nuclear translocation. Genes Cells 2007 Apr; 12 (4): 461-71.
(20) Eisenberg T, Büttner S, Kroemer G, Madeo F. The mitochondrial pathway in yeast apoptosis. Apoptosis 2007 May; 12 (5): 1011-23.
(21) Di Ventura B, Funaya C, Antony C, Knop M, Serrano L. Reconstitution of Mdm2-dependent post-translational modifications of p53 in yeast. PLoS ONE 2008 Jan;3 (1): e1507.
(22) Gudkov AV, Komarova EA. Prospective therapeutic applications of p53 inhibitors. Biochem Biophys Res Commun 2005 Jun; 331 (3): 726-36.
(23) Charlot JF, Nicolier M, Prétet JL, Mougin C. Modulation of p53 transcriptional activity by PRIMA-1 and Pifithrin-alpha on staurosporine-induced apoptosis of wild-type and mutated p53 epithelial cells. Apoptosis 2006 May; 11 (5): 813-27.
(24) Strom E, Sathe S, Komarov PG, Chernova OB, Pavlovska I, Shyshynova I et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. Nat Chem Biol 2006 Sep; 2 (9): 474-9.
(25) Hofmann J. The potential for isoenzyme-selective modulation of protein kinase C. FASEB J 11 (1997) 649-669.
(26) Swannie HC, Kaye SB. Protein kinase C inhibitors. Curr Oncol Rep 2002 Jan; 4 (1): 37-46.
(27) Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev 2008 Oct; 88 (4): 1341-78.

(28) Zeidman R, Löfgren B, Påhlman S, Larsson C. PKCepsilon, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. J Cell Biol 1999 May; 145 (4): 713-26.
(29) Nigro JM, Sikorski R, Reed SI, Vogelstein B. Human p53 and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae. Mol Cell Biol 1992 Mar; 12 (3): 1357-65.
(30) Zhao Y, Chaiswing L, Velez JM, Batinic-Haberle I, Colburn NH, Oberley TD et al. p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defence protein-manganese superoxide dismutase. Cancer Res 2005 May; 65 (9): 3745-50.
(31) Liu J, St Clair DK, Gu X. Zhao Y. Blocking mitochondrial permeability transition prevents p53 mitochondrial translocation during skin tumor promotion. FEBS Lett 2008 Apr; 582 (9): 1319-24.
(32) Breitkreutz D, Braiman-Wiksman L, Daum N, Denning MF, Tennenbaum T. Protein kinase C family: on the crossroads of cell signalling in skin and tumor epithelium. J Cancer Res Clin Oncol 2007 Nov; 133 (11): 793-808.
(33) Pereira C, Camougrand N, Manon S, Sousa MJ, Côrte-Real M. ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis. Mol Microbiol 2007 Nov; 66 (3): 571-82.

CHAPTER 4

Selective activation of protein kinase C-δ and -ε by 6,11,12,14-tetrahydroxyabieta-5,8,11,13-tetraene-7-one (coleon U)

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SELECTIVE ACTIVATION OF PROTEIN KINASE C-δ AND -ε BY 6,11,12,14-TETRAHYDROXYABIETA-5,8,11,13-TETRAENE-7-ONE (COLEON U)

4.1. ABSTRACT

6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U) is a diterpene compound isolated from *Plectranthus grandidentatus* with an antiproliferative effect on several human cancer cell lines. Herein, we studied the modulatory activity of coleon U on individual isoforms of the three protein kinase C (PKC) subfamilies, conventional (cPKC-α and -βI), novel (nPKC-δ and -ε) and atypical (aPKC-ζ), using a yeast PKC expression system. The results obtained showed that, whereas the PKC activator phorbol 12-myristate 13-acetate (PMA) activated every PKC tested except aPKC, coleon U not only had no effect on aPKC but also on cPKCs. Besides, the coleon U effect on nPKCs was higher than that exhibited by PMA. These data revealed that coleon U was a potent and selective activator of nPKCs. The isoform-selectivity of coleon U for nPKC-δ and -ε was further confirmed using an *in vitro* PKC assay. Most importantly, in opposition to PMA, which activated nPKCs inducing an isoform translocation from the cytosol to the plasma membrane and a G2/M cell cycle arrest, coleon U induced nPKCs translocation to the nucleus and a metacaspase- and mitochondrial-dependent apoptosis. This work therefore reconstitutes in yeast distinct subcellular translocations of a specific PKC isoform and the subsequent distinct cellular responses reported for mammalian cells. Together, our study identifies a new isoform-selective PKC activator with promising pharmacological applications. Indeed, since coleon U has no effect on cPKCs and aPKC, recognised as anti-apoptotic proteins, and selectively induces an apoptotic pathway dependent on nPKC-δ and -ε activation, it represents a promising compound for evaluation as an anti-cancer drug.

Keywords: Coleon U; Selective PKC activator; PKC-δ; PKC-ε; Apoptosis; Yeast

4.2. INTRODUCTION

6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U) is an abietane diterpene compound isolated from *P. grandidentatus*, shown to inhibit the growth of several human cancer cell lines such as MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), TK-10 (renal) and UACC-62 (melanoma) in a dose-dependent manner (1). However, the mechanisms of action responsible for this coleon U-induced growth inhibition have not been elucidated. In a later study, a dose-dependent antiproliferative effect of coleon U on T- and B-lymphocyte cells was also reported (2). Although this study established a relationship between the antiproliferative effect of coleon U and its capacity to induce apoptosis in lymphocyte cells, the molecular mechanisms associated with its antiproliferative effect remained unclear. However, the interference of this small-molecule with some cell signalling transduction kinases, such as protein kinase C (PKC) and/or protein tyrosine kinases, was hypothesized as a possible mechanism responsible for coleon U-induced growth inhibition (2). Since several diterpene compounds, such as phorbol esters, represent potent PKC activators (3,4), we questioned whether coleon U could indeed modulate PKC activity.

PKC is considered an important family of signalling serine/threonine kinases with at least 10 isoforms grouped into three subfamilies based on their primary structure and cofactors required for activation: the classical PKCs (cPKCs: α, βI, βII and γ), activated by the second messengers Ca2+ and diacylglycerol (DAG), the novel PKCs (nPKCs: δ, ε, η and θ) which respond only to DAG and the atypical PKCs (aPKCs: ζ and λι) not responsive to either of the second messengers (4). The PKC family is responsible for regulating a variety of physiological processes such as differentiation, proliferation, cell cycle and apoptosis, in an isoform specific manner. Hence, PKC isoforms represent key pharmacological targets for the treatment of numerous pathologies. For instance, some PKC isoforms have been recognised as important players in carcinogenesis, rendering them potentially suitable targets for anti-cancer therapy (3,5-7). While PKC-α, -β and -ζ are frequently associated with proliferative effects being involved in the tumorigenesis of various cancers, PKC-δ is often linked to anti-proliferative/pro-apoptotic effects in mammals (3,5-7). Consequently, selective activators of PKC-α, -β and -ζ are considered tumour promoters, while selective activators of PKC-δ represent promising anti-cancer drugs (3,5-7). Although PKC-δ and -ε display a high degree of homology and similar substrate specificity, suggesting similar targets in signal transduction pathways for both nPKCs, they are frequently described as mediating quite contrasting physiological effects

(3,5,6). However, although several studies suggest that PKC-ε favors life over death (3,5-7), recent research works also showed that PKC-ε activation can contribute to apoptosis. For example, it was demonstrated that ethanol induces apoptosis in hepatocytes via activation of the nPKC isoforms, PKC-δ and -ε (8).

In order to achieve the regulation of a particular PKC isoform, without affecting the activity of other isoforms also present in the cell, PKC isoform-selective activators are required. However, the complexity of the PKC family and the difficulty in carrying out independent analysis of an individual PKC isoform in mammalian cells may justify the low number of PKC isoform-selective modulators identified until now (3,7,9).

Based on these data, we exploited a yeast PKC expression system to study the modulatory activity of coleon U on individual PKC isoforms of the three PKC subfamilies, cPKCs (α and βI), nPKCs (δ and ε) and aPKCs (ζ), considered as major isoforms in carcinogenesis. The conservation of many pathways and cellular processes in yeast, such as apoptosis (10), has allowed transposing the knowledge obtained in yeast to mammalian cells and vice-versa. Additionally, yeast expressing human proteins has been used as a valuable tool to elucidate the role of these proteins in complex cellular processes, and to screen for their pharmacological modulators (11,12). Furthermore, since the yeast PKC (Pkc1p in *Saccharomyces cerevisiae*) is a structural but not a functional homologue of mammalian PKC isoforms (13), yeast has been considered a well-suited organism to study individual mammalian PKC isoforms. In fact, it was demonstrated that mammalian PKCs expressed in yeast have functional characteristics similar to those found in mammalian cells (14,15). It was also shown that the yeast PKC expression system was a potential in vivo assay for the screening of PKC modulators (14,16). Based on these data, this yeast PKC assay has been used by our group not only to study the role of PKC isoforms on apoptosis regulation (17), but also to search for isoform-selective PKC activators (18,19) and inhibitors (20,21). This simpler eukaryotic cell model, established and validated in such studies, allows the effect of small-molecules on each PKC isoform to be analysed without the genetic complexity of the PKC family, specifically the coexistence of multiple PKC isoforms in the same mammalian cells and the extensive cross-talk amongst numerous mammalian signalling pathways.

In the present study, we found that coleon U is a potent and selective activator of nPKC-δ and -ε. Besides, several experiments were carried out in order to elucidate the molecular mechanisms of action behind the observed coleon U-induced growth inhibition in yeast expressing the nPKC-δ or -ε. Another relevant point arising from this work was the validation of the yeast assay to reconstitute the distinct subcellular translocations of a

specific mammalian PKC isoform and the subsequent different cellular responses described in higher eukaryotes.

4.3. MATERIAL AND METHODS

4.3.1. Plant material, isolation and identification of coleon U

Details concerning the extraction, isolation and structure elucidation of the abietane diterpene coleon U from *P. grandidentatus* Gürke (Fig. 4.1) have previously been described (1,22).

4.3.2. Plasmids

Constructed yeast expression plasmids YEplac181-LEU2 with the cDNA encoding for bovine PKC-α, rat PKC-βI, or PKC-δ, mouse PKC-ε or PKC-ζ, under control of a galactose-inducible GAL1 promote, were kindly provided by Dr. Nigel Goode (The Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK). Constructed yeast expression plasmid pOW4-URA3 with the cDNA encoding for human Bcl-xL, under control of an ADH1 promoter, was kindly provided by Dr. Charles Rudin (Cancer Research Building, Baltimore, USA). Constructed yeast expression plasmid pCLbGFP-TRP3 with the cDNA encoding for mitochondria-localized green fluorescent protein (mt-GFP), under control of a GAL1-10 promoter, was kindly provided by Dr. Stéphen Manon (Université de Bordeaux, Bordeaux, France). All the plasmids used were amplified in Escherichia coli DH5α and confirmed by restriction analysis.

4.3.3. Yeast strain, growth conditions and yeast expression of a mammalian protein

For yeast expression studies *Saccharomyces cerevisiae* strain CG379 (α ade5 his7-2 leu2-112 trp1-289α ura3-52 [Kil-O]); Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed as reported (17). To ensure selection of transformed yeast, cells were routinely grown in a minimal selective medium. To induce yeast expression of a mammalian protein, cells were diluted to 0.05 measured at 600 nm (OD_{600}; Jenway 6310 Spectrophotometer, Jenway, Felsted, Dunmow, Essex, UK) in a 2% (w/v) galactose and raffinose (Sigma-Aldrich, Sintra, Portugal) selective medium and grown, at 30 ºC under continuous shaking, to 0.5 OD_{600} (mid-log phase; about 42 h incubation), as described (17). Yeast expression of a mammalian PKC isoform and/or human Bcl-xL was previously confirmed by Western blot analysis (17,19).

4.3.4. Effect of compounds on yeast cell growth

All compounds tested were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Sintra, Portugal). To analyse the effect of compounds on yeast growth, transformed cells were incubated in galactose selective medium with 0.1 – 10 μM coleon U, 1 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Sintra, Portugal) or 0.1% DMSO only to 0.5 OD_{600}. Cell growth was determined by colony forming unit (c.f.u.) counts, as described (17). The percentage of growth inhibition was estimated considering 100% growth as the number of c.f.u. obtained with yeasts incubated with DMSO only.

4.3.5. In vitro *PKC assay*

The *in vitro* PKC assay was performed using the non-radioactive kit MESACUP Protein Kinase Assay System and purified PKC enzymes from Upstate (Grupotaper, Sintra, Portugal): cPKCs (mixture of classical PKC isoforms, α, β and γ, obtained from rat brain; 5 ng per assay); nPKC-δ (50 ng/assay); nPKC-ε (12.5 ng/assay); aPKC-ζ (12.5 ng/assay), basically according to the kit procedure. Briefly, this ELISA-based detection method uses a peptide pseudosubstrate, pre-coated on a 96-well plate that can be phosphorylated by PKC. Samples containing the active PKC enzyme were transferred to the pseudosubstrate-coated wells. A biotinylated monoclonal antibody that recognises the phosphorylated form of the pseudosubstrate was added to the wells and detected using HRP-conjugated streptavidin. Colour intensity of reaction mixtures containing the endogenous PKC activator phosphatidylserine (PS), with or without 0.3 μM coleon U, was determined photometrically at 490 nm using a microplate spectrofluorometer (PowerWave™ S Microplate Spectrophotometer, Bio-TEK instruments, Inc., Highland Park, Winooski VT, USA). PKC activity is directly proportional to color intensity. OD490 obtained with the reaction mixture containing PS only (control) was considered as 100% PKC activation.

4.3.6. Cell cycle analysis

Flow cytometric analysis of DNA content was obtained using Sytox Green Nucleic Acid from Molecular Probes (Alfagene, Carcavelos, Portugal), basically as reported (23). Briefly, about 10^7 cells incubated in galactose selective medium with 1 μM PMA, 1 μM coleon U or DMSO only were fixed in 70% (v/v) ethanol overnight at 4 ºC, treated with 250 μg/ml RNase A (DNase-free; Sigma-Aldrich, Sintra, Portugal) for 3 h at 50 ºC and thereafter with 1 mg/ml Proteinase K (Sigma-Aldrich, Sintra, Portugal) for 3 h at 37 ºC.

Subsequently, cells were incubated with 10 μM Sytox Green overnight at 4 ºC. Fluorescence from at least 30,000 cells was analysed using the FL1 detector in linear amplification from a flow cytometer. Yeast cell cycle phases were quantified using ModFit LTTM software (Verity Software House, Inc., Topsham, USA).

4.3.7. Analysis of plasma membrane integrity, DNA fragmentation and chromatin condensation

Propidium iodide (PI) and TUNEL staining to monitor plasma membrane integrity and DNA fragmentation respectively were carried out as described (17). Briefly, about 10^7 cells incubated in galactose selective medium with 1 μM coleon U or DMSO only were collected and incubated with 5 μg/ml PI (Sigma-Aldrich, Sintra, Portugal) for 10 min at room temperature. TUNEL was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Amadora, Portugal). For chromatin condensation analysis, cells were incubated with 4 μg/ml DAPI (4,6-diamido-2-phenyl-indole; Sigma-Aldrich, Sintra, Portugal) for 15 min at room temperature, as described (24). At least 600 cells per sample were analysed under a fluorescence microscope.

4.3.8. Assessment of yeast metacaspase activity

Yeast metacaspase (Yca1p) activation was analysed basically as described (17). Flow cytometric analysis: about 10^6 cells incubated in galactose selective medium with 1 μM coleon U or DMSO only were collected and incubated with 12.5 μM of FITC-VAD-fmk from Promega (VWR International Material de Laboratório, Lda., Lisboa, Portugal) for 1 h at 30 ºC; fluorescence from at least 10,000 cells was analysed using the FL1-H detector from a flow cytometer. Cell death assay: cells were incubated in galactose selective medium with 1 μM coleon U and 20 μM of the caspase inhibitor z-VAD-fmk from Promega (VWR International Material de Laboratório, Lda., Lisboa, Portugal). Cell death was assessed by c.f.u. counts, considering 100% survival as the number of c.f.u. obtained with cells incubated with DMSO only.

4.3.9. Assessment of reactive oxygen species (ROS) production

ROS production was monitored by flow cytometry using dihydroethidium (DHE) from Molecular Probes (Alfagene, Carcavelos, Portugal) as described (25). About 10^6 cells, incubated in galactose selective medium with 1 μM coleon U or DMSO only, were

collected and incubated with 5 μg/ml DHE for 30 min at 30 ºC. Fluorescence from at least 10,000 cells was analysed using the FL2-H detector from a flow cytometer.

4.3.10. Assessment of mitochondrial membrane potential (Δψm)

Modification of Δψm in transformed yeast was monitored by flow cytometry using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) from Molecular Probes (Alfagene, Carcavelos, Portugal). About 10^6 cells, incubated in galactose selective medium with 1 μM coleon U or DMSO only were incubated with 1 nM $DiOC_6(3)$ for 30 min at 30 ºC. Thereafter, 2 μg/ml PI was added to exclude necrotic cells. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich, Sintra, Portugal) was used as positive control; after treatment with $DiOC_6(3)$, cells incubated with DMSO only were treated with 0.4 nM CCCP for 15 min at 30 ºC. Fluorescence from at least 10,000 cells was analysed using the FL1-H and FL2-H detectors from a flow cytometer.

4.3.11. Assessment of mitochondrial fragmentation

Analysis of mitochondrial fragmentation of transformed yeast was carried out using the plasmid pCLbGFP encoding for mt-GFP, as described (26). Cells incubated in galactose selective medium with 1 μM coleon U or DMSO only were observed under a fluorescence microscope.

4.3.12. Immunofluorescence assay

About 10^7 cells grown in galactose selective medium with 1 μM PMA, 1 μM coleon U or DMSO only were fixed with 3.7% formaldehyde (Sigma-Aldrich, Sintra, Portugal) for 30 min at 30 ºC. For spheroplasts formation, cells were treated with zymolyase solution, containing 0.1M potassium phosphate pH 7.5, 2 μl/ml 2-mercaptoethanol (Sigma–Aldrich, Sintra, Portugal), 1 mg/ml zymolyase 20 T (MP Biomedicals Solon, Fountain Parkway Solon, USA), at 37 ºC for 25 min. Spheroplasts were transferred to polylysine-coated glass slides (Sigma-Aldrich, Sintra, Portugal) and permeabilised with 1% Triton X-100 (Sigma-Aldrich, Sintra, Portugal) for 2 min at 4 ºC before incubation with the mouse monoclonal antibody anti-PKC-δ or anti-PKC-ε (1:50) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal) for 2 h at room temperature. This was then followed by incubation with the anti-mouse Alexa Fluor 488 secondary antibody (1:200) from Molecular Probes (Alfagene, Carcavelos, Portugal) for 2 h at room temperature. Mounting

medium containing 1.5 μg/ml DAPI was used to visualize nuclear DNA. Samples were observed under a fluorescence microscope.

4.3.13. Flow cytometric data acquisition and analysis

Flow cytometric analysis was performed using a FACSCaliburTM flow cytometer and the CellQuest software (BD Biosciences, San José, CA, USA).

4.3.14. Fluorescence microscopy

For fluorescent microscopic examination, samples were observed under an Eclipse E400 fluorescence microscope (Nikon, Japan) equipped with a 100 W mercury lamp and appropriate filter setting. Yeast cells were observed with an oil immersion lens (Plan Fluor 100/1.30) and images were captured by a Digital Sight camera system (Nikon DS-5Mc, Japan) carrying built-in software for image acquisition (Nikon ACT-2U, Japan).

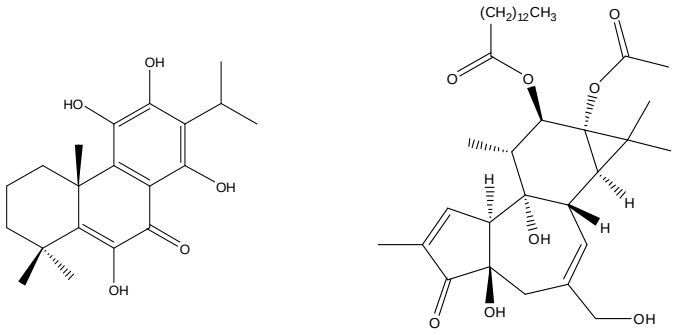
4.3.15. Statistical analysis

Data were analysed statistically using the SigmaStat 3.1 programme (SYSTAT® Software, Inc., Mountain River, CA, USA). Differences between means were tested for significance using the unpaired Student´s t-test. *P* values of 0.05 or lower were considered statistically significant. Results are expressed as the mean ± s.e.m. of the indicated number of experiments.

4.4. RESULTS

4.4.1. Coleon U is a potent and selective activator of nPKC-δ and -ε

In order to analyse a possible modulation of PKC isoforms by coleon U (Fig. 4.1), we used a yeast PKC expression system that consists of the use of yeast cells expressing an individual mammalian PKC-α, -βI, -δ, -ε or -ζ. In this assay, it was demonstrated that PKC activators inhibit the growth of yeast expressing a PKC isoform without interfering with the growth of yeast transformed with the empty vector (control yeast) (14,18,19).



Coleon U **PMA**

Fig. 4.1. Chemical structures of coleon U, an abietane diterpene isolated from *P. grandidentatus,* and phorbol 12-myristate 13-acetate (PMA).

Hence, we began by analysing the effect of several concentrations of coleon U, 0.1 – 10 μM, on the growth of yeast expressing PKC-α, -βI, -δ, -ε or -ζ and control yeast (Fig. 4.2A). Yeast cell growth was analysed by c.f.u. counts and the effects obtained with coleon U were compared to those obtained with the standard PKC activator, PMA (Fig. 4.1; an activator of cPKCs and nPKCs). We detected that 0.1 and 1 μM coleon U significantly inhibited the growth of yeast expressing PKC-δ or -ε without affecting the growth of control yeast and yeast expressing PKC-α, -βI or -ζ (Fig. 4.2A). This selectivity of coleon U to inhibit the growth of yeast expressing PKC-δ or -ε was particularly evident for 1 μM (Fig. 4.2B). In fact, for this concentration, and in opposition to PMA that activated every PKC tested except aPKC-ζ, coleon U had no effect on cPKCs as well as on aPKC-ζ (Fig. 4.2B,C). In addition, the effect of coleon U on yeast expressing the nPKCs, PKC-δ or -ε, was shown to be higher than that obtained with PMA (Fig. 4.2B,C). These results therefore indicated that coleon U was a potent and selective activator of the two nPKCs, PKC-δ and -ε.

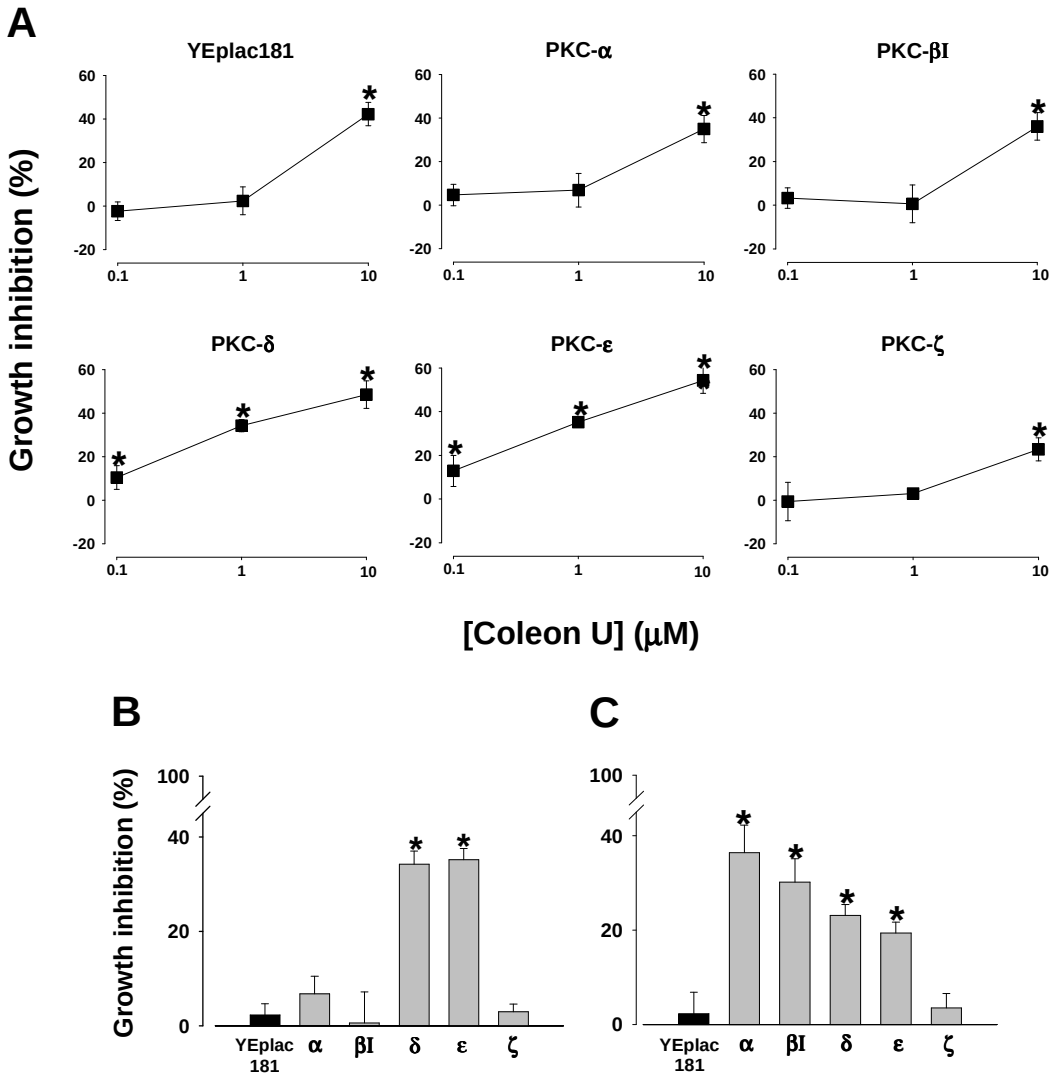


Fig. 4.2. Effect of PMA and coleon U on the growth of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with coleon U, PMA or DMSO only to 0.5 OD_{600} (about 42 h incubation). The percentage of growth inhibition was estimated by c.f.u. counts, considering 100% growth as the number of c.f.u. obtained with yeast incubated with DMSO only. (**A**) Concentration–response curves for the coleon U effect on the growth of transformed yeast. Effect of (**B**) 1 μM coleon U and (**C**) 1 μM PMA on the growth of transformed yeast. Data are the mean ± s.e.m. of 4 - 8 independent experiments with 6 replicates each. Values significantly different from those obtained with (**A**) DMSO and (**B**, **C**) control yeast, **P* <= 0.001.

Direct activation of PKC-δ and -ε by coleon U and the selectivity of this small-molecule for these nPKCs was further confirmed using an *in vitro* protein kinase assay system and purified PKC enzymes: cPKCs (mixture of classical PKC isoforms, α, β and γ), nPKC-δ, nPKC-ε and aPKC-ζ. In the *in vitro* PKC assay, PKC activators increase the phosphatidylserine (PS; an endogenous PKC activator) effect. In fact, in this assay, it was

observed that 0.3 μM coleon U significantly increased the PS effect on nPKC-δ and -ε. In opposition, 0.3 μM coleon U did not interfere with the PS effect on cPKCs and aPKC-ζ (Fig. 4.3).

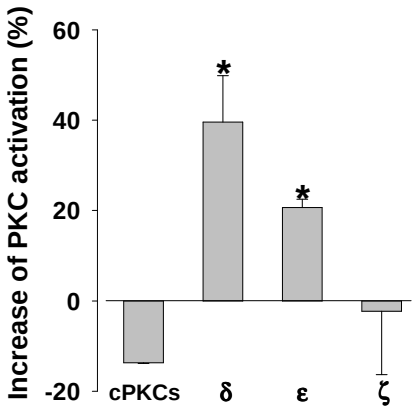


Fig. 4.3. *In vitro* PKC assay. Direct activation of nPKC-δ and -ε by coleon U and its selectivity for these nPKCs was confirmed *in vitro* using a protein kinase assay system and purified PKC enzymes: cPKCs (mixture of classical PKC isoforms, α, β and γ); nPKC-δ; nPKC-ε and aPKC-ζ. Coleon U was tested at the final concentration of 0.3 μM. Colour intensity of reaction mixtures containing the endogenous PKC activator phosphatidylserine (PS) with and without coleon U was determined photometrically at 490 nm using a microplate spectrofluorometer. Data represent the increase in the percentage of PKC activation obtained when coleon U was added to PS, considering 100% PKC activation the OD_{490} obtained with the reaction mixture containing PS only (control), and correspond to the mean ± s.e.m. of 2 independent experiments. Values significantly higher than those obtained with the control, **P* < 0.05.

4.4.2. Coleon U-induced growth inhibition in yeast expressing nPKC-δ or -ε is associated with the occurrence of a metacaspase- and mitochondrial-dependent apoptotic cell death

It was showed that the expression of bovine PKC-α in *S. cerevisiae* causes growth inhibition associated with the occurrence of a G2/M arrest, which is markedly increased by treatment of these cells with PMA (15). However, as previously reported by us (17), under our experimental conditions and with the yeast strain used, single expression of a mammalian PKC-α, -δ, -ε or -ζ in yeast did not significantly interfere with cell growth and survival. Even so, in agreement with that reported for PKC-α (15), treatment of yeast expressing nPKC-δ or -ε with PMA caused significant growth inhibition related to a G2/M cell cycle arrest (Fig. 4.4A, B). This blockage in cell cycle progression by PMA was not accompanied by the occurrence of cell death, as revealed by the preservation of plasma membrane integrity, the absence of DNA fragmentation and chromatin condensation in PMA-treated yeast cells (data not shown).

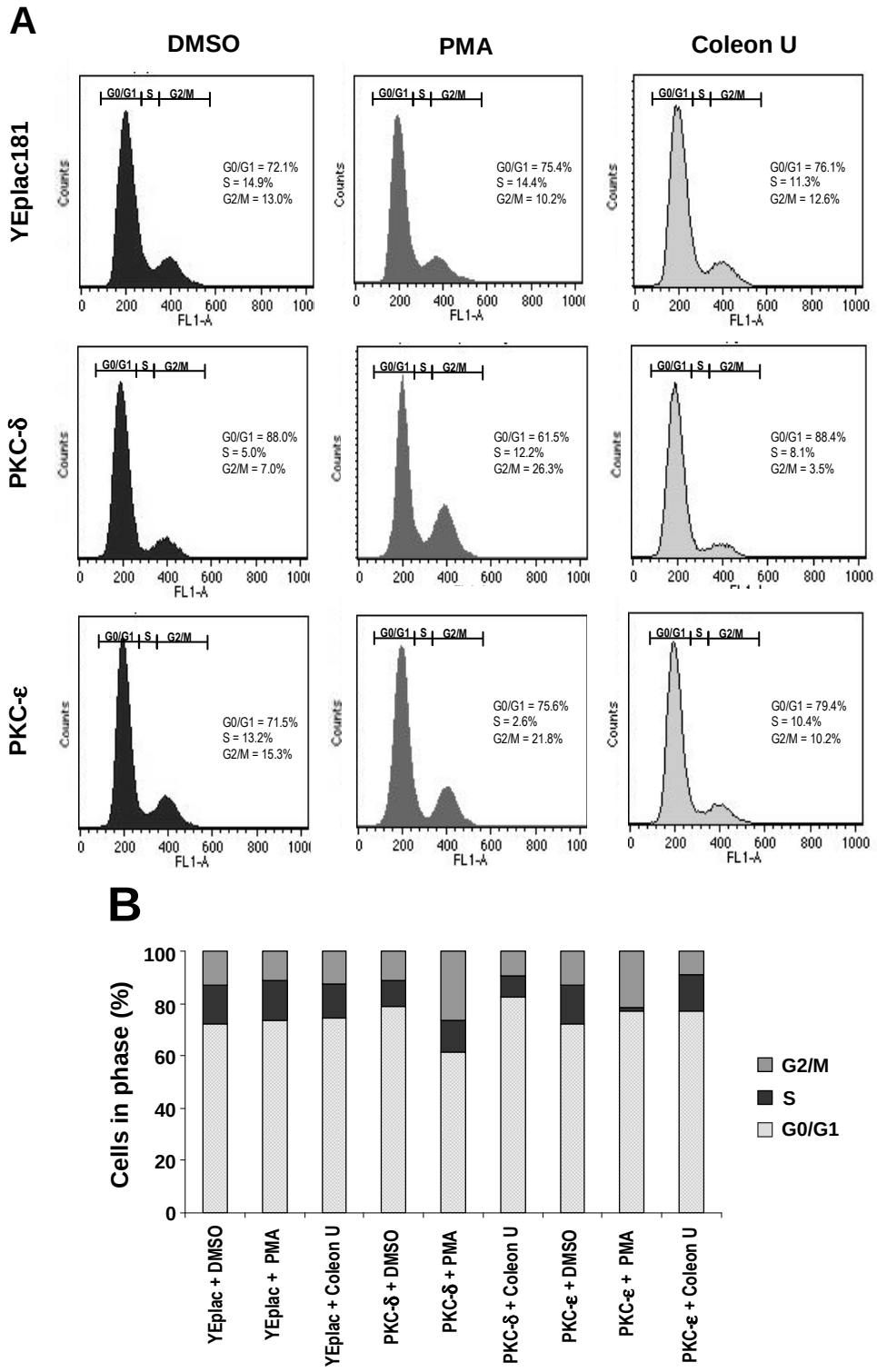


Fig. 4.4. Effect of PMA and coleon U on the cell cycle progression of control yeast and yeast expressing nPKC-δ or -ε. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U, 1 μM PMA or DMSO only to 0.5 OD_{600} (about 42 h incubation). Analysis of DNA content was carried out by flow cytometry using Sytox Green. (**A**) Histograms represent 1 of 3 independent experiments. (**B**) Quantification of yeast cell cycle phases; data are the mean of 3 independent experiments.

With a view to elucidating the molecular mechanisms behind coleon U-induced growth inhibition in yeast cells expressing nPKC-δ or -ε, we began by analysing the effect of this small-molecule on the cell cycle progression of these transformed yeast. In contrast to PMA, coleon U only slightly interfered with the cell cycle progression of yeast expressing nPKC-δ or -ε (Fig. 4.4A,B). This small effect of coleon U on cell cycle could not explain per se the marked growth inhibition induced by this small-molecule on yeast expressing nPKC-δ or -ε.

Hence, we next addressed whether coleon U-induced growth inhibition could reflect cell death. With this goal, several apoptotic markers were investigated. We verified that 1 μM coleon U caused in yeast expressing nPKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ, a marked increase in the percentage of cells with chromatin condensation and DNA fragmentation (TUNEL-positive cells), without loss of plasma membrane integrity as revealed by the low percentage of PI-positive cells (absence of necrosis) (Fig. 4.5A-C). These results indicated that coleon U-induced growth inhibition in yeast expressing PKC-δ or -ε was linked to the activation of an apoptotic cell death.

Additionally, flow cytometric analysis of FITC-VAD-fmk stained cells revealed the occurrence of yeast metacaspase (Yca1p) activation in coleon U-treated yeast cells expressing PKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ (Fig. 4.6A,B). This was further confirmed by the marked decrease in the percentage of dead cells expressing nPKC-δ or -ε obtained when coleon U treatment was carried out in the presence of the caspase inhibitor z-VAD-fmk (Fig. 4.6C).

We further observed that 1 μM coleon U markedly increased mitochondrial ROS production (Fig. 4.7A,B), decreased $\Delta\psi m$ (Fig. 4.8A,B) and increased mitochondrial fragmentation (Fig. 4.8C) in yeast expressing nPKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ.

It was showed that human Bcl-xL is also a mitochondrial anti-apoptotic protein in yeast, inhibiting yeast apoptosis induced by stress stimuli such as hydrogen peroxide, menadione (27) and acetic acid (17). Moreover, a previous work performed by our group showed that co-expression of human Bcl-xL with a mammalian PKC isoform did not significantly interfere with yeast cell growth and survival (17). Based on these data, the interference of human Bcl-xL with coleon U-induced cell death in yeast expressing PKC-α, -βI, -δ, -ε or -ζ and control yeast was analysed. The results obtained showed that Bcl-xL completely abolished the coleon U-induced apoptosis in yeast expressing nPKC-δ or -ε,

without interfering with the coleon U effect in control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ (Fig. 4.8D).

Together, the results obtained corroborate the activation by coleon U of a metacaspase- and mitochondrial-dependent apoptotic pathway dependent on nPKC-δ or -ε expression in yeast.

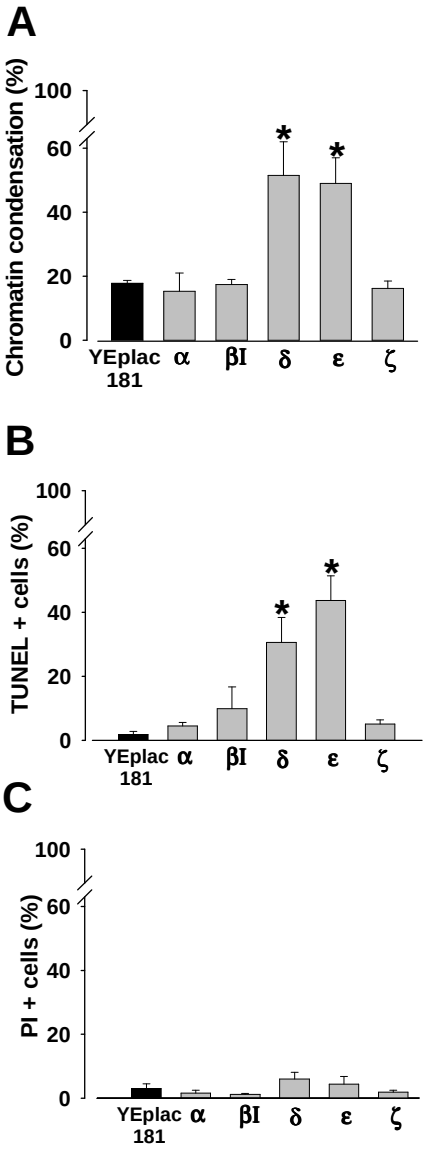


Fig. 4.5. Effect of coleon U on chromatin condensation, DNA fragmentation and plasma membrane integrity of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U to 0.5 OD_{600} (about 42 h incubation). (**A**) Chromatin condensation was analysed with DAPI. (**B**) Cells with DNA fragmentation are indicated as TUNEL + cells. (**C**) Necrotic cells are indicated as PI + cells. Data are the mean ± s.e.m. of 3 – 4 independent experiments; means correspond to counts of at least 600 cells per sample analysed by fluorescence microscopy. Values significantly different from those obtained with the control yeast, **P*<0.05.

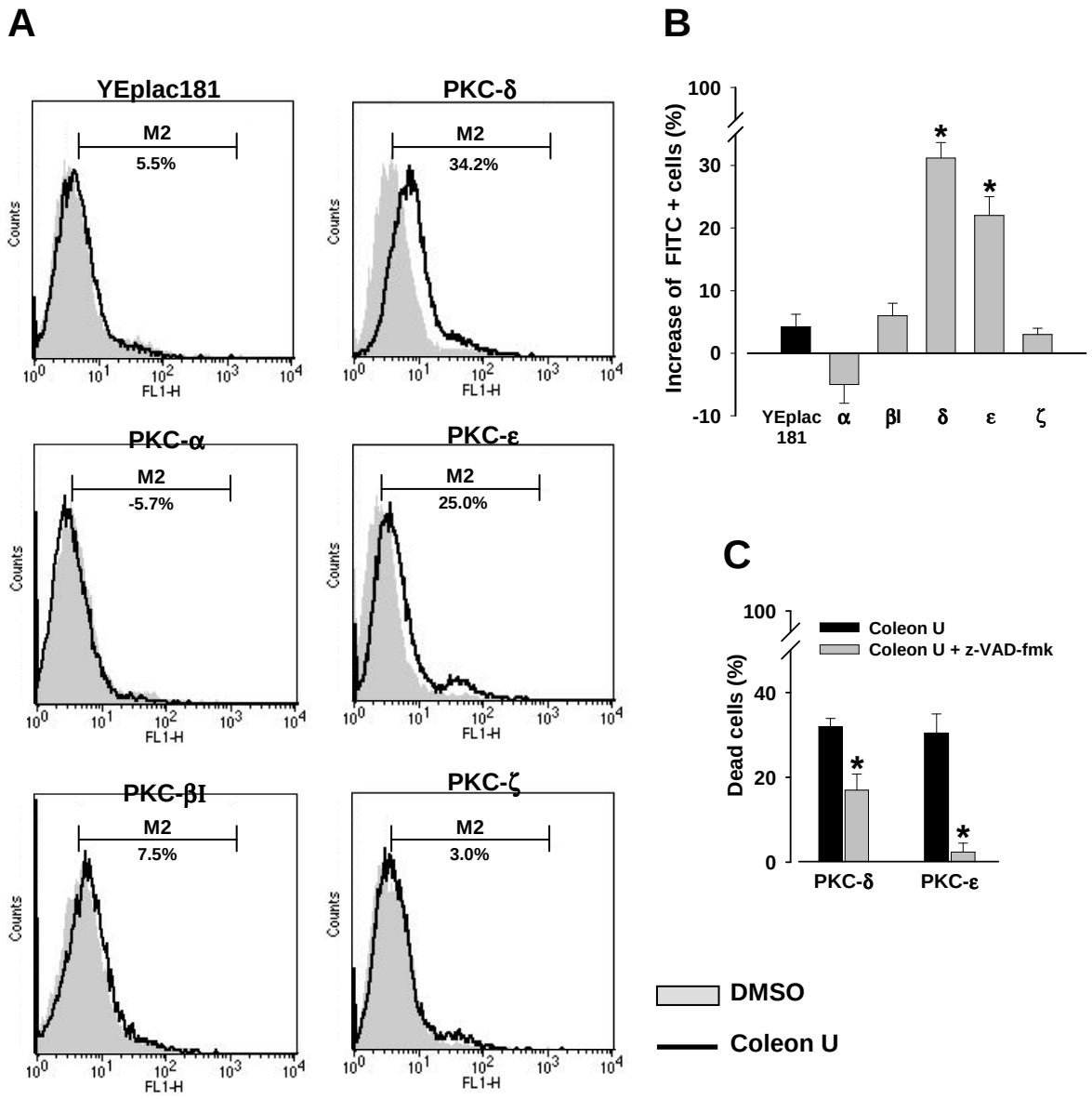


Fig. 4.6. Effect of coleon U on metacaspase activation of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U or DMSO only to 0.5 OD_{600} (about 42 h incubation). (**A**, **B**) Yeast metacaspase activation was monitored by flow cytometry using FITC-VAD-fmk. (**A**) Overlays of green fluorescence histograms were obtained with cells incubated with coleon U and DMSO only. M2 values correspond to the increase in the percentage of FITC-VAD-fmk positive cells obtained when cells were incubated with coleon U. Data represent 1 of 2 independent experiments. (**B**) Data are the mean ± s.e.m. of M2 values obtained from 2 independent experiments. Values significantly different from those obtained with the control yeast, **P*<0.05. (**C**) Effect of the caspase inhibitor z-VAD-fmk on the survival of coleon U-treated yeast cells expressing nPKC-δ or -ε. Cells were incubated in galactose selective medium with 1 μM coleon U, 1 μM coleon U and 20 μM of z-VAD-fmk or DMSO only to 0.5 OD_{600}. The percentage of dead cells was estimated by c.f.u. counts, considering 100% survival the number of c.f.u. obtained with cells incubated with DMSO only. Data are the mean ± s.e.m. of 4 independent experiments with 6 replicates each. Values significantly different from those obtained with yeast incubated with coleon U only, $^{*}P < 0.05$.

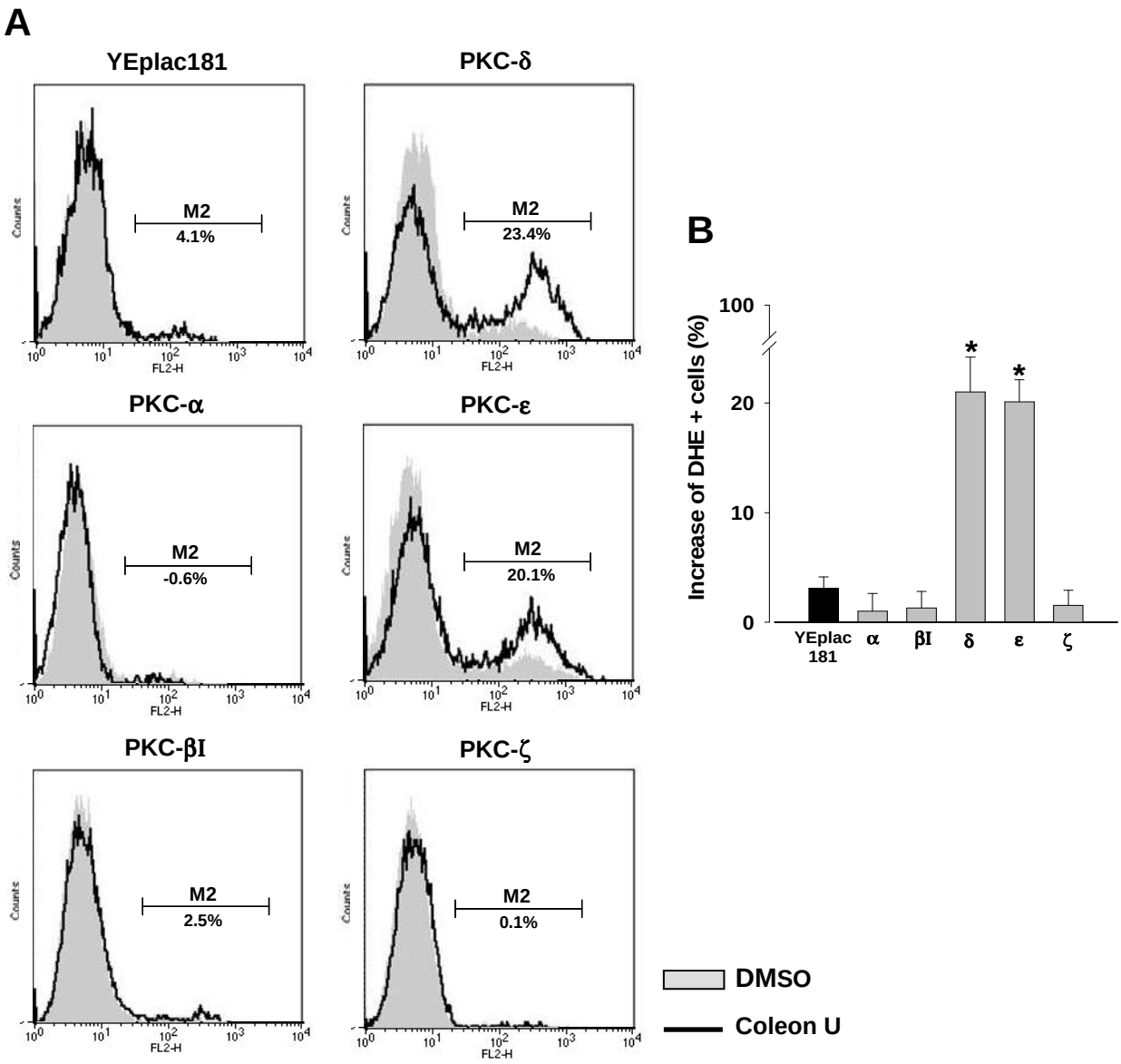


Fig. 4.7. Effect of coleon U on mitochondrial ROS production of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U or DMSO only to 0.5 OD_{600} (about 42 h incubation). ROS production was analysed by flow cytometry using DHE. (**A**) Overlays of red fluorescence histograms obtained with yeast incubated with coleon U or DMSO only. M2 values correspond to the increase in the percentage of DHE positive cells obtained when cells were incubated with coleon U. Data represent 1 of 2 independent experiments. (**B**) Data are the mean ± s.e.m. of M2 values obtained from 2 independent experiments. Values significantly different from those obtained with the control yeast, **P*<0.05.

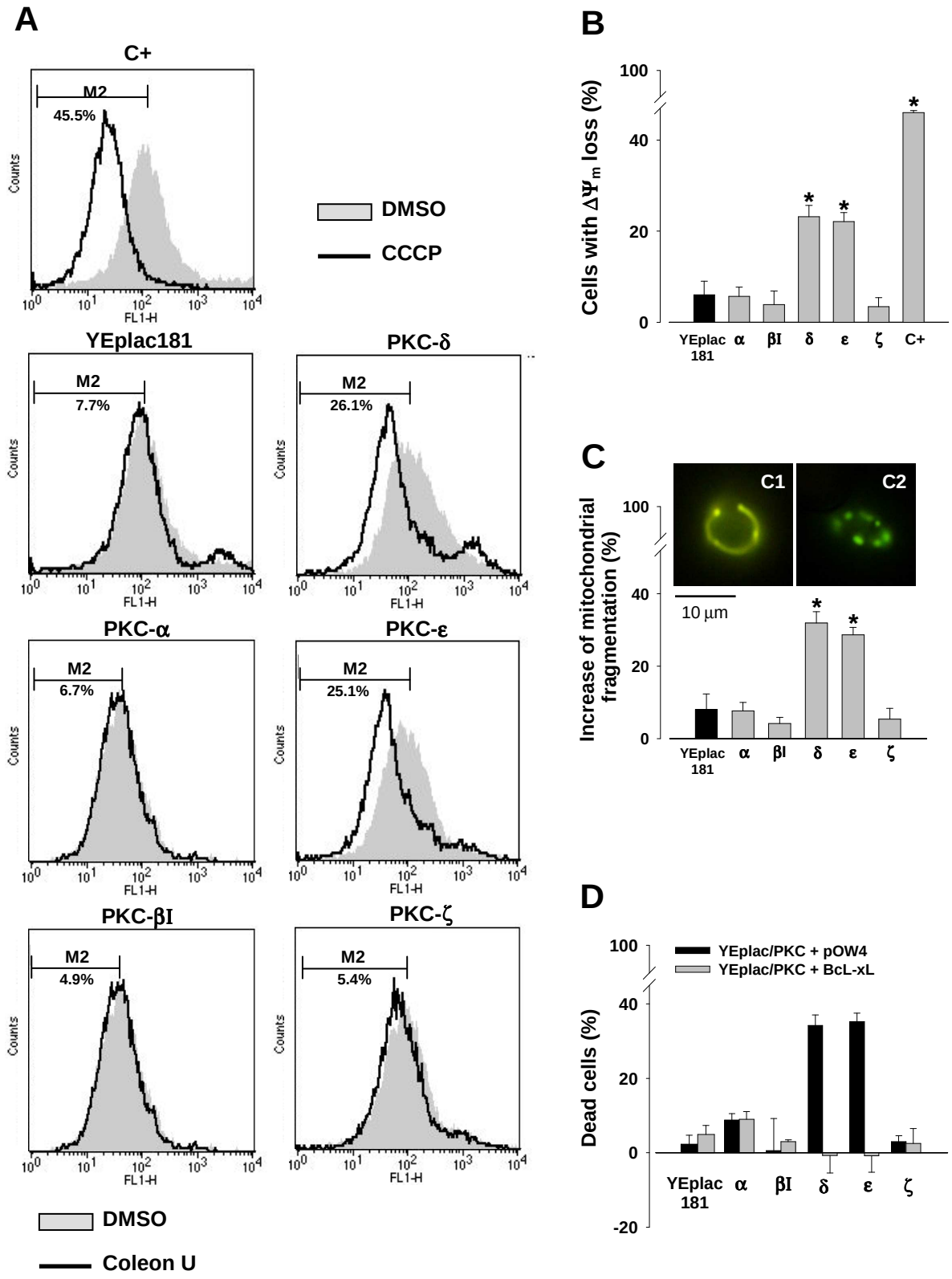


Fig. 4.8. Effect of coleon U on $\Delta\psi_m$ and mitochondrial fragmentation of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ and interference of human Bcl-xL with the coleon U-induced yeast cell death. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U or DMSO only to 0.5 OD_{600} (about 42 h incubation). (**A**, **B**) $\Delta\psi_m$ was monitored by flow cytometry

using $DiOC_6(3)$. (**A**) Overlays of green fluorescence histograms obtained with yeast incubated with coleon U and DMSO only. C+, positive control, control yeast treated with CCCP. M2 values correspond to the increase in the percentage of cells with $\Delta\psi_m$ loss obtained when cells were incubated with coleon U. Data represent 1 of 2 independent experiments. (**B**) Data are the mean ± s.e.m. of M2 values obtained from 2 independent experiments. Values significantly different from those obtained with the control yeast, **P*<0.05. (**C**) Mitochondrial fragmentation was analysed using yeasts expressing mt-GFP. Values correspond to the percentage of cells incubated with coleon U or DMSO only presenting mitochondrial fragmentation. Data are the mean ± s.e.m. of 3 independent experiments; means correspond to counts of at least 100 cells per sample analysed by fluorescence microscopy. Values significantly different from those obtained with the control yeast, **P*<0.05. C1: Normal tubular mitochondria; C2: Mitochondrial fragmentation observed with coleon U-treated yeast expressing nPKC-δ or -ε. (**D**) The interference of Bcl-xL with the coleon U effect was analysed by c.f.u. counts, considering 100% survival as the number of c.f.u. obtained with DMSO. Data are the mean ± s.e.m. of 4 independent experiments with 6 replicates each.

4.4.3. Coleon U-induced apoptosis is associated with the translocation of nPKC-δ and -ε from the cytosol to the nucleus of yeast cells

In mammalian cells, it was reported that distinct subcellular translocations of a specific PKC isoform lead to different cellular responses (28). Since PMA and coleon U induced in yeast expressing nPKC-δ or -ε different cellular responses, we questioned whether these two PKC activators could also induce distinct subcellular translocations of these nPKCs in yeast cells. With this goal, immunofluorescence studies were carried out with yeast cells expressing nPKC-δ or -ε treated with 1 μM PMA, 1 μM coleon U or DMSO only. Since the results obtained with PKC-ε were not significantly different from those obtained with PKC-δ, the PKC-δ results were used as representative of the subcellular distribution obtained with the two nPKCs (Fig. 4.9A,B). The results showed that in the absence of activators, nPKCs were predominantly located at the cytosol of yeast cells (Fig. 4.9A,B; DMSO). In agreement with that reported for mammalian cells (28), PMA caused a marked translocation of nPKCs to the yeast plasma membrane (Fig. 4.9A,B; PMA). On the other hand, coleon U caused a pronounced translocation of nPKCs to the nucleus of yeast cells (Fig. 4.9A,B; coleon U).

Together, the results obtained showed that PMA and coleon U induced translocation of nPKCs to distinct yeast subcellular compartments.

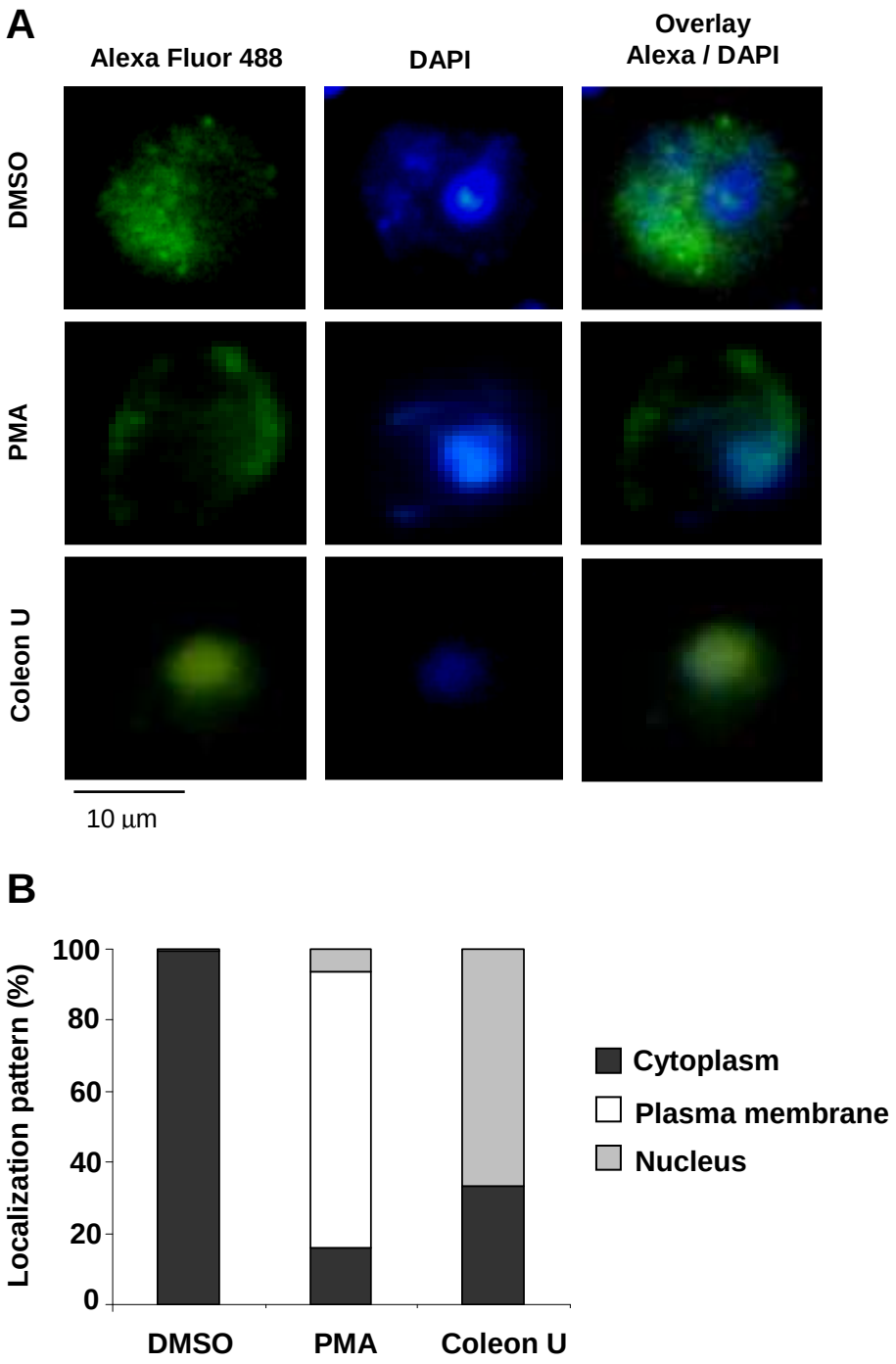


Fig. 4.9. Effect of PMA and coleon U on yeast subcellular localization of PKC-δ. Analysis of PKC-δ localization in yeast was carried out by immunofluorescence microscopy. (**A**) Cytoplasmic localization of PKC-δ observed when yeasts were incubated with DMSO only; localization of PKC-δ at the plasma membrane observed when yeasts were incubated with 1 µM PMA; nuclear localization of PKC-δ observed when yeasts were incubated with 1 µM coleon U. (**B**) Quantification of subcellular distribution of PKC-δ in yeast cells incubated with PMA, coleon U or DMSO only; values correspond to the mean of 3 independent experiments.

4.5. DISCUSSION

Previous studies from our group exploited the yeast PKC expression system not only in searching for isoform-selective PKC modulators (18-21), but also in studying the role of several PKC isoforms in apoptosis regulation (17). In the present work, this yeast PKC assay was used to study the modulatory activity of coleon U on several PKC isoforms of the three PKC subfamilies, cPKCs (α and βI), nPKCs (δ and ε) and aPKC (ζ), considered as the most relevant isoforms in carcinogenesis. With this cell system, it was possible to analyse the effect of coleon U on each PKC isoform expressed in the same cellular background and without the genetic complexity of mammalian pathways and interference from other PKC family isoforms.

In agreement with that reported for human cells (1,2), we observed that 0.1 - 1 μM coleon U also induced yeast growth inhibition but only in cells expressing the nPKC-δ or -ε. This isoform-selectivity was further observed for 0.3 μM coleon U using an *in vitro* PKC assay. Together, these results led us to discover that coleon U is a selective activator of nPKC-δ and -ε. This work also studied the molecular mechanisms of action underlying coleon U-induced growth inhibition in yeast expressing nPKC-δ or -ε. We demonstrated that this coleon U antiproliferative effect was not linked to the induction of cell cycle arrest, but instead to the activation of an apoptotic cell death. This is in agreement with a previous study performed in lymphocyte cells, where detection of PS externalisation by annexin V allowed the antiproliferative effect of coleon U to be associated with apoptosis induction (2). Herein, we have further shown that this coleon U-induced apoptosis involved the activation of a metacaspase- and mitochondrial-dependent pathway in yeast expressing nPKC-δ or -ε, as revealed by the increase in mitochondrial dysfunctions such as fragmentation, depolarization and ROS accumulation and by the complete abolishment of coleon U cytotoxic effect by the mitochondrial anti-apoptotic protein Bcl-xL.

Another relevant point from this work is the corroboration that a specific PKC isoform can induce, in the same cellular background, different cellular responses depending on the stimulus applied. In fact, comparing the effects of PMA and coleon U, we detected that these two PKC activators induced different effects in yeast due to the activation of the same PKC isoform. While PMA caused a G2/M cell cycle arrest, coleon U induced an apoptotic cell death in yeast expressing nPKC-δ or -ε. This can be explained by the ability of different stimuli to selectively translocate a PKC isoform to distinct subcellular compartments. In fact, it is believed that translocation of a specific PKC isoform to a cellular compartment is the major determinant of its specificity and function

(28). Indeed, similarly to results from mammalian cells (28,29), we found that in the absence of activators both nPKC-δ and -ε were localized in the cytosol of yeast cells. Additionally, as reported for PKC-δ (28,29) and PKC-ε (29) in mammalian cells, treatment of yeast cells with PMA induced translocation of these isoforms to the plasma membrane. On the other hand, coleon U treatment stimulated translocation of nPKC-δ and -ε to the nucleus of yeast cells. In accordance with the results obtained in yeast, recent studies performed with mammalian cells revealed that several pro-apoptotic kinases, specifically PKC-δ, undergo cytoplasmic-nuclear shuttling in response to DNA damage (30-32). These studies demonstrated that upon exposure to a genotoxic stress, such as etoposide, PKC-δ accumulates in the nucleus. Whereas retention of PKC-δ in the cytoplasm is compatible with cell survival, its nuclear retention is required for commitment to apoptosis, showing that cellular localization of PKC-δ regulates the survival/death pathway (30-32). Indeed, nuclear targeting of kinases such as PKC-δ is considered a new and essential regulatory mechanism that directly influences the induction of apoptosis (32). Thus, while modulation of nPKC-δ and -ε nuclear translocation by coleon U remains an open issue that deserves further studies, we corroborated previous data from mammalian cells showing that the correct cellular localization is critical to the function of these two kinases.

Though several evidences supporting the involvement of mitochondria in coleon U-induced apoptosis are presented herein, we could not demonstrate mitochondrial accumulation of nPKC-δ or -ε after coleon U treatment. Interestingly, other authors demonstrated the involvement of mitochondria in PKC-δ-induced apoptosis in a rat vascular smooth muscle cell line based on the decrease of apoptosis by Bcl-2 and loss of $\Delta\psi m$ without detection of PKC-δ mitochondrial localization (33). Another work also showed that although induction of apoptosis via activation of PKC-δ and -ε increased cytochrome c release and accumulation of ROS, no PKC-δ and -ε were detected in mitochondria (8). Supporting these data, we showed that also in yeast, under apoptotic conditions, PKC-δ and -ε seem to activate a mitochondrial pathway without directly interfering with this organelle. As stressed by Yoshida (32), the molecular devices that allow various stimuli, such as coleon U, to be transmitted from the nucleus into the mitochondrion and which represents a point of integration for the different apoptotic signalling cascades are still unclear. Further studies are underway in yeast to clarify this issue.

In conclusion, this work identifies a new isoform-selective small-molecule with potential pharmacological applications. Indeed, as a potent and selective activator of the nPKC-δ and -ε, coleon U represents a promising tool to further understand the nPKC-δ

and -ε cellular signalling pathways in mammalian cells. Furthermore, the absence of an effect on cPKC-α and -β and aPKC-ζ, reported as anti-apoptotic proteins, and its selectivity to induce an apoptotic pathway dependent on nPKC-δ and -ε activation, indicate that coleon U is a promising compound for evaluation as an anti-cancer drug. This work also shows, for the first time, that the yeast PKC expression system allows reconstituting distinct subcellular translocations of a specific PKC isoform and the subsequent different cellular responses previously reported for mammalian systems. Considering these similarities and the advantages provided by the genetic tractability of yeast, a broader outcome of this study is the validation of this cell model to unravel the intra-organelle communication systems and their roles in the PKC isoform apoptotic signalling network, as well as to study the molecular mechanisms of action of compounds with potential therapeutic applications.

4.6. ACKNOWLEDGMENTS

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4.7. REFERENCES

(1) Marques C, Pedro M, Simões M, Nascimento MSJ, Pinto M, Rodríguez B. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. Planta Med 2002 Sep; 68 (9): 839-40.

(2) Cerqueira F, Cordeiro-Da-Silva A, Gaspar-Marques C, Simões F, Pinto MMM, Nascimento MSJ. Effect of abietane diterpenes from *Plectranthus grandidentatus* on T- and B-lymphocyte proliferation. Bioorg Med Chem 2004 Jan 2; 12 (1): 217-23.

(3) Hofmann J. Protein kinase C isozymes as potential targets for anti-cancer therapy. Curr Cancer Drug Targets 2004 Mar; 4 (2): 125-46.

(4) Battaini F, Mochly-Rosen D. Happy birthday protein kinase C: Past, present and future of asuperfamily. Pharmacol Res 2007 Jun; 55 (6): 461-6.

(5) Gutcher I, Webb PR, Anderson NG. The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci 2003 Jun; 60 (6): 1061-70.

(6) Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. Cancer Lett 2006 Apr 8; 235 (1): 1-10.

(7) Irie K, Nakagawa Y, Ohigashi H. Toward the development of new medicinal leads with selectivity for protein kinase C isozymes. Chem Rec 2005; 5 (4): 185-95.

(8) Zhang Y, Venugopal SK, He S, Liu P, Wu J, Zern MA. Ethanol induces apoptosis in hepatocytes by a pathway involving novel protein kinase C isoforms. Cell Signal 2007 Nov; 19 (11): 2339-50.

(9) Budas GR, Churchill EN, Mochly-Rosen D. Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury. Pharmacol Res 2007 Jun; 55 (6): 523-36.

(10) Madeo F, Herker E, Wissing S, Jungwirth H, Eisenberg T, Fröhlich KU. Apoptosis in yeast. Curr Opin Microbiol 2004 Dec; 7 (6): 655-60.

(11) Barberis A, Gunde T, Berset C, Audetat S, Lu U. Yeast as a screening tool. Drug Discovery Today: Technologies 2005; 2 (2): 187-92.

(12) Petranovic D, Nielsen J. Can yeast systems biology contribute to the understanding of human disease?. Trends Biotechnol 2008 Nov ;26 (11) :584-90.

(13) Perez P, Calonge TM. Yeast protein kinase C. J Biochem 2002 Oct; 132 (4): 513-7.

(14) Parissenti AM, Riedel H. Yeast as a Host to Screen for Modulators and Regulatory Regions of Mammalian Protein Kinase C Isoforms. Methods Mol Biol 2003; 233: 491-516.

(15) Sprowl JA, Villeneuve DJ, Guo B, Young AJ, Hembruff SL, Parissenti AM. Changes in expression of cell wall turnover genes accompany inhibition of chromosome segregation by bovine protein kinase C alpha expression in *Saccharomyces cerevisiae*. Cell Biol Int 2007 Oct; 31 (10): 1160-72.

(16) Keenan C, Goode N, Pears C. Isoform specificity of activators and inhibitors of protein kinase C gamma and delta. FEBS Lett 1997 Sep; 415 (1): 101-8.

(17) Saraiva L, Silva RD, Pereira G, Gonçalves J, Côrte-Real M. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci 2006 Aug; 119 (15): 3171-81.

(18) Saraiva L, Fresco P, Sousa E, Pinto E, Pinto M, Gonçalves J. Synthesis and in vivo modulatory activity of protein kinase C of xanthone derivatives. Bioorg Med Chem 2002 Oct; 10 (10): 3219-27

(19) Saraiva L, Fresco P, Pinto E, Gonçalves J. Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay. Eur J Pharmacol 2004 May; 491 (2-3): 101-10.

(20) Saraiva L, Fresco P, Sousa E, Pinto E, Pinto M, Gonçalves J. Inhibition of protein kinase C by synthetic xanthone derivatives. Bioorg Med Chem 2003 Apr; 11 (7): 1215-25.

(21) Saraiva L, Fresco P, Pinto E, Sousa E, Pinto M, Gonçalves J. Inhibition of alpha, betaI, delta, eta and zeta protein kinase C isoforms by xanthonolignoids. J Enzyme Inhib Med Chem 2003 Aug; 18 (4): 357-70.

(22) Teixeira AP, Batista O, Simões MF, Nascimento J, Duarte A, de la Torre MC, et al. Abietane diterpenoids from *Plectranthus grandidentatus*. Phytochemistry 1997 Jan; 44 (2): 325-7.

(23) Fortuna M, Sousa MJ, Côrte-Real M, Leão C, Salvador A, Sansonetty F. Cell cycle analysis of yeasts using Syber Green I. In: J.P. Robinson, editor. Curr Protocols in Cytometry. New York: John Wiley & Sons; 2000.

(24) Silva RD, Sotoca R, Johansson B, Ludovico P, Sansonetty F, Silva MT, et al. Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. Mol Microbiol 2005 Nov; 58 (3): 824-34.

(25) Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, et al. Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 1999 May; 145 (4): 757-67.

(26) Okamoto K, Perlman PS, Butow RA. The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. J Cell Biol 1998 Aug; 142 (3): 613-23.

(27) Chen SR, Dunigan DD, Dickman MB. Bcl-2 family members inhibit oxidative stress-induced programmed cell death in *Saccharomyces cerevisiae*. Free Radic Biol Med 2003 May; 34 (10): 1315-25.

(28) Shirai Y, Saito N. Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. J Biochem 2002 Nov; 132 (5): 663-8.
(29) Wang QJ, Lu G, Schlapkohl WA, Goerke A, Larsson C, Mischak H, et al. The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-delta and –epsilon. Mol Cancer Res 2004 Feb; 2 (2): 129-40.
(30) DeVries-Seimon TA, Ohm AM, Humphries MJ, Reyland ME. Induction of apoptosis is driven by nuclear retention of protein kinase C delta. J Biol Chem 2007 Aug; 282 (31): 22307-14.
(31) Reyland ME. Protein kinase Cdelta and apoptosis. Biochem Soc Trans 2007 Nov; 35 (5): 1001-4.
(32) Yoshida K. Nuclear trafficking of pro-apoptotic kinases in response to DNA damage. Trends Mol Med 2008 Jul; 14 (7): 305-13.
(33) Goerke A, Sakai N, Gutjahr E, Schlapkohl WA, Mushinski JF, Haller H, et al. Induction of apoptosis by protein kinase C delta is independent of its kinase activity. J Biol Chem 2002 Aug; 277 (35): 32054-62.

CHAPTER 5

Aspartic vinyl sulfones: inhibitors of a caspase-3-dependent pathway

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ASPARTIC VINYL SULFONES: INHIBITORS OF A CASPASE-3-DEPENDENT PATHWAY

5.1. ABSTRACT

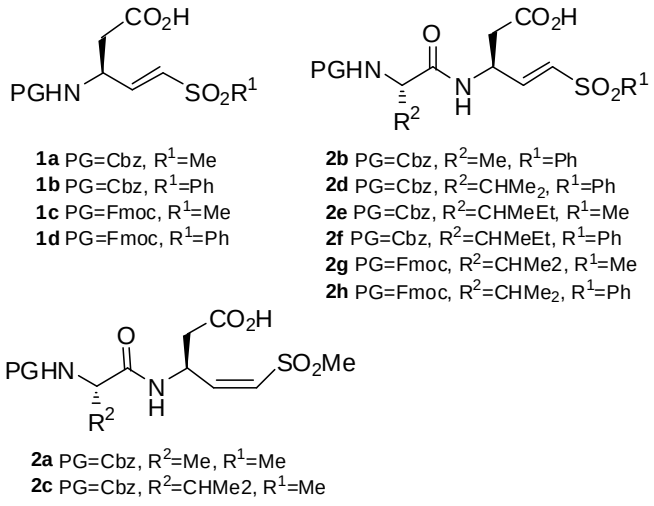
In this article we describe an expanded structure-activity relationship study for vinyl sulfones as caspase-3 inhibitors, a topic virtually unexplored in the existing literature. Most remarkably, and to our surprise, tripeptidyl vinyl sulfones were not active for caspase-3, opposite to other examples described in literature for peptidyl vinyl sulfones as potent cysteine protease inhibitors of clan CA. Moreover, the caspase-3 inhibitory activity of vinyl sulfones using an in vitro assay was then confirmed using a yeast cell-based assay. The results show that Fmoc-protected vinyl sulfones containing only the Asp moiety are inhibitors of a caspase-3-dependent pathway and the IC_{50} values obtained in the yeast assay are in the same order of magnitude of that obtained with the caspase-3 inhibitor tetrapeptidyl chloromethyl ketone, Ac-DEVD-CMK. This observation is consistent with appropriate cell permeability properties displayed by the vinyl sulfone inhibitors, as reflected by log *P* values ranging from 1.1 to 3.4. Overall, these results suggest that vinyl sulfones containing Asp at P1 should be considered for further optimization as caspase inhibitors and modulators of caspase-3-dependent pathways.

Keywords: Vinyl sulfone; Caspase-3 inhibitor; Michael acceptor; Irreversible inhibitor; Yeast cell-based assay.

5.2. INTRODUCTION

Caspases are a family of cysteine endoproteases involved in cytokine maturation and apoptosis. They represent one of the most specific protease families yet described, since they have an almost absolute requirement for an aspartic acid residue in the P_1 position of their substrate (1-3). To date, only a few caspase inhibitors have entered preclinical studies with animal models of human diseases and their major disadvantage is the lack of selectivity. As a consequence, the search for effective caspase inhibitors as possible therapeutic agents against different diseases caused by excessive apoptosis, such as neurodegenerative disorders, is an important area of research (4-6).

One type of irreversible cysteine proteases inhibitors that has received special attention in the last few years are the ones based on Michael acceptor scaffolds. This class of inhibitors includes vinyl sulfones, which have been developed as highly potent inhibitors of many clan CA cysteine proteases (7). Recently, we described the first structure-activity relationship study of peptidyl vinyl sulfones as caspase-3 inhibitors, enzymes that belong to clan CD (8). Vinyl sulfones containing Asp at P_1 **1a-d** and **2a-h** (Fig. 5.1) were shown to be only moderate inhibitors of caspase-3, with IC_{50} values in the µM range. Dipeptidyl vinyl sulfones displayed improved activity over their counterparts containing Asp as the single amino acid residue, with this effect being particularly noticeably for the Fmoc-protected compounds. The most active compound, Fmoc-VD-VSMe **2g**, presented an IC_{50} value of 29 µM and was selective for caspase-3 over caspase-7 (8).



1a PG=Cbz, R^1=Me
1b PG=Cbz, R^1=Ph
1c PG=Fmoc, R^1=Me
1d PG=Fmoc, R^1=Ph

2b PG=Cbz, R^2=Me, R^1=Ph
2d PG=Cbz, R^2=$CHMe_2$, R^1=Ph
2e PG=Cbz, R^2=CHMeEt, R^1=Me
2f PG=Cbz, R^2=CHMeEt, R^1=Ph
2g PG=Fmoc, R^2=CHMe2, R^1=Me
2h PG=Fmoc, R^2=$CHMe_2$, R^1=Ph

2a PG=Cbz, R^2=Me, R^1=Me
2c PG=Cbz, R^2=CHMe2, R^1=Me

Fig. 5.1. Vinyl sulfone caspase-3 inhibitors.

In this study, in order to expand the structure-activity relationships (SARs) and to improve the inhibitory potency of vinyl sulfones, in the present study, we decided to extend the recognition structure by incorporating a third amino acid to derivatives **2**. The *in vitro* caspase-3 inhibition for other Fmoc dipeptidyl vinyl sulfones was also studied. Finally, the caspase-3 inhibitory effect of the vinyl sulfones active *in vitro* was also analysed using a yeast cell-based assay.

In order to search for modulators of caspase-3, an independent analysis of this caspase is required. However, the identification of at least 14 caspase family members, coupled with the complex patterns of caspase gene expression, the tightly synchronized cascade of activation and the extensive cross-talk amongst numerous signalling pathways in mammalian cells, have hampered the pharmacological analysis of individual caspases in a cell environment (9). To address this issue, yeast expressing human caspase-3 can be used to analyse the caspase-3 inhibitory effect of small molecules in a simpler eukaryotic cell system. In fact, many researchers have used yeast expressing human caspase-3 to uncover the mechanisms of endogenous regulation of this caspase (10-17).

Indeed, the high degree of conservation of many pathways and cellular processes among yeast and human cells led several researchers to use yeast as a valuable cell model for functional studies of human proteins and as a drug screening tool (18-24). Additionally, though yeast has a metacaspase (Yca1p), it was shown that the activity of human caspase-3 in yeast is independent of Yca1p activity (17). Finally, it was shown that expression of an active form of caspase-3 in yeast caused a marked growth inhibition (10-17), which was reverted by endogenous inhibitors of caspase-3, such as mammalian IAPs (inhibitors of apoptosis) homologues (MIH) MIHA, MIHB and MIHC, and baculoviral caspase inhibitors p35 and p49 (12-16). These studies established a correlation between yeast cell growth and caspase-3 activity.

5.3. MATERIAL AND METHODS

5.3.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. High resolution mass spectra (HMRS) were performed in Unidade de Espectrometria de Masas, Santiago de Compostela. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental Analyser for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200-400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield (400 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (J) in hertz.

*5.3.1.1. General procedure for the preparation of vinyl sulfones **4a-d***

To a suspension of NaH 60% (1.8 mmol, 1.1 equiv.) in THF (5 ml), at 0 ºC, was added 1 equiv of the appropriate phosphonate. The resulting solution was stirred at temperature room for 30 minutes. A solution of 1 equiv of the appropriate aldehyde **3** in THF (16 ml) was added and stirred at room temperature for 2h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. The organic solution was washed with brine, dried, and concentrated. The resulting residue was flash chromatographed.

*5.3.1.2. General procedure for the preparation of vinyl sulfones **6a-d***

A solution of the appropriate vinyl sulfone **5** (8) (1 mmol, 1 equiv.) in a 1:1 mixture of MeCN/Et_2NH (5 ml) was stirred at room temperature for 2h. After this time, the solvent was removed under vacuum and the resulting residue was dissolved in DMF (3 ml). To this solution was added the protected dipeptide (FmocValValOSu, 1 equiv.) and the mixture stirred for 12h at room temperature. The reaction mixture was diluted with H_2O, extracted with AcOEt (3×) and the organic layers combined, dried under Na_2SO_4 and the solvent removed under vacuum. The resulting residue was flash chromatographed.

*5.3.1.3. General procedure for the preparation of vinyl sulfones **2i-m** and **7a**, **7b***

The appropriate vinyl sulfone was treated with TFA at 0 ºC for 1 h. TFA was removed under vacuum and the final products were recrystallized from ethyl acetate/hexane as white solids.

*5.3.1.3.1. FmocAlaAspVSMe (**2i**)*

Obtained in 93% yield. M.p. 97-98ºC; IR (NaCl): 3421, 3318, 3074, 2959, 1714, 1682, 1510 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (1H, d, *J* = 8.0 Hz), 7.77 (2H, d, *J* = 8.0 Hz), 7.59 (2H, d, *J* = 8.0 Hz), 7.42 (2H, t, *J* = 8.0 Hz), 7.28 (2H, t, *J* = 8.0 Hz), 6.63 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Me), 6.56 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Me), 5.50 (1H, m), 5.21 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.12 (3H, s), 2.51 (1H, m), 2.48 (1H, m), 1.24 (3H, d, *J* = 8.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.86, 171.90, 160.65, 158.68, 144.95, 141.31, 139.57, 128.78, 127.56, 124.34, 122.93, 66.68, 50.69, 50.33, 48.17, 39.89, 38.13, 18.44; HRMS-ESI-TOF: *m*/*z* calcd $C_{24}H_{26}N_2O_7SNa$ (M^++Na) 509.1358, found 509.1364.

*5.3.1.3.2. FmocAlaAspVSPh (**2j**)*

Obtained in 91% yield. M.p. 99-100ºC; IR (NaCl): 3421, 3305, 3061, 2932, 1714, 1657, 1510 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (1H, d, *J* = 8.0 Hz), 7.74 (2H, d, *J* = 8.0 Hz), 7.56-7.23 (11H, m), 6.72 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.66 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Ph), 5.29 (1H, m), 5.22 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.12 (3H, s), 2.81 (1H, m), 2.75 (1H, m), 1.25 (3H, d, *J* = 8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.72, 171.76, 160.51, 144.81, 140.75, 139.43, 136.77, 135.44, 133.04, 129.08, 128.63, 128.14, 127.41, 124.20, 122.79, 66.54, 50.54, 50.18, 48.03, 39.75, 18.29; HRMS-ESI-TOF: *m*/*z* calc $C_{29}H_{28}N_2O_7SNa$ (M^++Na) 571.1515, found 571.1523.

*5.3.1.3.3. FmocIleAspVSMe (**2l**)*

Obtained in 89% yield. M.p. 87-88ºC; IR (NaCl): 3305, 2971, 1727, 1657, 1523 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (1H, d, *J* = 8.0 Hz), 7.59 (2H, d, *J* = 8.0 Hz), 7.45 (2H, d, *J* = 8.0 Hz), 7.41 (2H, t, *J* = 8.0 Hz), 7.32 (2H, t, *J* = 8.0 Hz), 6.73 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Me), 6.66 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Me), 5.50 (1H, m), 5.23 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.02 (3H, s), 2.72 (1H, m), 2.60 (1H, m), 1.51 (1H, sl), 1.23 (1H, sl), 0.97 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.20, 171.85, 160.83,

158.64, 144.91, 141.27, 139.53, 128.73, 127.51, 124.30, 122.89, 66.63, 60.57, 50.28, 48.12, 39.85, 38.08, 35.66, 25.05, 16.37, 11.66; HRMS-ESI-TOF: *m*/*z* calc $C_{27}H_{32}N_2O_7SNa$ (M^++Na) 551.1828, found 551.1835.

*5.3.1.3.4. FmocIleAspVSPh (**2m**)*

Obtained in 88% yield. M.p. 111-112ºC; IR (NaCl): 3429, 2974, 1705, 1523 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.00 (1H, d, *J* = 8.0 Hz), 7.75 (2H, d, *J* = 8.0 Hz), 7.61-7.23 (11H, m), 6.73 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.63 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Ph), 5.46 (1H, m), 5.24 (1H, sl), 4.49 (2H, m), 4.23 (1H, m), 4.15 (1H, m), 2.73 (1H, m), 2.58 (1H, m), 1.51 (1H, sl), 1.27 (1H, sl), 0.68 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.11, 171.76, 160.73, 144.81, 140.75, 139.43, 136.77, 135.44, 133.04, 129.08, 128.63, 128.14, 127.41, 124.20, 122.79, 66.54, 60.47, 50.18, 48.03, 39.75, 35.56, 24.95, 16.27, 11.56; HRMS-ESI-TOF: *m*/*z* calc $C_{32}H_{34}N_2O_7SNa$ (M^++Na) 613.1984, found 613.1993.

*5.3.1.3.5. FmocValValAspVSMe (**7a**)*

Obtained in 69% yield. M.p. 96-97ºC; IR (NaCl): 3283, 2949, 1741, 1700, 1655, 1522 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.23 (1H, sl), 8,12 (1H, sl), 7.76 (2H, d, *J* = 8.0 Hz), 7.41-7.33 (6H, m), 6.74 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Me), 6.47 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Me), 4.70 (2H, d, *J* = 8.0 Hz), 4.60 (1H, sl), 4.54 (1H, sl), 4.47 (2H, m), 3.28 (1H, sl), 2.93 (3H, s), 2.78 (1H, dd, *J* = 8.0, 4.0 Hz), 2.57 (1H, dd, *J* = 8.0, 4.0 Hz), 2.03 (1H, m), 1.85 (1H, m), 1.07 (6H, d, *J* = 8.0 Hz), 1.00 (6H, d, *J* = 8.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.39, 172.21, 171.81, 160.79, 158.60, 144.87, 144.87, 141.23, 139.49, 128.69, 127.47, 124.26, 122.85, 66.54, 59.11, 58.33, 50.19, 48.03, 39.76, 37.99, 30.48, 19.04; HRMS-ESI-TOF: *m*/*z* calc $C_{31}H_{39}N_3O_8SNa$ (M^++Na) 636.2356, found 636.2363.

*5.3.1.3.6. FmocValValAspVSPh (**7b**)*

Obtained in 73% yield. M.p. 86-87ºC; IR (NaCl): 3314, 2992, 1740, 1702, 1666, 1516 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.50 (1H, sl), 7.88 (2H, d, *J* = 8.0 Hz), 7.79 (2H, d, *J* = 8.0 Hz), 7.71 (1H, m), 7.58 (2H, m), 7.43 (4H, m), 7.33 (2H, m), 7.16 (1H, sl), 6.50 (1H, dd, *J* = 16.0, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.25 (1H, d, *J* = 16.0 Hz, CH=C*H*SO$_2$Ph), 4.70 (2H, d, *J* = 8.0 Hz), 4.60 (1H, sl), 4.46 (1H, m), 4.39 (2H, sl), 4.15 (1H, sl), 2.83 (1H, dd, *J*

= 8.0, 4.0 Hz), 2.62 (2H, m), 2.27 (1H, m), 1.11 (6H, d, *J* = 8.0 Hz), 0.91 (6H, d, *J* = 8.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.34, 172.16, 171.76, 160.73, 144.81, 140.75, 139.43, 136.77, 135.44, 133.03, 129.09, 128.64, 128.64, 127.42, 124.21, 122.79, 66.56, 59.13, 58.35, 50.22, 48.05, 39.78, 30.50, 19.06; HRMS-ESI-TOF: *m/z* calc $C_{36}H_{41}N_3O_8SNa$ (M^++Na) 698.2512, found 698.2516.

5.3.2. Caspase-3 in vitro assays

Caspase-3 fluorimetric assay was used, which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC) moiety. Briefly, 5 μl of stock solution in assay buffer (10 U/ μl) of caspase-3 (caspase-3, human, recombinant, Calbiochem) were added to 5 μl of the tested inhibitors at various concentrations. The reaction was initiated by the addition of 190 μl of substrate to a final concentration of 200 μM in assay buffer (20 mM HEPES, 2mM EDTA, 0.1% CHAPS, and 5 mM DTT, pH 7.4). Liberation of AMC was monitored continuously at 37ºC using a Tecan infinite M200 (Tecan, Switzerland) 96-well plate reader (white plates from Greiner bio-one, Germany) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Ac-DEVD-CMK, Calbiochem).

5.3.3. Plasmids, yeast strain, transformation and growth conditions

Constructed yeast expression plasmid pGALL-*LEU2* encoding human reverse caspase-3, an active form of human caspase-3, and the respective empty vector were kindly provided by Dr. C. J. Hawkins (Children's Cancer Centre, Royal Children's Hospital, Parkville, Australia). Plasmids have a galactose-inducible *GAL1/10* promoter.

Saccharomyces cerevisiae CG379 (α *ade5 his7-2 leu2-112 trp1-289 ura3-52 [Kil-O];* Yeast Genetic Stock Center) strain was transformed by the lithium acetate method as described (24). Transformed cells were routinely grown in a minimal selective medium with 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and all the amino acids required for yeast growth (50 μg/ml) except leucine, at 30ºC, with mechanical shaking (200 r.p.m.) to approximately 1 optical density (OD_{600}; *Jenway 6310 Spectrophotometer*). To induce expression of human caspase-3, yeast cells were diluted to 0.05 OD_{600} in a minimal selective medium with 2% (w/v) galactose and raffinose,

instead of glucose, and 3% (v/v) glycerol, and grown at 30ºC with mechanical shaking (200 r.p.m.) to 0.3 OD_{600}.

5.3.3.1. Western blot analysis

Preparation of yeast extracts and Western blot analysis were performed basically as described (24). Expression of human caspase-3 in yeast was detected using the anti-caspase-3 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), followed by the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). For loading control, membranes were stripped and reprobed with the anti-Pgk1p mouse monoclonal antibody (1:5000; Molecular probes) followed by the anti-mouse HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Immunoblots were developed by enhanced chemiluminescence.

5.3.3.2. Yeast caspase-3 assay

To analyse the effect of compounds on the growth of yeast expressing human caspase-3 and control yeast (transformed with the vector without the cDNA encoding human caspase-3; pGALL), transformed cells were incubated in galactose selective medium with 5 – 100 µM vinyl sulfones, 5 – 50 µM Ac-DEVD-CMK (positive control; Caspase-3 inhibitor III; Calbiochem) or 0.1% DMSO only to 0.3 OD_{600}. Cell growth was analysed by counting the number of colony-forming units (CFU) per ml obtained after 2 days incubation, at 30 ºC, on Sabouraud Dextrose Agar plates. The percentage of growth stimulation of yeast expressing caspase-3 (proportional to the degree of caspase-3 inhibition) caused by compounds was calculated considering the number of CFU obtained with yeast expressing caspase-3 incubated only with DMSO as 100% growth. Concentration-response curves for 5 – 100 µM vinyl sulfones and 5 - 50 µM Ac-DEVD-CMK were obtained, considering the maximal growth stimulation achieved with 25 µM Ac-DEVD-CMK as 100% caspase-3 inhibition, and the lowest concentration that caused 50% caspase-3 inhibition (IC_{50}) calculated.

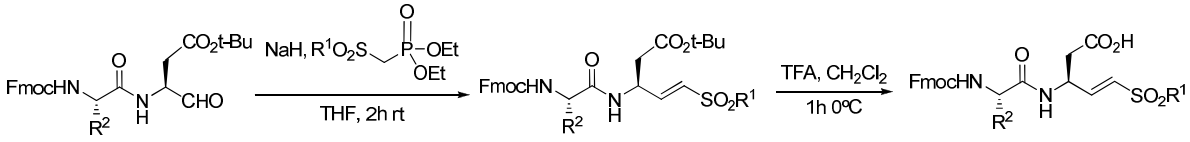
5.3.3.3. Statistical analysis

Data were analysed statistically using the *SigmaStat 3.5 software*. Differences between means were tested for significance using the unpaired Student´s *t* test ($P < 0.05$).

5.4. RESULTS AND DISCUSSION

5.4.1. Chemistry

Dipeptidyl aldehydes **3a** and **3b** were synthesized as reported before from the correspondent dipeptide acids using Weinreb chemistry and used without further purification. After Horner-Wadsworth-Emmons reaction between the dipeptidyl aldehydes **3a** and **b** with the correspondent phosphonate, vinyl sulfones **4a-d** were obtained with yields of 36-53%. Deprotection of vinyl sulfones **4a-d** with TFA, afforded vinyl sulfones **2i-m** with yields of 88-93% (Scheme 1).



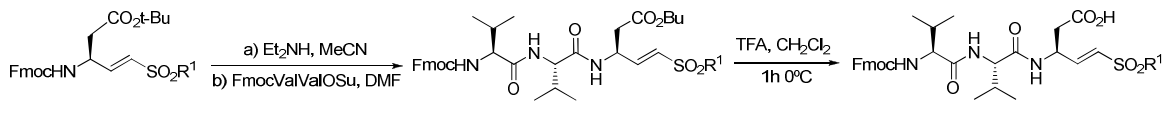
3a R^2=Me
3b R^2=CHMeEt

4a R^2=Me, R^1=Me, 36%
4b R^2=Me, R^1=Ph, 44%
4c R^2=CHMeEt, R^1=Me, 53%
4d R^2=CHMeEt, R^1=Ph, 38%

2i R^2=Me, R^1=Me, 93%
2j R^2=Me, R^1=Ph, 91%
2l R^2=CHMeEt, R^1=Me, 89%
2m R^2=CHMeEt, R^1=Ph, 88%

Scheme 1. Synthesis of Fmoc-dipeptidyl vinyl sulfones **2i-m**.

To improve the potency of the inhibitors, we decided to extend the molecular recognition by incorporating a third amino acid, Valine, since it has been shown experimentally to be favourable at the P_3 position (25). We had tried before, without success, to obtain the dipeptidyl vinyl sulfones **2** by Fmoc deprotection of the nitrogen atom of the vinyl sulfones **5a** and **b**, using several different basic conditions (piperidine, triethylamine, DBU) (8). However, recently this Fmoc deprotection was accomplished in the presence of Et_2NH (26). Using the same methodology, vinyl sulfones **6a** and **b** were obtained in a one pot reaction, by *N*-deprotection of vinyl sulfones **5a** and **b** using Et_2NH in acetonitrile, followed by coupling with the dipeptide FmocValValOSu in DMF with yields of 54-57%. Deprotection of vinyl sulfones **6a** and **b** with TFA, afforded vinyl sulfones **7a** and **b** with yields of 69-73% (Scheme 2).



5a R^1=Me
5b R^1=Ph

6a R^1=Me, 54%
6b R^1=Ph, 57%

7a R^1=Me, 73%
7b R^1=Ph, 69%

Scheme 2. Synthesis of Fmoc-tripeptidyl vinyl sulfones **7a** and **b**.

All of the proposed structures were established by NMR (^{1}H, ^{13}C, COSY and HMQC), IR, and MS. The stereochemistry around the double bond was established using the corresponding ^{1}H NMR coupling constant, where a double doublet (J = 16.0 Hz and 4 Hz) and a doublet (J = 16.0 Hz) are observed for the β and α proton relative to the sulfone group, respectively, confirming the presence of the *E* isomer on vinyl sulfones **2i-m** and **7a**, **7b.**

5.4.2. Biological activity

The vinyl sulfones synthesized were examined for their ability to inhibit the activity of recombinant human effector caspase-3. The IC_{50} values were determined using a fluorometric assay and the tetrapeptidyl chloromethyl ketone Ac-DEVD-CMK was used as positive control. Fmoc dipeptidyl vinyl sulfones with Ala at position P_2 **2i** and **2j** were inactive against caspase-3 for the tested concentrations (<200 μM), as we had observed previously for the Cbz-Ala-Asp vinyl sulfones **2a** and **2b** counterparts.

Surprisingly, the presence of Ile on vinyl sulfones **2l** and **2m** did not improve the activity related to the Fmoc-Val-Asp vinyl sulfones **2g** and **2h**, as was observed before for the Cbz dipeptidyl series. Most remarkably, and to our surprise, tripeptidyl vinyl sulfones were not active for caspase-3, opposite to other examples described in literature for peptidyl vinyl sulfones as potent cysteine protease inhibitors of clan CA (7). In fact, the presence of a third amino acid on vinyl sulfones **2g** and **2h** also resulted in lost of activity against caspase-3, as observed for the Fmoc-tripeptidyl vinyl sulfones **7a** and **b**.

The vinyl sulfones that were active *in vitro* were then studied using a yeast cell-based assay (Table 5.1). In this assay, inhibitors of caspase-3 are those compounds that stimulate the growth of yeast expressing human caspase-3 without interfering with the growth of control yeast (transformed with the vector without the cDNA encoding human caspase-3).

To implement this assay, we firstly confirmed that, as previously reported by other authors (10-17), yeast expression of human reverse caspase-3, an active form of human caspase-3 (Fig. 5.2) caused a marked growth inhibition, when compared to control yeast (pGALL) (Fig. 5.3). Additionally, the reduction of this caspase-3-induced yeast growth inhibition by the commercial caspase-3 inhibitor, Ac-DEVD-CMK, (Fig. 5.3) corroborated the correlation, between stimulation of growth of yeast expressing caspase-3 and caspase-3 inhibition, and validated this assay to search for small molecule inhibitors of caspase-3.

Table 5.1. Inhibition of human caspase-3 using a yeast cell system by vinyl sulfones (PG-N-X-CH=CHSO$_2$R)

Compound	PG	X	R	IC_{50} (μM)[a]	Growth Stimulation (%)[b]
				Cell System	(Control Yeast)
1c	Fmoc	Asp	Me	23.7±4.5	1.7 ± 3.2
1d	Fmoc	Asp	Ph	16.2±2.0	2.8 ± 2.3
2c	Cbz	Val-Asp	Me	35.3±3.3*	-1.0 ± 0.4
2e	Cbz	Ile-Asp	Me	30.5±3.5*	-3.3 ± 2.1
2f	Cbz	Ile-Asp	Ph	28.3±8.9	1.9 ± 2.2
2g	Fmoc	Val-Asp	Me	34.0±4.3*	-7.0 ± 0.1
2h	Fmoc	Val-Asp	Ph	29.9±3.4*	1.3 ± 3.9
Ac-DEVD-CMK	—	—	—	13.4±2.8	1.7 ± 3.2

The percentage of growth stimulation was calculated considering 100% growth the number of CFU obtained with transformed yeast incubated with DMSO only.

[a] IC_{50} values, were obtained from concentration-response curves of 5-100 μM vinyl sulfones and 5-50 μM Ac-DEVD-CMK, which were obtained considering the maximal growth stimulation obtained with 25 μM Ac-DEVD-CMK as 100% caspase-3 inhibition. Data are means ± s.e.m. of IC_{50} values obtained from 4-5 independent experiments with 6 replicates each. IC_{50} value significantly higher than that obtained with Ac-DEVD-CMK, *P< 0.05.

[b] Each compound was tested in control yeast at the concentration that caused the maximal effect on yeast expressing caspase-3. Positive values indicate stimulation of yeast growth. Data are means ± s.e.m. of 4 independent experiments with 6 replicates each.

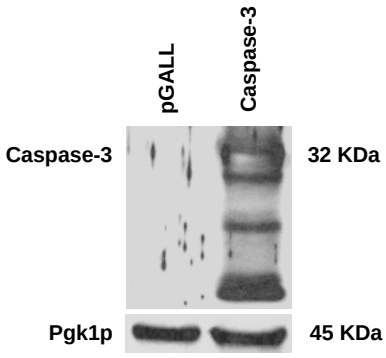


Fig. 5.2. Expression of human reverse caspase-3 in yeast was confirmed by Western blot analysis. Control yeast (pGALL) and yeast expressing caspase-3 were incubated in galactose selective medium to 0.3 OD_{600}. Full length procaspase (32 kDa) and the respective cleaved fragments (processed caspase-3) were detected using an anti-caspase-3 polyclonal antibody. Detection of Pgk1p was used as loading control. Immunoblots were developed by chemiluminescence.

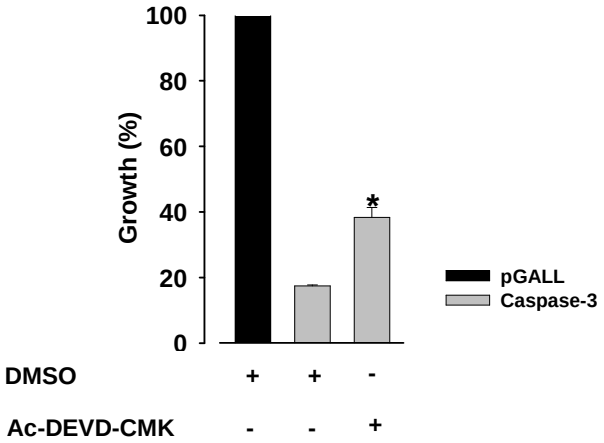


Fig. 5.3. Expression of human caspase-3 caused a marked yeast growth inhibition that was significantly reduced by the commercial caspase-3 inhibitor Ac-DEVD-CMK. Transformed yeast cells were incubated in galactose selective medium with 25 µM Ac-DEVD-CMK or DMSO only to 0.3 OD_{600}. Yeast growth was evaluated by CFU counts, considering the number of CFU obtained with control yeast (pGALL) incubated only with DMSO as 100% growth. Data are means ± s.e.m. of 4 independent experiments with 6 replicates each. Value of yeast expressing caspase-3 incubated with Ac-DEVD-CMK significantly higher than that obtained with DMSO only, **P* < 0.05.

Subsequently, the effect of vinyl sulfones **1c**, **1d**, **2c**, **2e-f** and **2g-h** on caspase-3 activity was also analysed using the yeast caspase-3 assay. For that, concentration-response curves for 5 - 100 µM vinyl sulfones and 5 - 50 µM Ac-DEVD-CMK (positive control) were obtained, considering the maximal growth stimulation achieved with 25 µM Ac-DEVD-CMK as 100% caspase-3 inhibition (Fig. 5.4A), and the IC_{50} value (lowest concentration of compound that caused 50% caspase-3 inhibition) was determined for each compound. The results obtained showed that all vinyl sulfones active *in vitro* behaved as inhibitors of a caspase-3 pathway in the cell-based assay (Table 5.1). In fact, vinyl sulfones **1c**, **1d**, **2c**, **2e-f** and **2g-h**, did not interfere with the growth of control yeast. This indicates that these vinyl sulfones had no effect on endogenous yeast proteins, such as yeast caspase-like. However, they inhibited human caspase-3-induced yeast growth inhibition. This reveals that they inhibited caspase-3 directly or indirectly by inhibition of a caspase-3-dependent pathway.

Based on Table 5.1, it was observed that the IC_{50} values for vinyl sulfones **1c**, **1d**, **2c**, **2e-h** determined in the yeast assay ranged from 16 to 35 µM and were within the same order of magnitude of that determined for Ac-DEVD-CMK (13 µM - Table 5.1). A remarkable increase in the activity of some vinyl sulfones, identified as weak inhibitors of caspase-3 in the *in vitro* assay, was observed in the yeast-cell based assay. In particular, vinyl sulfone **1d**, exhibited a proliferative effect (reduction of caspase-3-induced growth

inhibition) in the same order of magnitude of the commercial caspase-3 inhibitor, Ac-DEVD-CMK (Fig. 5.4A,B and Table 5.1). These results indicate that, in spite of their poor activity as inhibitors of caspase-3 (8), vinyl sulfones **1c**, **1d**, **2c**, **2e-f** and **2h** are inhibitors of a caspase-3-dependent pathway. In fact, since these compounds have a lower potency *in vitro* than in the yeast-cell based assay, we hypothesize that although they inhibit caspase-3 their main effect can be ascribed to the inhibition of a caspase-3-dependent pathway by modulation of other proteins of its pathway that indirectly inhibit caspase-3. In fact, in the case of Fmoc-Asp vinyl sulfones **1c** and **1d** the lower selectivity for caspase-3, can be explained because the recognition structure is less extended than the one of the dipeptide counterparts.

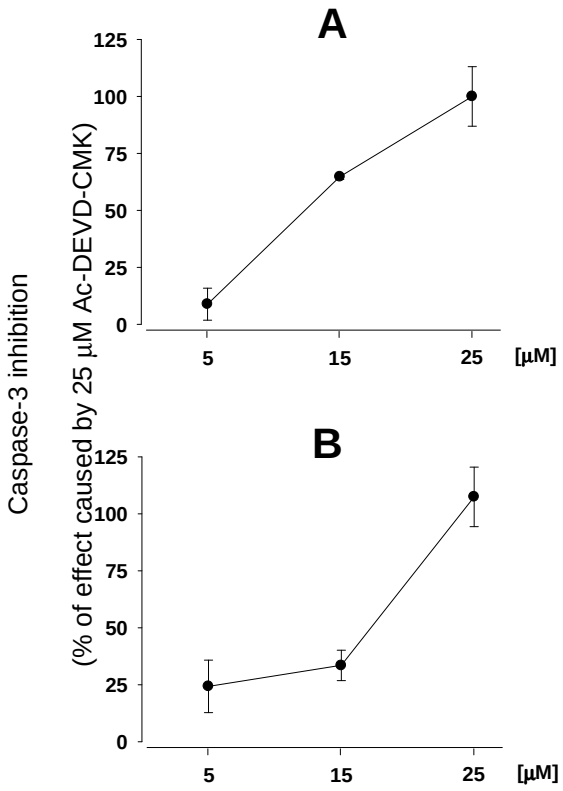


Fig. 5.4. Concentration-response curves for (**A**) Ac-DEVD-CMK and (**B**) vinyl sulfone **1d**. The percentage of growth stimulation caused by compounds was calculated considering the number of CFU obtained with yeast expressing caspase-3 incubated with DMSO only as 100% growth. Concentration-response curves were obtained considering the maximal growth stimulation obtained with 25 µM Ac-DEVD-CMK as 100% caspase-3 inhibition. Data are mean ± s.e.m. 4 to 5 independent experiments.

Overall, the results obtained from the yeast cell-based assay strongly suggest that the vinyl sulfones reported in the present study are inhibitors of a caspase-3-dependent pathway. Importantly, the calc log *P* values (27) for vinyl sulfones range from

1.1 to 3.4 (Table 5.2), i.e. significantly higher than that of Ac-DEVD-CMK (calc log *P* = -0.6), suggesting that they present appropriate cell permeability properties.

Table 5.2. Relevant parameters for preliminary permeation properties of vinyl sulfones **1c**, **1d**, **2c** and **2e-h** (data for Ac-DEVD-CMK are also included for comparison).

Compound	PG	X	R	log*P*[a]
1c	Fmoc	Asp	Me	2.22
1d	Fmoc	Asp	Ph	2.34
2c	Cbz	Val-Asp	Me	1.16
2e	Cbz	Ile-Asp	Me	1.53
2f	Cbz	Ile-Asp	Ph	2.38
2g	Fmoc	Val-Asp	Me	2.33
2h	Fmoc	Val-Asp	Ph	3.43
Ac-DEVD-CMK	—	—	—	-0.63

[a] Estimated by the ALOGPS 2.1 algorithm (27).

5.5. CONCLUSION

The comprehensive SAR study of vinyl sulfones as caspase-3 inhibitors, incorporating one, two and three amino acid residues, reveals that introduction of a third amino acid in dipeptidyl vinyl sulfones (i.e. converting **2g** and **2h** into **7a** and **7b**, respectively), did not improve the inhibitory activity determined using the biochemical assay. For all the series of vinyl sulfones synthesized, the compounds active against caspase-3 were then tested in a yeast cell system expressing this enzyme. This yeast assay revealed that Fmoc vinyl sulfones containing Asp at P1 **1c** and **1d** were inhibitors of a caspase-3-dependent pathway in a cell system, with IC_{50} values similar to that obtained with the positive control (Ac-DEVD-CMK). In spite of their lack of selectivity to caspase-3, they can be considered potent modulators of a caspase-3-dependent pathway and therefore interesting compounds to be explored *in vivo*. Additionally, they can be used as lead compounds for the development of more potent and selective caspase-3 inhibitors. Further work must be carried out in order to identify the molecules of the caspase-3 pathway also modulated by these compounds.

5.6. ACKNOWLEDGMENTS

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5.7. REFERENCES

(1) Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999; 68: 383-424.

(2) Denault JB, Salvesen GS. Caspases: keys in the ignition of cell death. Chem Rev. 2002 Dec; 102 (12): 4489-500.

(3) Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J. 2004 Dec; 384 (2): 201-32.

(4) Fleischer A, Ghadiri A, Dessauge F, Duhamel M, Rebollo MP, Alvarez-Franco F et al. Modulating apoptosis as a target for effective therapy. Mol Immunol. 2006 Mar; 43 (8): 1065-79.

(5) Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008 Mar; 9 (3): 231-41.

(6) Castro RE, Santos MM, Glória PM, Ribeiro CJ, Ferreira DM, Xavier JM, Moreira R, Rodrigues CM. Cell death targets and potential modulators in Alzheimer's disease. Curr Pharm Des. 2010; 16 (25): 2851-64.

(7) Santos MM, Moreira R. Michael acceptors as cysteine protease inhibitors. Mini Rev Med Chem. 2007 Oct; 7 (10): 1040-50.

(8) Newton AS, Glória PM, Gonçalves LM, dos Santos DJ, Moreira R, Guedes RC et al. Synthesis and evaluation of vinyl sulfones as caspase-3 inhibitors. A structure-activity study. Eur J Med Chem. 2010 Sep; 45 (9): 3858-63.

(9) Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. J Clin Invest. 2005 Oct; 115 (10): 2665-72.

(10) Kang JJ, Schaber MD, Srinivasula SM, Alnemri ES, Litwack G, Hall DJ et al. Cascades of mammalian caspase activation in the yeast Saccharomyces cerevisiae. J Biol Chem. 1999 Jan; 274 (5): 3189-98.

(11) Wright ME, Han DK, Carter L, Fields S, Schwartz SM, Hockenbery DM. Caspase-3 inhibits growth in Saccharomyces cerevisiae without causing cell death. FEBS Lett. 1999 Mar; 446 (1): 9-14.

(12) Wright ME, Han DK, Hockenbery DM. Caspase-3 and inhibitor of apoptosis protein(s) interactions in Saccharomyces cerevisiae and mammalian cells. FEBS Lett. 2000 Sep; 481 (1): 13-8.

(13) Hawkins CJ, Silke J, Verhagen AM, Foster R, Ekert PG, Ashley DM. Analysis of candidate antagonists of IAP-mediated caspase inhibition using yeast reconstituted with the mammalian Apaf-1-activated apoptosis mechanism. Apoptosis. 2001 Oct ;6 (5): 331-8.

(14) Silke J, Ekert PG, Day CL, Hawkins CJ, Baca M, Chew J, Pakusch M, Verhagen AM, Vaux DL. Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP. EMBO J. 2001 Jun; 20 (12): 3114-23.

(15) Jabbour AM, Ekert PG, Coulson EJ, Knight MJ, Ashley DM, Hawkins CJ. The p35 relative, p49, inhibits mammalian and Drosophila caspases including DRONC and protects against apoptosis. Cell Death Differ. 2002 Dec; 9 (12): 1311-20.

(16) Ho PK, Jabbour AM, Ekert PG, Hawkins CJ. Caspase-2 is resistant to inhibition by inhibitor of apoptosis proteins (IAPs) and can activate caspase-7. FEBS J. 2005 Mar; 272 (6): 1401-14.

(17) Puryer MA, Hawkins CJ. Human, insect and nematode caspases kill Saccharomyces cerevisiae independently of YCA1 and Aif1p. Apoptosis. 2006 Apr; 11 (4): 509-17.

(18) Barberis A, Gunde T, Berset C, Audetat S, Lu U. Yeast as a screening tool. Drug Discovery Today: Technologies 2005; 2 (2): 187-92.

(19) Sturgeon CM, Kemmer D, Anderson HJ, Roberge M. Yeast as a tool to uncover the cellular targets of drugs. Biotechnol J. 2006 Mar; 1 (3): 289-98.

(20) Miller-Fleming L, Giorgini F, Outeiro TF. Yeast as a model for studying human neurodegenerative disorders. Biotechnol J. 2008 Mar; 3 (3): 325-38.

(21) Petranovic D, Nielsen J. Can yeast systems biology contribute to the understanding of human disease?. Trends Biotechnol 2008 Nov ;26 (11) :584-90.

(22) Greenwood MT, Ludovico P. Expressing and functional analysis of mammalian apoptotic regulators in yeast. Cell Death Differ. 2010 May; 17 (5): 737-45.

(23) Coutinho I, Pereira G, Simões MF, Côrte-Real M, Gonçalves J, Saraiva L. Selective activation of protein kinase C-delta and -epsilon by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U). Biochem Pharmacol. 2009 Sep; 78 (5): 449-59.

(24) Saraiva L, Silva RD, Pereira G, Gonçalves J, Côrte-Real M. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci. 2006 Aug; 119 (15): 3171-81.

(25) Guo Z, Xian M, Zhang W, McGill A, Wang PG. N-nitrosoanilines: a new class of caspase-3 inhibitors. Bioorg Med Chem. 2001 Jan; 9 (1): 99-106.

(26) Lee JT, Chen DY, Yang Z, Ramos AD, Hsieh JJ, Bogyo M. Design, syntheses, and evaluation of Taspase1 inhibitors. Bioorg Med Chem Lett. 2009 Sep; 19 (17): 5086-90.

(27) ALOGPS 2.1, Virtual Computational Chemistry Laboratory. Available in http://www.vcclab.org/lab/alogps/ (accessed 15.02.11).

CHAPTER 6

Selective activation of human caspase-7 by prenylated flavonoids, revealed by yeast assays

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SELECTIVE ACTIVATION OF HUMAN CASPASE-7 BY PRENYLATED FLAVONOIDS, REVEALED BY YEAST ASSAYS

6.1. ABSTRACT

Several works showed that prenylated flavonoids have potent antiproliferative activities in distinct human tumour cells. In order to identify the molecular targets involved in the antiproliferative effects of these compounds, their modulatory activities on three key regulators of cell proliferation and death, caspase-3, caspase-7 and p53, were analysed. For this purpose, yeast assays based on the heterologous expression of these human target proteins were used. As previously reported for caspase-3 and wild-type p53, the expression in yeast of an active form of human caspase-7, caused a marked growth inhibition which was stimulated or reverted by activators or inhibitors, respectively. Using these yeast phenotypic assays for human caspase-3, caspase-7 and p53, we identified three prenylated flavonoids, 5,6-dihydroxy-7-prenyloxyflavone, 3-hydroxy-7-geranyloxyflavone and artelastin, that activated caspase-7 with a higher potency than the standard activator of caspase-3 and -7, procaspase activating compound-1 (PAC-1). We also showed that the yeast growth inhibition induced by expression of caspase-7 was associated to cell cycle arrest in G2/M and S phases and apoptosis. Moreover the stimulation of this inhibitory effect by PAC-1 and the three prenylated flavonoids was associated with an increase in the percentage of cells in S phase. These flavonoids, in contrast to PAC-1, did not interfere with the activity of caspase-3 and had no effect on p53 activity. Altogether, in this work, three potent activators of caspase-7, 5,6-dihydroxy-7-prenyloxyflavone, 3-hydroxy-7-geranyloxyflavone and artelastin, are identified. Promising pharmacological applications can be therefore envisaged for these prenylated flavonoids, particularly as anticancer agents in tumours without caspase-3 or a functional p53 pathway, or with elevated levels of caspase-7.

Keywords: Caspase-3; Caspase-7; p53; Prenylated flavonoids; Yeast

6.2. INTRODUCTION

Flavonoids are widely known for their potent antitumor activity. In fact, these compounds have been described as potent apoptotic inducers in human tumour cells (1, 2). In conformity with this, baicalein and 3,7-dihydroxyflavone exhibited antiproliferative effects in several human tumour cell lines (3-5). Moreover, it was recently shown that the suitable addition of prenyl side chains to baicalein and 3,7-dihydroxyflavone, increased the growth inhibitory effect of these flavonoids (6). These data indicate that prenylated flavonoids may represent a promising group of compounds to search for anticancer agents. In fact, the natural prenylated flavonoid artelastin, isolated from *Artocarpus elasticus* (7), integrated the "*In vitro* Anticancer Drug Discovery Screen" (NSC 710340) from the National Cancer Institute (NCI), potently inhibiting the growth of distinct human tumour cells (8). However, though the antiproliferative effect of some of these prenylated flavonoids have been attributed to the induction of apoptosis and/or cell cycle arrest (6, 8), the molecular mechanism of action behind these activities remains to be uncovered.

Previous reports showed the induction of a caspase-dependent apoptosis in several human tumour cells by baicalein (3, 4) and 3,7-dihydroxyflavone (5). The caspase family of cysteine proteases is composed by 15 members that are grouped into the inflammatory and apoptotic sub-families. Apoptotic caspases can be further divided into two functional groups based on their position in the apoptotic cascade, the apical or initiator caspases (caspase-2, -8, -9 and -10) and the executioner or downstream caspases (caspase-3, -6 and -7) [reviewed in (9)]. It is widely established that cleaved apoptotic caspase substrates exert major roles in the apoptotic pathway [reviewed in (9, 10)]. However, low or transient levels of caspase activation may also lead to non-apoptotic responses, such as cell proliferation and differentiation (11-13). Due to their crucial roles in cell proliferation and death, caspases, particularly the executioners, are major therapeutic targets in pathologies where these cellular processes are dysregulated, such as cancer and neurodegeneration [reviewed in (14, 15)]. In this context, small molecule activators and inhibitors of caspases represent promising therapeutic agents to stimulate and prevent apoptosis in cancer and neurodegenerative disorders, respectively. Among the three apoptotic executioner caspases, caspase-3 and -7 had been considered functionally redundant proteases. However, several data sustain that these caspases have also distinct roles in apoptosis [reviewed in (10, 16)]. In fact, mice deficient in either caspases exhibit distinct phenotypes (17). Moreover, caspase-3 and -7 exhibit distinct activities towards multiple substrates. In fact, while caspase-3 is responsible for the cleavage of the majority of substrates during the demolition phase of apoptosis, caspase-

7 is more selective (18). This emphasizes the therapeutic value of selective caspase-7 small molecule modulators.

The activation of a p53-dependent apoptosis by baicalein has been also reported (3, 4). The p53 tumour suppressor protein acts as a major defence against cancer, and altered p53 activity is often observed in human cancers. In fact, about 50% of all reported cancers have a mutation in the *TP53* gene. Among the remaining cancers that retain a wild-type (wt) p53, the p53 pathway is often inactivated due to multiple defects in p53 responses. Hence, restoring p53 function has been considered a promising strategy in anticancer therapy (19, 20). Thus, small molecule activators of p53 are also considered promising anticancer agents.

Yeast expressing a given human disease-related protein has widely contributed to the discovery of promising therapeutic agents (21-23). Though mammalian cells provide a more adequate physiological model system, yeast allows a clean read-out in a null- or almost null-background environment for the expression of the human target protein. Even when yeast possesses an orthologue of the human protein expressed most often it displays structural but not functional homology, and therefore does not respond to the same compounds that act as modulators in human cells. Moreover, the analysis of drug targets in a eukaryotic environment with a considerable lower redundancy level, such as in yeast, allows most often the unambiguous measurement of a specific effect on a given target (21-23). Several examples illustrating the advantages of yeast as a drug screening tool can be referred. For instance, a potent and selective activator of novel protein kinase C (PKC) δ and ε was identified by our group using yeast cells expressing individual members of mammalian PKC family (24). In another recent work, we also identified selective inhibitors of human caspase-3 by testing a library of vinyl sulfones (25).

Similarly to human wt p53 (26), the expression of active forms of human caspase-3 and -7 in yeast causes a marked growth inhibition that is proportional to the degree of human caspase activation (27-29). This inhibitory effect of recombinant human proteins expressed in yeast has been used in the establishment of drug screening assays based on the direct assessment of the yeast cell growth. Though no orthologues of human p53 have been found in yeast, in the case of caspases, a possible interference of the yeast metacaspase (Yca1p) has been considered. However, the activity of human caspases in yeast was shown to be independent of Yca1p (29).

In this work, several prenylated derivatives of baicalein and 3,7-dihydroxyflavone, and the natural prenylated flavonoid artelastin, with antiproliferative activities in several human tumour cells (6, 8), were studied in order to identify the molecular targets involved

in their growth inhibitory effects. With this goal, the modulatory activity of these compounds on the three major regulators of cell proliferation and death, caspase-3, caspase-7 and p53, was tested. To this end, yeast assays based on the heterologous expression of these human target proteins were used. It was found that 5,6-dihydroxy-7-prenyloxyflavone, 3-hydroxy-7-geranyloxyflavone and artelastin are potent activators of caspase-7.

6.3. MATERIAL AND METHODS

6.3.1. Plasmids

Constructed yeast expression plasmid pGALL-(*LEU2*) encoding auto-activating forms of human caspase-3 (reverse (rev)-caspase-3; procaspase-3 with the small subunit preceding the prodomain and large subunit) or caspase-7 (caspase-7^{53}; procaspase-7 lacking the N-terminal 1-53 amino acid prodomain) and the empty vector were kindly provided by Dr. Christine J. Hawkins (Department of Haematology and Oncology, Royal Children's Hospital, Australia); pLS89-(*TRP1*) encoding human wt p53 and the empty vector were kindly provided by Dr. Richard Iggo (Swiss Institute for Experimental Cancer Research, Switzerland). Plasmids used have a galactose-inducible *GAL1-10* promoter. Construction of these yeast plasmids was previously described (30-32).

6.3.2. Yeast strain, transformation and growth conditions

Saccharomyces cerevisiae CG379 was transformed as reported (33). To ensure selection of transformed yeast, cells were routinely grown in glucose minimal selective medium. To induce expression of human caspase-3, caspase-7 or wt p53, yeast cultures were diluted to 0.05 optical density at 600 nm (OD_{600}) in 2% (w/v) galactose and raffinose selective medium and grown at 30 ºC under continuous shaking (33). Effects of expression of caspase-3 (25) and wt p53 (26) on yeast growth were analysed as previously described. Yeast expressing caspase-7 and control yeast (transformed with the empty vector, pGALL) were grown for up to 50 hours for growth curves experiments. For the analysis of the effect of caspase-7 on yeast growth, cells were incubated for 36 hours (time required by yeast expressing caspase-7 to achieve approximately 0.40 OD_{600}). Yeast growth was analysed by counting the number of colony-forming units per ml (CFU/ml) after 2 days incubation at 30 ºC on Sabouraud Dextrose Agar plates.

6.3.3. Western blot analysis

Western blot analysis to confirm expression of human caspase-3 (25) and human wt p53 (26) in yeast were previously reported. Western blot analysis to confirm the expression in yeast of human caspase-7 was performed basically as described (33). To analyse the expression of human caspase-7, a mouse monoclonal anti-caspase-7 antibody, which detects caspase-7 full length precursor and the large subunit (LS) of cleaved caspase-7 (Santa Cruz Biotechnology), followed by an anti-mouse horseradish-

peroxidase (HRP)-conjugated secondary antibody (Southern Biotechnology), were used. For loading control, membranes were stripped and reprobed with a mouse monoclonal anti-yeast phosphoglycerate kinase (Pgk1p) antibody (Molecular probes). Immunoblots were developed by chemiluminescence.

6.3.4. Cell death markers

Propidium iodide (PI) staining and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) staining, to monitor plasma membrane integrity and DNA fragmentation, respectively, were performed basically as described (33). For PI staining, cells were incubated with 5 µg/ml PI (Molecular Probes) for 10 min at room temperature; fluorescence was monitored by flow cytometry. TUNEL assay was performed using the *In Situ Cell Death Detection Kit*, Fluorescein (Roche Applied Science); samples were observed under a fluorescence microscope.

6.3.5. Reactive oxygen species (ROS) accumulation

Analysis of mitochondrial ROS accumulation was performed basically as described (24). Briefly, cells were incubated with 5 µg/ml dihydroethidium (DHE; Sigma-Aldrich) for 30 min at 30 ℃, and fluorescence was monitored by flow cytometry.

6.3.6. Cell cycle

DNA content was monitored by flow cytometry, as described (24). Briefly, about 10^7 cells were fixed in 70% (v/v) ethanol, digested with 250 µg/ml RNase A (DNase-free; Sigma–Aldrich) and 1 mg/ml Proteinase K (Sigma–Aldrich), and afterwards incubated with 10 µM Sytox Green Nucleic Acid (Alfagene). Yeast cell cycle phases were identified and quantified using ModFit LT software (VeritySoftware House Inc.).

6.3.7. Effects of PAC-1 and flavonoids on yeast growth

All compounds were prepared in dimethyl sulfoxide (DMSO; Sigma). Details concerning synthesis of baicalein (**1**) and 3,7-dihydroxyflavone (**2**) prenylated derivatives, namely 5,6-dihydroxy-7-prenyloxyflavone (**1a**) and 3-hydroxy-7-geranyloxyflavone (**2a**) (Fig. 6.1), have been previously reported (6). Isolation of artelastin (**3**) (Fig. 6.1) have been previously described (7). In yeast caspase-3/-7 assays, procaspase activating compound 1 (PAC-1; Calbiochem), a standard activator of caspase-3 and -7, was used as

positive control. To analyse the effect of compounds on yeast cell growth, transformed cells were incubated in galactose selective medium with 0.1 - 25 μM PAC-1, 0.1 - 10 μM flavonoids or 0.1% DMSO only, at 30 ºC under continuous shaking to approximately 0.4 OD_{600}. Cell growth was analysed as described above. For each transformant, the percentage of drug-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with yeast incubated with DMSO only. EC_{50} values were obtained from concentration-response curves of 0.1 – 25 μM PAC-1 and 0.1 – 10 μM flavonoids, and correspond to the concentration that caused 50% of the maximal stimulation of growth inhibition obtained with 25 μM PAC-1 on yeast expressing caspase-7.

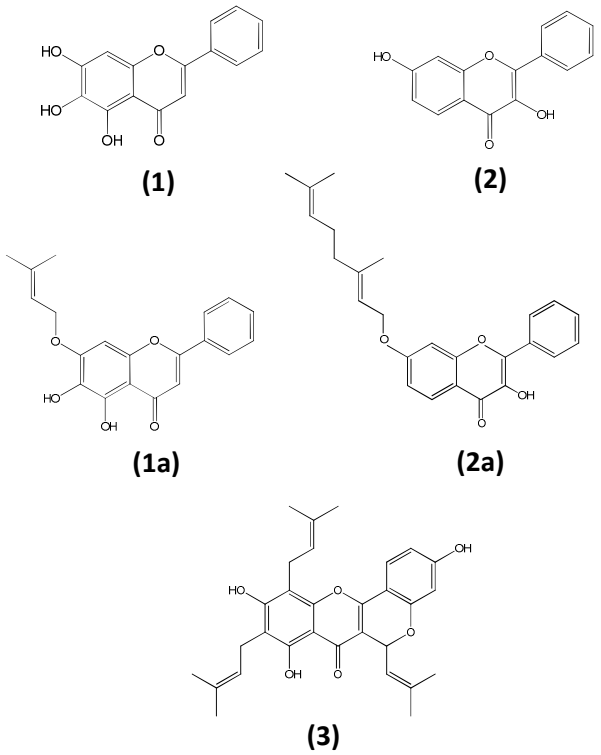

Figure 6.1. Chemical structures of baicalein (**1**), 3,7-dihydroxyflavone (**2**), 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**).

6.3.8. Fluorescence microscopy and flow cytometric data acquisition and analysis

For fluorescent microscopic examination, samples were observed under an Eclipse E400 fluorescence microscope (Nikon) equipped with a 100 W mercury lamp and appropriate filter setting. Cells were observed with an oil immersion lens (Plan Fluor 100/1.30). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) and the CellQuest software (BD Biosciences).

6.3.9. Statistical analysis

Data were analysed statistically using the SigmaStat 3.5 software. Differences between means were tested for significance using the unpaired Student´s *t*-test ($P<0.05$).

6.4. RESULTS

6.4.1 Expression of human caspase-7^{53} in yeast induces growth inhibition associated with cell cycle arrest in G2/M and S phases and apoptosis

Human caspase-7^{53} is an auto-activated form of caspase-7 both in yeast (27, 28) and mammalian cells (34). In this work, expression of human caspase-7^{53} in yeast was confirmed by Western blot analysis. Using an antibody that recognizes the 34 kDa caspase-7 full-length precursor (procaspase) and the 19 kDa large subunit (LS) of cleaved caspase-7, we also detected the absence of the 34 kDa band (Fig. 6.2A). This indicated that human caspase-7^{53} was successfully processed into an active form in our yeast strain and under our growth conditions. Moreover, similarly to human rev-caspase-3 (25) and wt p53 (26), the previously reported growth inhibition induced by expression of an active caspase-7 in yeast (27, 28) was confirmed (Fig. 6.2B).

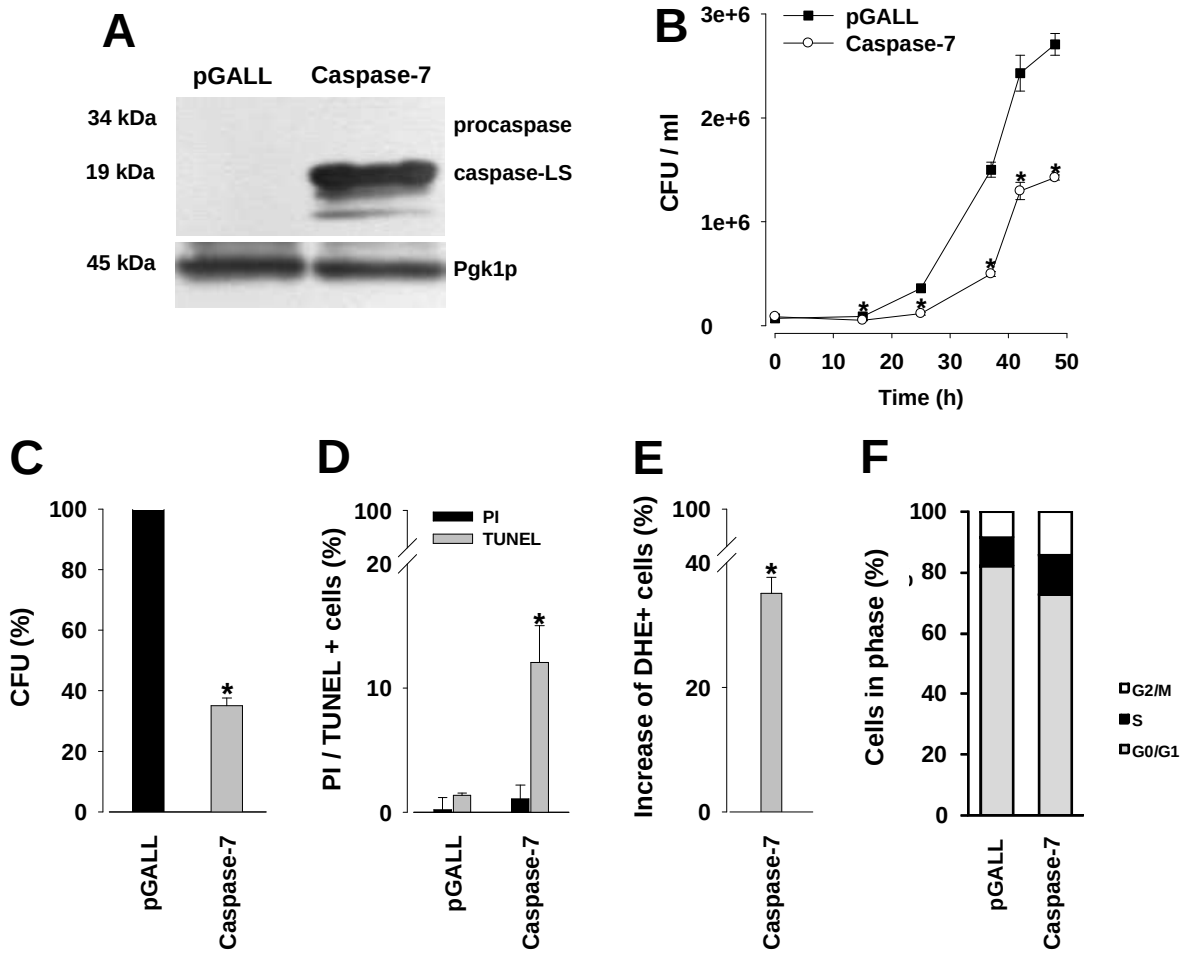

Figure 6.2. Expression of human caspase-7 in yeast induces growth inhibition associated with apoptosis and cell cycle arrest. Yeast expressing human caspase-7 and control yeast (pGALL) were grown in galactose selective medium for up to 50 hours for growth curves experiments or for 36 hours (time required by yeast

expressing caspase-7 to achieve ~0.40 OD_{600}) in all other experiments. (**A**) Expression of human caspase-7 in yeast was confirmed by Western blot analysis. Full length procaspase-7 (34 kDa) and the large subunit (LS) of cleaved caspase-7 (19 kDa) were detected using an anti-caspase-7 monoclonal antibody. Pgk1p was used as a loading control. Immunoblots were developed by chemiluminescence and represent 1 of 2 experiments. (**B**) Growth curves obtained by CFU counts. Data are means ± s.e.m. of 6 independent experiments with 6 replicates each. (**C**) Percentage of growth obtained for 36 h incubation and considering the number of CFU obtained with control yeast as 100% growth. Data are means ± s.e.m. of 6 independent experiments with 6 replicates each. (**D**) DNA fragmentation (TUNEL + cells) and loss of plasma membrane integrity (PI + cells). Data are means ± s.e.m. of 3 independent experiments. (**E**) Mitochondrial ROS accumulation. Values correspond to the increase in the percentage of DHE + cells obtained with yeast expressing caspase-7 when compared to control yeast. Data are means ± s.e.m. of 3 independent experiments. In **B - E**, values significantly different from control yeast: **P*< 0.05. (**F**) Analysis of DNA content using Sytox Green. Values correspond to the quantification of yeast cell cycle phases and are means of 3 independent experiments.

This inhibitory effect was maximal after approximately 36 h incubation (Fig. 6.2B,C). For this incubation time, the caspase-7-induced growth inhibition was accompanied by a significant increase in DNA fragmentation with no loss of plasma membrane integrity (Fig. 6.2D), and by a pronounced increase in mitochondrial ROS accumulation (Fig. 6.2E). These data indicated the activation in yeast of an apoptotic cell death involving mitochondrial ROS accumulation by human caspase-7. Moreover, it was detected the interference of human caspase-7 with the yeast cell cycle progression. In fact, the expression of human caspase-7 in yeast increased the percentage of cells in G2/M and S phases (Fig. 6.2F). Altogether, these results indicated that this active form of human caspase-7 induced yeast growth inhibition which is associated with cell cycle arrest in G2/M and S phases and apoptosis.

6.4.2. Development of a yeast assay to search for small molecule modulators of human caspase-7

In our previous works, it was shown that expression of human wt p53 in yeast induced growth inhibition, which was markedly increased by the known natural p53 activator PKCδ and inhibited by the selective p53 inhibitor pifithrin-α (26, 35). In another work, we confirmed that expression of human rev-caspase-3 in yeast also induced growth inhibition, which was reverted by the standard caspase-3 inhibitor, Ac-DEVD-CMK (25). In these studies, a correlation between yeast cell growth and degree of activity of the expressed human protein was confirmed. These works therefore established yeast phenotypic assays to search for modulators of human caspase-3 and p53, which are based on the direct assessment of the yeast cell growth. In these assays, activators and

inhibitors of the human target protein will increase and reduce, respectively, the yeast growth inhibition induced by the expressed human protein, without affecting the growth of control yeast (transformed with the empty vector). In fact, using this strategy, promising inhibitors of human caspase-3 were already identified by our group by testing a library of vinyl sulfones (25).

In the present work, the heterologous expression of the activated form of human caspase -7 described above was exploited and validated as a yeast phenotypic assay for the screening of small molecule modulators of human caspase-7. For that, the effect of the standard activator of caspase-3 and -7, PAC-1 (36) was tested on yeast cells expressing human caspase-7. As expected, for 0.1 – 25 μM, PAC-1 stimulated caspase-7-induced growth inhibition in a dose-dependent manner, without affecting the growth of control yeast (Fig. 6.3). A correlation between stimulation of growth inhibition and caspase-7 activation was therefore established. The suitability of the previously established yeast caspase-3 assay (25) to search for activators of human caspase-3 was further corroborated in this study by testing PAC-1. As expected, PAC-1 also stimulated caspase-3-induced growth inhibition, without interfering with the growth of control yeast. Though PAC-1 has been reported as a more potent activator of caspase-3 than caspase-7 in mammalian cells (36), in our yeast assays the maximal effect obtained with PAC-1 was similar on both caspases (Table 6.1).

Table 6.1. Increase of human caspase-3-, caspase-7- and p53-induced yeast growth inhibition by PAC-1, 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**).

	Drug-induced growth inhibition (%)		
	Caspase-3	**Caspase-7**	**p53**
PAC-1	24.8 ± 2.6*	25.1 ±3.2*	*NT*
1a	3.7 ± 7.0	23.0 ±1.9*	6.9 ± 3.6
2a	6.9 ± 0.9	22.9 ± 4.9*	6.8 ± 2.6
3	5.0 ± 4.4	28.1 ± 4.5*	-0.7 ± 5.4

Values correspond to the maximal increase on human caspase-3-, caspase-7- and p53-induced growth inhibition achieved with 25 μM PAC-1 and 1 μM 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**)**.** 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 ± s.e.m. of 6 independent experiments with six replicates each; values significantly higher than control yeast, **P*< 0.05. *NT*: effect of compound was not tested.

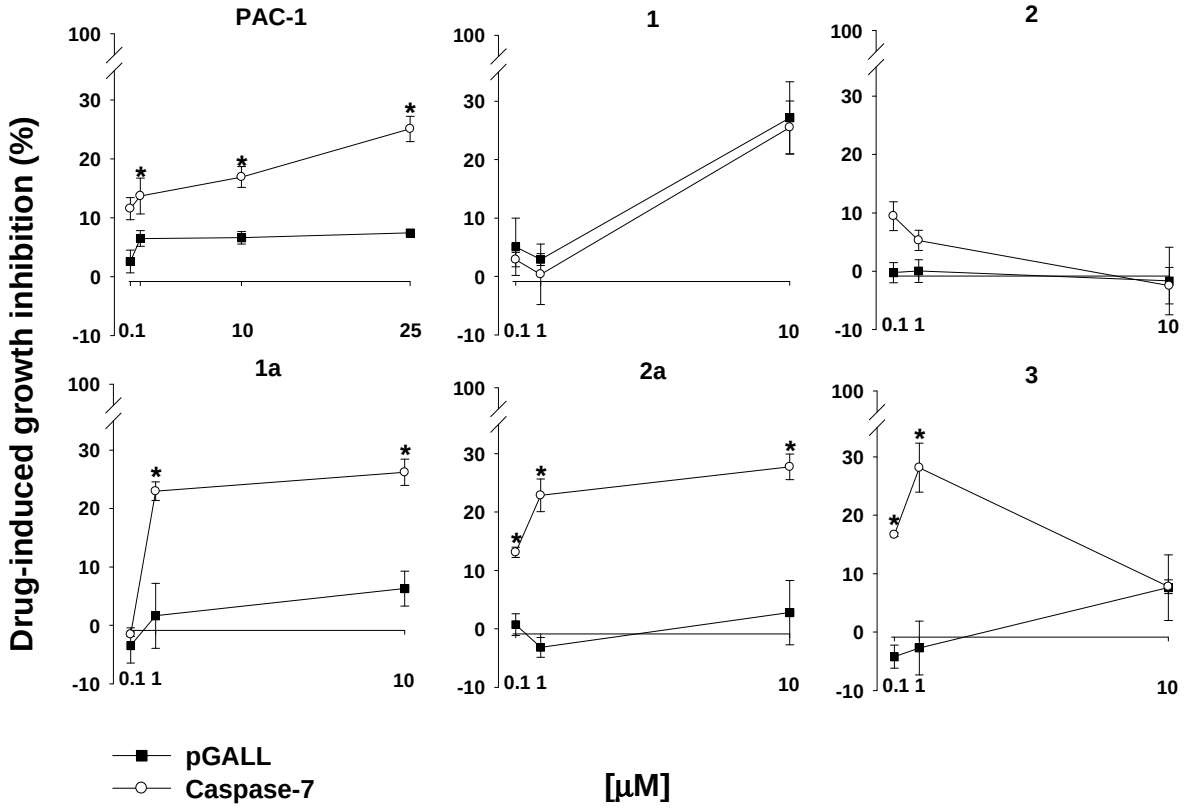

Figure 6.3. Concentration-response curves for the effects of PAC-1, baicalein (**1**), 3,7-dihydroxyflavone (**2**), 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**) on the growth of yeast expressing human caspase-7 and control yeast (pGALL). Transformed yeasts were incubated in galactose selective medium with 0.1 - 25 μMPAC-1, 0.1 - 10 μM flavonoids or 0.1% DMSO only, to ~0.4 OD_{600}. For each transformant, the percentage of drug-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with yeast incubated with DMSO only. Data are means ± s.e.m. of 6 independent experiments with 6 replicates each; values significantly different from control yeast: **P*< 0.05.

*6.4.3. 5,6-Dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**) increased human caspase-7 activity, with no effects on the activity of human caspase-3 and p53*

The modulatory activity of baicalein (**1**) and 3,7-dihydroxyflavone (**2**) prenyl-derivatives (**1a** and **2a**) and artelastin (**3**) on human caspase-3, caspase-7 and wt p53 was analysed using the yeast phenotypic assays developed for each human protein.

When 5,6-dihydroxy-7-prenyloxyflavone (**1a**), a monoprenylated derivative of baicalein (**1**) (Fig. 6.1), was tested, a remarkable increase of caspase-7-induced growth inhibition was obtained for 1 – 10 μM (Fig. 6.3). Since this compound did not interfere with the control yeast, this result indicated that flavonoid **1a** was an activator of caspase-7.

Though the maximal effects obtained with PAC-1 and flavonoid **1a** on caspase-7 were similar (Table 6.1), the estimated EC_{50} values indicated that flavonoid **1a** was a more potent activator of caspase-7 than PAC-1 (Table 6.2). However, contrarily to that obtained with caspase-7, flavonoid **1a** did not interfere with caspase-3- and p53-induced growth inhibition. In fact, the maximal effects obtained with this compound on these human proteins were not significant (Table 6.1). These results indicated that flavonoid **1a** potently and selectively activated caspase-7. Interestingly, when the effect of the non-prenylated analogue of flavonoid **1a**, baicalein (**1**), was tested in yeast cells expressing caspase-7, a non-specific effect of this compound was observed. In fact, baicalein (**1**) inhibited the growth of yeast expressing caspase-7 as well as of control yeast (pGALL) (Fig. 6.3).

Monogeranylated derivative of 3,7-dihydroxyflavone (**2**), 3-hydroxy-7-geranyloxyflavone (**2a**), and artelastin (**3**) (Fig. 6.1), behaved similarly to flavonoid **1a**. In fact, 0.1 – 10 μM flavonoid **2a** and 0.1 – 1 μM flavonoid **3** markedly increased caspase-7-induced growth inhibition without affecting the growth of control yeast (Fig. 6.3). It is also interesting to note the absence of effect of 3,7-dihydroxyflavone (**2**), the non-prenylated analogue of flavonoid **2a**, on yeast expressing caspase-7 (Fig. 6.3). Additionally, as obtained with flavonoid **1a**, flavonoids **2a** and **3** also did not interfere with caspase-3- and p53-induced yeast growth inhibition (Table 6.1), and presented lower EC_{50} values than PAC-1 on caspase-7 (Table 6.2).

Table 6.2. EC_{50} values obtained for PAC-1, 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**) on human caspase-7.

	EC_{50} (μM)
PAC-1	0.65 ± 0.18
1a	0.26 ± 0.03*
2a	0.14 ± 0.03*
3	0.1 ± 0.01*

EC_{50} values were obtained from concentration-response curves of 0.1 – 25 μM PAC-1 and 0.1 – 10 μM 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**), and correspond to the concentration that caused 50% of the maximal stimulation of growth inhibition obtained with 25 μM PAC-1 on caspase-7. 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 ± s.e.m. of EC_{50} values obtained from 6 independent experiments; EC_{50} value significantly lower than PAC-1, *P< 0.05.

Moreover, in this work, it was observed that, similarly to PAC-1, the stimulation of caspase-7-induced yeast growth inhibition by flavonoids **1a**, **2a** and **3** was not associated with an increase in the percentage of apoptotic cells, as assessed by TUNEL and PI staining (not shown), but instead to effects on the yeast cell cycle. In fact, when compared to yeast cells expressing caspase-7 incubated with DMSO only, it was observed that PAC-1 and flavonoids **1a**, **2a** and **3** increased the percentage of cells in S phase (Fig. 6. 4).

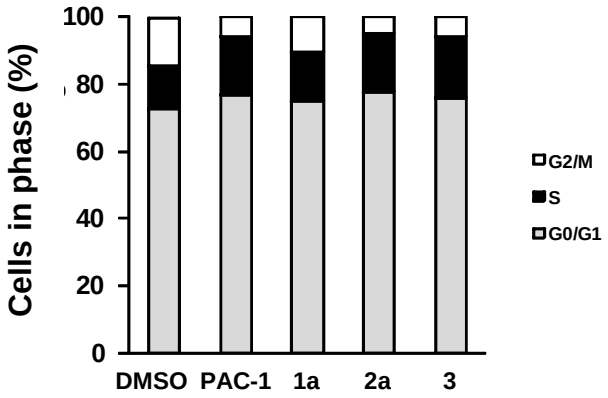

Figure 6.4. Effects of PAC-1, 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**) on the cell cycle progression of yeast cells expressing human caspase-7. Yeast expressing caspase-7 was incubated with 25 μM PAC-1, 1 μM flavonoids or 0.1% DMSO only for ~0.4 OD_{600}. Analysis of DNA content was monitored using Sytox Green. Values correspond to the quantification of cell cycle phases and represent means of 3 independent experiments; with PAC-1, flavonoids **2a** and **3,** S and G2/M values are significantly different from DMSO only: $P< 0.05$.

Together, these results indicated that 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**) are activators of caspase-7. Additionally, in comparison with the positive control PAC-1, the higher potency obtained with flavonoids **1a**, **2a** and **3** on caspase-7 also indicated that they are potent activators of caspase-7.

6.5. DISCUSSION

A recent work showed that prenylated derivatives of baicalein (**1**) and 3,7-dihydroxyflavone (**2**) had potent antiproliferative activities in several human tumour cell lines (6). Supporting the promising anticancer activity of prenylated flavonoids, an earlier study showed that the natural prenylated flavonoid artelastin (**3**) potently inhibited the growth of distinct tumour cells (8, 37). In spite of this finding, the molecular mechanisms mediating the antiproliferative effects of these compounds are still uncovered. The main goal of the present work was therefore to identify the molecular targets involved in the growth inhibitory effects of prenylated flavonoids. Since previous works have reported the involvement of caspases and/or p53 in the apoptotic cell death induced by baicalein (**1**) and 3,7-dihydroxyflavone (**2**) in human tumour cells (3-5), the modulatory activity of these prenylated flavonoids on the three major regulators of cell proliferation and death, caspase-3, caspase-7 and p53, was therefore analysed.

To achieve this goal, yeast cells expressing human caspase-3, caspase-7 or wt p53 were used. Previous works performed by our group established yeast phenotypic assays, based on the direct assessment of the yeast cell growth, to search for inhibitors of human caspase-3 (25) and for activators and inhibitors of p53 (26, 35). In the present work, a yeast phenotypic assay to search for activators of human caspase-7 is developed. We started to confirm that the expression of an active form of human caspase-7 induced inhibition of yeast growth as previously reported (27, 28). Moreover, we showed that this growth inhibitory effect of human caspase-7 in yeast was associated with an apoptotic cell death and a G2/M and S cell cycle arrest. Though it is well-established that caspase-7 is a major executioner of apoptosis, its involvement in non-apoptotic responses, namely in cell cycle progression, has also been reported in mammalian cells (12). This work represents the first report for a regulatory effect of human caspase-7 on yeast cell cycle progression. Since the role of caspase-7 in the regulation of cell cycle progression is still largely unclear, this work opens the way to the use of the yeast model system in the elucidation of this issue. Additionally, by testing the standard activator of caspase-3 and -7, PAC-1, we validated the yeast caspase-7 assay and the previously reported yeast caspase-3 assay (25) to the search for small molecule activators of human caspase-3 and -7. Moreover, the capability of PAC-1 to potently activate both caspases, reported in mammalian cells (36), was further confirmed in yeast.

Subsequently, by using the established yeast screening assays, we identified three prenylated flavonoids, 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-

geranyloxyflavone (**2a**) and artelastin (**3**) that activated caspase-7 with a higher potency than PAC-1. It was also shown that the stimulation of caspase-7-induced yeast growth inhibition by PAC-1 and flavonoids **1a**, **2a** and **3** was associated with an increase in the percentage of cells in S phase. Interestingly, the antiproliferative effects of flavonoids **1a** and **3** in human tumour cells were also attributed respectively to a G1 (6) and S (8) cell cycle arrest. Additionally, in opposition to PAC-1, these flavonoids did not interfere with the activity of human caspase-3. This absence of effect of flavonoids **1a**, **2a** and **3** on human caspase-3 is in accordance with previous results obtained in human tumour cells (6, 8). In fact, similar antiproliferative activities of flavonoids **1a**, **2a** and **3** were obtained in NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) with caspase-3 and MCF-7 (breast adenocarcinoma) without caspase-3. Besides, none of these prenylated flavonoids interfered with the activity of human p53 in yeast.

It is also interesting to note that, in opposition to flavonoid **1a**, its non-prenylated analogue, baicalein (**1**), markedly inhibited the growth of control yeast. This indicated the conservation in yeast of the molecular targets of baicalein (**1**). The identification of these molecular targets, and respective orthologues in mammals, could therefore advance our knowledge about the antiproliferative effect of this flavonoid in human tumour cells. These results also indicate that the monoprenylation is a structural requirement for the selectivity of flavonoid **1a** to caspase-7, since when absent in baicalein (**1**) non-specific effects in yeast were observed. Moreover, it was verified that, contrarily to flavonoid **2a**, its non-prenylated analogue 3,7-dihydroxyflavone (**2**) had no effect on caspase-7. This can therefore indicate that the monogeranylation is a structural requirement for caspase-7 activation by flavonoid **2a**. Important data are therefore provided by this work concerning structural requirements for the activity and selectivity of these prenylated flavonoids towards caspase-7.

As a whole, the present work identify three potent activators of caspase-7, 5,6-dihydroxy-7-prenyloxyflavone (**1a**), 3-hydroxy-7-geranyloxyflavone (**2a**) and artelastin (**3**). 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, flavonoids **1a**, **2a** and **3** may represent an alternative strategy for personalized cancer treatment, namely against tumours without caspase-3 (e.g. breast adenocarcinoma) or a functional p53 pathway (e.g. chronic myelocytic leukemia), or with elevated levels of caspase-7 (e.g. prostate cancer).

6.6. ACKNOWLEDGEMENTS

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6.7. REFERENCES

(1) Lopez-Lazaro M. Flavonoids as anticancer agents: structure-activity relationship study. Curr Med Chem Anticancer Agents 2002 Nov; 2 (6): 691-714.

(2) Kale A, Gawande S, Kotwal S. Cancer phytotherapeutics: role for flavonoids at the cellular level. Phytother Res 2008 May; 22 (5): 567-77.

(3) Lin YT, Yang JS, Lin HJ, Tan TW, Tang NY, Chaing JH, et al. Baicalein induces apoptosis in SCC-4 human tongue cancer cells via a Ca2+-dependent mitochondrial pathway. In Vivo 2007 Nov-Dec; 21 (6): 1053-8.

(4) Li YC, Lin HJ, Yang JH, Yang JS, Ho HC, Chang SJ, et al. Baicalein-induced apoptosis via endoplasmic reticulum stress through elevations of reactive oxygen species and mitochondria dependent pathway in mouse-rat hybrid retina ganglion cells (N18). Neurochem Res 2009 Mar; 34 (3): 418-29.

(5) Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N, Martinez-Irujo JJ. Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. Nutr Cancer 2004; 50 (1): 90-100.

(6) Neves MP, Cidade H, Pinto M, Silva AM, Gales L, Damas AM, et al. Prenylated derivatives of baicalein and 3,7-dihydroxyflavone: Synthesis and study of their effects on tumor cell lines growth, cell cycle and apoptosis. Eur J Med Chem 2011 Jun; 46 (6): 2562-74.

(7) Kijjoa A, Cidade H, Pinto M, Gonzalez M, Anantachoke C, Gedris T, et al. Prenylflavonoids from Artocarpus elasticus. . Phytochemistry 1996; 43: 691-4.

(8) Pedro M, Ferreira MM, Cidade H, Kijjoa A, Bronze-da-Rocha E, Nascimento MS. Artelastin is a cytotoxic prenylated flavone that disturbs microtubules and interferes with DNA replication in MCF-7 human breast cancer cells. Life Sci 2005 Jun; 77 (3): 293-311.

(9) Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. J Biol Chem 2009 Aug; 284 (33): 21777-81.

(10) Luthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. Cell Death Differ 2007 Apr; 14 (4): 641-50.

(11) Feinstein-Rotkopf Y, Arama E. Can't live without them, can live with them: roles of caspases during vital cellular processes. Apoptosis 2009 Aug; 14 (8): 980-95.

(12) Hashimoto T, Yamauchi L, Hunter T, Kikkawa U, Kamada S. Possible involvement of caspase-7 in cell cycle progression at mitosis. Genes Cells 2008 Jun; 13 (6): 609-21.

(13) Lamkanfi M, Festjens N, Declercq W, Vanden Berghe T, Vandenabeele P. Caspases in cell survival, proliferation and differentiation. Cell Death Differ 2007 Jan; 14 (1): 44-55.

(14) Bulat N, Widmann C. Caspase substrates and neurodegenerative diseases. Brain Res Bull 2009 Oct; 80 (4-5): 251-67.

(15) Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and cancer: mutations within caspase genes. Journal of Medical Genetics 2009; 46 (8): 497-510.

(16) Lamkanfi M, Kanneganti TD. Caspase-7: a protease involved in apoptosis and inflammation. Int J Biochem Cell Biol 2010 Jan; 42 (1): 21-4.

(17) Lakhani SA, Masud A, Kuida K, Porter GA, Jr., Booth CJ, Mehal WZ, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 2006 Feb; 311 (5762): 847-51.

(18) Walsh JG, Cullen SP, Sheridan C, Luthi AU, Gerner C, Martin SJ. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci U S A 2008 Sep; 105 (35): 12815-9.

(19) Cheok CF, Verma CS, Baselga J, Lane DP. Translating p53 into the clinic. Nat Rev Clin Oncol 2011 Jan; 8 (1): 25-37.

(20) Wang W, El-Deiry WS. Restoration of p53 to limit tumor growth. Curr Opin Oncol 2008 Jan; 20 (1): 90-6.

(21) Barberis A, Gunde T, Berset C, Audetat S, Lüthi U. Yeast as a screening tool. Drug Discovery Today: Technologies 2005 Summer 2005; 2 (2): 187-92.

(22) Simon JA, Bedalov A. Yeast as a model system for anticancer drug discovery. Nat Rev Cancer 2004 Jun; 4 (6): 481-92.

(23) Khurana V, Lindquist S. Modelling neurodegeneration in Saccharomyces cerevisiae: why cook with baker's yeast? Nat Rev Neurosci 2010 Jun; 11 (6): 436-49.

(24) Coutinho I, Pereira G, Simoes MF, Corte-Real M, Goncalves J, Saraiva L. Selective activation of protein kinase C-delta and -epsilon by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U). Biochem Pharmacol 2009 Sep; 78 (5): 449-59.

(25) Gloria PM, Coutinho I, Goncalves LM, Baptista C, Soares J, Newton AS, et al. Aspartic vinyl sulfones: Inhibitors of a caspase-3-dependent pathway. Eur J Med Chem 2011 Jun; 46 (6): 2141-6.

(26) Coutinho I, Pereira G, Leao M, Goncalves J, Corte-Real M, Saraiva L. Differential regulation of p53 function by protein kinase C isoforms revealed by a yeast cell system. FEBS Lett 2009 Nov; 583 (22): 3582-8.

(27) Jabbour AM, Ekert PG, Coulson EJ, Knight MJ, Ashley DM, Hawkins CJ. The p35 relative, p49, inhibits mammalian and Drosophila caspases including DRONC and protects against apoptosis. Cell Death Differ 2002 Dec; 9 (12): 1311-20.

(28) Ho P-k, Jabbour AM, Ekert PG, Hawkins CJ. Caspase-2 is resistant to inhibition by inhibitor of apoptosis proteins (IAPs) and can activate caspase-7. FEBS Journal 2005; 272 (6): 1401-14.

(29) Puryer MA, Hawkins CJ**.** Human, insect and nematode caspases kill Saccharomyces cerevisiae independently of YCA1 and Aif1p. Apoptosis 2006; 11 (4): 509-17.
(30) Hawkins CJ, Wang SL, Hay BA**.** A cloning method to identify caspases and their regulators in yeast: identification of Drosophila IAP1 as an inhibitor of the Drosophila caspase DCP-1. Proc Natl Acad Sci U S A 1999 Mar; 96 (6): 2885-90.
(31) Hawkins CJ, Silke J, Verhagen AM, Foster R, Ekert PG, Ashley DM**.** Analysis of candidate antagonists of IAP-mediated caspase inhibition using yeast reconstituted with the mammalian Apaf-1-activated apoptosis mechanism. Apoptosis 2001 Oct; 6 (5): 331-8.
(32) Scharer E, Iggo R**.** Mammalian p53 can function as a transcription factor in yeast. Nucleic Acids Res 1992 Apr; 20 (7): 1539-45.
(33) Saraiva L, Silva RD, Pereira G, Goncalves J, Corte-Real M**.** Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci 2006 Aug; 119 (Pt 15): 3171-81.
(34) Denault JB, Salvesen GS**.** Human caspase-7 activity and regulation by its N-terminal peptide. J Biol Chem 2003 Sep; 278 (36): 34042-50.
(35) Coutinho I, Pereira C, Pereira G, Goncalves J, Corte-Real M, Saraiva L**.** Distinct regulation of p53-mediated apoptosis by protein kinase Calpha, delta, epsilon and zeta: Evidence in yeast for transcription-dependent and -independent p53 apoptotic mechanisms. Exp Cell Res 2011 May; 317 (8): 1147-58.
(36) Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland MS, Kwon JT, et al. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nat Chem Biol 2006 Oct; 2 (10): 543-50.
(37) Pedro M, Lourenco CF, Cidade H, Kijjoa A, Pinto M, Nascimento MS**.** Effects of natural prenylated flavones in the phenotypical ER (+) MCF-7 and ER (-) MDA-MB-231 human breast cancer cells. Toxicol Lett 2006 Jun; 164 (1): 24-36.

CHAPTER 7

General discussion

PKC isoforms, p53 tumour suppression protein, caspase-3 and -7 have crucial roles in different cellular processes namely, cell proliferation and death. Therefore, these cellular players represent key therapeutic targets in human diseases where these processes are dysregulated, such as cancer and neurodegeneration. The present thesis focused on the use of yeast to study functional, molecular and pharmacological aspects of these mammalian proteins.

7.1. REGULATION OF P53 ACTIVITY IN CELL PROLIFERATION AND DEATH BY PKC ISOFORMS: TRANSCRIPTION-DEPENDENT AND -INDEPENDENT P53 MECHANISMS IN YEAST

Though several works had already reported the p53 phosphorylation and the activation of the p53 transcriptional activity by PKCδ (1, 2), the role of other PKC isoforms in the regulation of p53 activity was still largely unknown. Hence, with the present thesis, we aimed to clarify this relevant issue. For this purpose, we first attempted to reconstitute in yeast a mammalian PKC isoform-p53 network which could allow the direct assessment of the function of specific PKCα, δ, ε and ζ (reported as major PKC isoforms in carcinogenesis) in the regulation of p53 activity in cell proliferation and death.

Before setting up in yeast the co-expression of p53 and individual PKC isoforms, we confirmed that expression of the human wt p53 in yeast induced growth inhibition as previously reported by Amor and colleagues (3). However, in our case this effect was not associated with cell death but instead with the induction of a cell cycle arrest. Moreover, the regulation of yeast cell growth by p53 through a transcription-dependent mechanism was also pointed by us. Together, these observations supported the establishment of a yeast p53 phenotypic assay to perform functional and pharmacological studies of p53. Subsequently, we further exploited this yeast p53 assay to elucidate the regulation of p53 activity in cell proliferation by PKC isoforms.

The results obtained, in unstressed cells, show a differential regulation of p53-induced growth inhibition by PKC isoforms with the identification of positive and negative regulators among the tested PKC isoforms. In fact, whereas PKCα reduced the p53-induced yeast growth inhibition, PKCδ and ε phosphorylated p53 and markedly increased its effect. Moreover, PKCζ had no effect on p53 activity. Interestingly, similar results were

obtained for PKCδ, ε and ζ under stress conditions. In fact, while PKCα and ζ had no effect on p53 activity, PKCδ and ε stimulated p53-mediated apoptosis. Together, this work provides a new pharmacological strategy of p53 regulation through modulation of PKCδ and ε, with the identification of these kinases as key therapeutic targets in tumours with a wt p53.

Moreover, as described in mammalian cells (4-6), this work also reveals in yeast the cooperation between transcription-dependent and -independent p53 mechanisms to ultimately cause an apoptotic cell death. In fact, it provides the first evidence for the conservation in yeast of a transcription-independent p53-mediated apoptosis characterized by the p53 translocation to mitochondria. Moreover, relevant insights about this p53 transcription-independent mechanism are revealed. Since mitochondrial localization of p53 was only detected under an apoptotic scenario, this study corroborates the hypothesis raised by others in mammalian cells (5, 6) that the p53 mitochondrial localization determines whether cells die or arrest growth. Additionally, it shows that, besides the reported activation of a p53 transcriptional activity, PKCδ also triggers the p53 translocation to mitochondria. It also identifies PKCε as an additional activator of this transcription-independent p53 apoptotic mechanism. The identification of activators of p53 translocation to mitochondria, achieved with this work, advances our knowledge about an unknown issue concerning the regulation of p53 transcription-independent mechanisms. Finally, it has been proposed that physical and functional interactions of p53 with various members of the Bcl-2 family are involved in the transcription-independent route of p53-mediated cell death. However, the underlying mechanisms are not completely clarified. The extensive and successful exploitation of yeast to study the structural and functional properties of members of Bcl-2 family (7) allied to the observation of p53 translocation to yeast mitochondria open new perspectives to the understanding of how interactions between p53 and Bcl-2 family members promote MOMP. Though yeast was early considered devoid of members of the Bcl-2 family, a Bcl-xL interacting protein harbouring a Bcl-2 homology (BH3) domain, the Ybh3p, which translocates to mitochondria in response to a lethal stimulus mediating the disruption of mitochondrial membrane potential, was recently identified in *S. cerevisiae* (8). In this context the yeast model system emerges as a valuable tool that may provide relevant clues to the design of promising alternative strategies for p53-based cancer therapy.

A schematic illustration of the regulation of transcription-dependent and -independent p53 mechanisms by nPKCδ and ε in yeast is presented in Fig. 7.1.

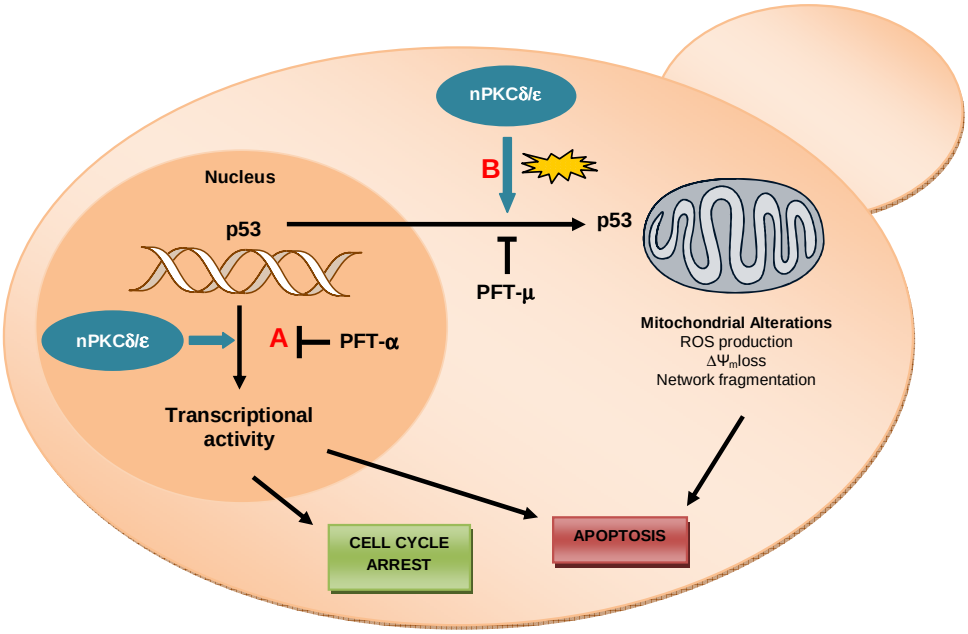

Fig. 7.1. Regulation of transcription-dependent and -independent p53 mechanisms by nPKCδ and ε in yeast. (**A**) Human wt p53 expressed in yeast has nuclear localization and induces growth inhibition associated with a S-phase cell cycle arrest, which is abolished by the selective inhibitor of p53 transcriptional activity, PFT-α, and markedly increased by nPKCδ and ε. (**B**) Under stress conditions induced by H_2O_2 (represented with a yellow flash), nPKCδ and ε stimulate the p53 translocation to mitochondria and a mitochondrial-dependent apoptotic pathway characterized by an increase on mitochondrial ROS production, $\Delta\Psi_m$ loss and mitochondrial network fragmentation. Mitochondrial p53 translocation in yeast is inhibited by the selective inhibitor of mitochondrial p53 translocation, PFT-μ .

7.2. ROLE OF PKC ISOFORMS IN APOPTOSIS: PHYSIOLOGICALLY RELEVANT SUBSTRATES

In the last years, yeast has emerged as an important cell model to study the role of PKC isoforms in apoptosis. In fact, using this cell system, important data have been provided in this area with the identification of relevant apoptotic substrates of individual PKC isoforms. An additional contribution to advance our knowledge in this area is provided by this thesis.

Concerning PKCα, several reports indicate an anti-apoptotic activity for this isoform (9-11). Additionally, some contradictory results suggesting a pro-apoptotic role for PKCα have also been reported (12-14). This pro-apoptotic function of PKCα was corroborated in previous works performed in yeast. In fact, this isoform stimulated acetic

acid-induced apoptosis (15), abolished the Bcl-xL anti-apoptotic effect (15), and stimulated the pro-apoptotic activity of Bax (16). However, in the scope of the studies developed in this thesis, we found that PKCα does not stimulate H_2O_2-induced yeast apoptosis. Similarly to that reported in mammalian cells (17, 18), this result corroborates that the apoptotic function of a specific PKC isoform, namely PKCα, is highly dependent on the stimulus applied. In fact, it was shown that distinct stimuli can induce the translocation of a specific PKC isoform to distinct subcellular compartments. Subsequently, this is associated to the phosphorylation of distinct substrates, and therefore to distinct cellular responses. In the case of H_2O_2, the PKCα substrates do not seem to be conserved in yeast. Moreover, we also found that PKCα does not modulate p53 apoptotic activity. This may therefore suggest that p53 is not a substrate of PKCα.

Concerning PKCδ, the well-known pro-apoptotic function of this isoform in mammalian cells (19) was widely confirmed in yeast. In fact, a previous study showed the stimulation of acetic acid-induced apoptosis by PKCδ (15). This study also revealed that PKCδ does not regulate the anti-apoptotic protein Bcl-xL. During this thesis, we found that PKCδ also stimulates coleon U-induced apoptosis. Additionally, similarly to that reported in mammalian cells (2, 20-22), we confirmed that PKCδ regulates the p53 apoptotic activity. Interestingly, stimulation of H_2O_2-induced apoptosis by PKCδ in yeast was only achieved in the presence of p53. This corroborates similar studies performed in mammalian cells, in which the stimulation of H_2O_2-induced apoptosis was practically abolished in the presence of rottlerin (a selective inhibitor of PKCδ) and in null-p53 cells (21). This may suggest that, for this stimulus, the pro-apoptotic activity of PKCδ is highly dependent on a specific substrate that seems to be p53.

Concerning PKCε, this isoform is frequently regarded as having anti-apoptotic properties in mammalian cells (19, 23). Although the mechanisms responsible for its anti-apoptotic function are still not well clarified, it seems to involve the regulation of Bcl-2 family proteins, namely Bax and Bad (23). In fact, the yeast cell model revealed that PKCε also regulates Bcl-xL, markedly increasing its activity (15). Although several studies suggest that PKCε favours life over death, others exist reporting the involvement of this isoform in apoptosis promotion (24). In fact, a previous study in yeast showed the stimulation of acetic acid-induced apoptosis by PKCε (15). In the present thesis, it is also shown the stimulation of coleon U- and H_2O_2-induced yeast apoptosis by this isoform. Curiously, for H_2O_2, the pro-apoptotic function of PKCε seems to be conserved in yeast, contrarily to that observed with the other isoforms tested. This work also shows that PKCε phosphorylates p53 and stimulates the p53 apoptotic activity. This may suggest that p53

is a substrate of PKCε. Together, these results obtained in yeast suggest that the apoptotic function of PKCε seems to be highly dependent on its accessibility to key apoptotic proteins, such as Bcl-xL and p53. The translocation of PKCε to distinct subcellular compartments, for instance due to distinct stimuli applied, may expose the PKC isoform to distinct substrates and this can be responsible for the distinct responses elicited by PKCε.

Concerning PKCζ, it is also well-accepted the anti-apoptotic role of this isoform in mammalian cells (19). However, it was previously shown in yeast the stimulation of acetic acid-induced apoptosis by PKCζ (15). In spite of this, when PKCζ was co-expressed with Bcl-xL in yeast, it markedly enhanced the Bcl-xL anti-apoptotic activity with a complete abolishment of acetic acid-induced apoptosis (15). PKCζ was therefore identified in yeast as a regulator of the anti-apoptotic protein Bcl-xL. The present work also corroborates an anti-apoptotic activity for PKCζ. In fact, it shows that PKCζ did not stimulate H_2O_2-induced yeast apoptosis. Additionally, it reveals that in yeast PKCζ does not regulate p53 apoptotic activity. This may therefore suggest that p53 is not a substrate of PKCζ.

7.3. IDENTIFICATION OF A SELECTIVE SMALL MOLECULE ACTIVATOR OF NPKCδ AND ε

The high complexity of the PKC-signalling pathway in mammalian cells has hampered the identification of isoform-selective PKC modulators. In fact, the development of new therapeutic strategies involving the modulation of PKC activity by many identified PKC modulators has being discarded in early phases of clinical trials due to their lack of selectivity (25, 26). Potent and selective modulators of individual PKC isoforms are therefore highly required in the PKC research field.

In this thesis, we used yeast cells individually expressing PKCα, βI, δ, ε or ζ to elucidate the molecular mechanism of action of coleon U, a diterpene compound isolated from *Plectranthus grandidentatus* reported to induce apoptosis in human cells (27), although the molecular mechanism of action associated with this coleon U-induced apoptosis have was not elucidated. Since several diterpene compounds, such as phorbol esters, had been recognised as potent PKC activators, the yeast PKC expression system was exploited to study the modulatory activity of coleon U on individual PKC isoforms. As

reported for human cells, coleon U also induced an apoptotic cell death in yeast, involving the mitochondrial pathway and metacaspase activation, but only in yeast cells expressing PKCδ or ε. The results obtained led to the discovery of a potent and selective activator of PKCδ and ε, which activity was thereafter confirmed in human cells (28). This compound therefore represents a promising pharmacological probe to study PKC-signalling pathways involving these isoforms in mammalian cells, as performed by Maghzal and colleagues (28). Furthermore, the stimulation of an apoptotic pathway independent of PKCα, βI and ζ, commonly reported as anti-apoptotic proteins, confers to coleon U promising applications in anticancer therapy.

Interestingly, in this work, we also showed that while PMA induced PKCδ/ε translocation from the cytosol to the plasma membrane and a G2/M cell cycle arrest, coleon U induced PKCδ/ε translocation from the cytosol to the nucleus and an apoptotic cell death. These results confirm in yeast cells previous evidences from mammalian cells (17, 18, 29) and reveal that different stimuli can induce the translocation of a same PKC isoform to distinct cellular compartements, which may be subsequently associated with different cellular responses. Moreover, they support the notion that the nuclear retention of PKC isoforms, particularly of PKCδ/ε, may be a required factor for commitment to apoptosis (30, 31).

A schematic illustration of the molecular mechanism of action of PMA and coleon U in yeast cells expressing PKCδ/ε is presented in Fig. 7.2.

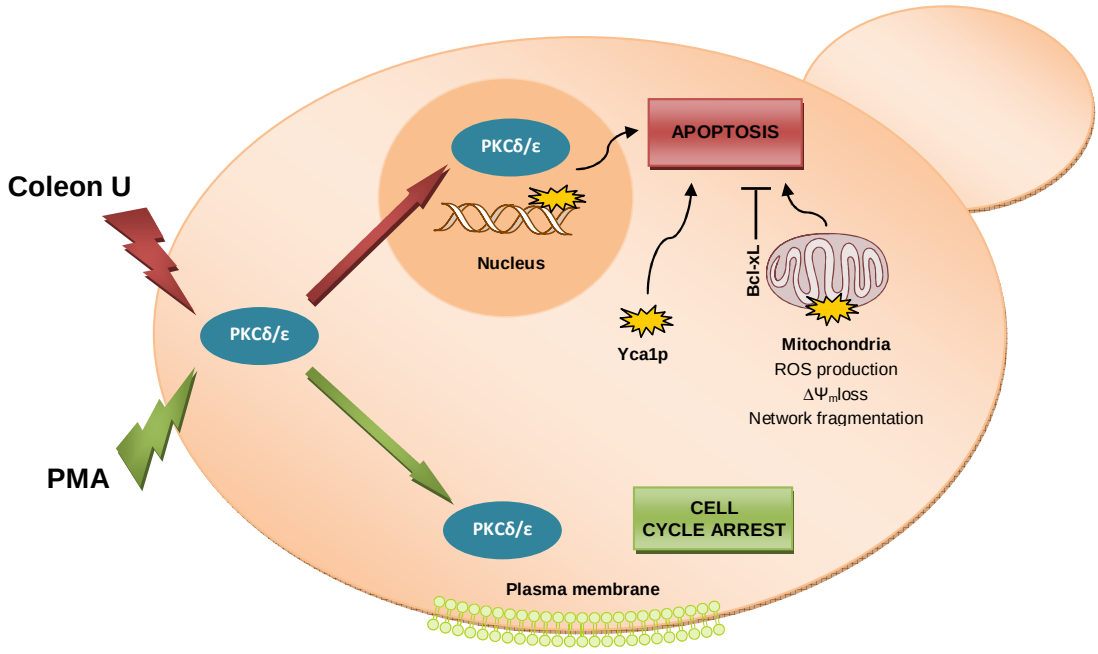

Fig. 7.2. Molecular mechanism of action of PMA and coleon U in yeast cells expressing nPKCδ/ε. nPKCδ and ε expressed in yeast have cytoplasmic localization. PMA induces the translocation of nPKCδ/ε to the plasma

membrane and a G2/M cell cycle arrest. On the contrary, coleon U induces the translocation of nPKCδ/ε to the nucleus and a metacaspase (Yca1p)- and mitochondrial-dependent apoptosis, which is abolished by the co-expression in yeast of the human anti-apoptotic protein Bcl-xL. DNA fragmentation, Yca1p activation and mitochondrial alterations (increase on mitochondrial ROS production, $\Delta\Psi_m$ loss and network fragmentation) induced by coleon U are represented with yellow flashes.

7.4. IDENTIFICATION OF SELECTIVE SMALL MOLECULE MODULATORS OF CASPASE-3 AND -7

As major regulators of cell proliferation and death, caspase-3, caspase-7 and p53 are therefore considered key therapeutic targets in human diseases where these cellular processes are dysregulated, such as cancer and neurodegeneration. In this context, small molecule activators and inhibitors of these proteins may be used to stimulate and prevent apoptosis in cancer and neurodegenerative disorders, respectively (32-37). However, similarly to PKC family, the high complexity of p53 and caspase-signalling pathways in mammalian cells has hampered the identification of selective modulators of these proteins.

With this thesis, we aimed to develop yeast assays for the screening of small molecule modulators of human caspase-3 and caspase-7. With this goal, we firstly confirmed the previously reported growth arrest induced by expression of active forms of human caspase-3 or -7 in yeast. In the case of caspase-7, this growth inhibitory effect was further associated with apoptosis and cell cycle arrest in G2/M and S phases. A correlation between yeast cell growth and human caspase activity was thereafter established using standard caspase activators and inhibitors. Using the yeast caspase-3 assay, new promising caspase-3 inhibitors were subsequently identified by testing a chemical library of vinyl sulfones (from the iMed.UL group), which are described as inhibitors of many cysteine proteases from clan CA (papain superfamily) (38).

The yeast assays developed in this thesis to search for modulators of human wt p53, caspase-3 and caspase-7 were also used to identify the molecular targets involved in the antiproliferative effect of several prenylated flavonoid derivatives of baicalein and 3,7-dihydroxyflavone and of the natural prenylated flavonoid artelastin (from the CEQUIMED-UP group) on distinct human tumour cell lines. (39, 40). With this study, three potent activators of caspase-7, 6-dihydroxy-7-prenyloxyflavone, 3-hydroxy-7-geranyloxyflavone and artelastin, were identified. These prenylated flavonoids have promising

pharmacological applications not only as probes in the study of caspase-signalling pathways, but also as anticancer agents. In fact, these flavonoids may represent an alternative therapeutic strategy against tumours without caspase-3 (e.g. breast adenocarcinoma) and/or a functional p53 pathway (e.g. chronic myelocytic leukemia), or with elevated levels of caspase-7 (e.g. prostate cancer).

Besides the promising therapeutic applications in cancer (caspase-7 activators) and neurodegeneration (caspase-3 inhibitors), the small molecule modulators of caspase-3 and -7 identified in this thesis have been used by the Medicinal Chemistry groups involved in the referred studies as lead compounds in the synthesis of new modulators of caspase-3 and -7 with improved potency and selectivity.

7.5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the present thesis, valuable data about the biology and pharmacology of major protein regulators of cell proliferation and death are provided using the yeast model system. Additionally, new targets and therapeutic opportunities are also revealed. In fact, the yeast assays herein developed and exploited in the screening of activators and inhibitors of the disease-related proteins also led to the discovery of promising pharmacological tools and therapeutic agents against devastating diseases, such as cancer and neurodegeneration.

The yeast research performed in this thesis confirmed the tremendous potential of this model system towards the study of PKC isoforms, p53 and caspase family members. We anticipate therefore that new promising discoveries can be done in these areas using this model organism. In fact, new ways were opened and several relevant questions were raised with this work that would be interesting to cover in future works, namely:

a) The identification of endogenous yeast pro-apoptotic substrates and respective orthologues in mammalian cells would certainly help in the identification of PKC isoform-specific substrates;

b) Since the p53 phosphorylation pattern observed under an apoptotic scenario was similar to that observed in unstressed cells, it would be important to determine whether p53 phosphorylation by PKCδ and ε is in fact a relevant factor for the stimulation of p53-mediated apoptosis by these PKC isoforms;

c) Further studies could be performed to ascertain if p53 interacts with the already identified yeast orthologues involved in MOMP. Future studies addressing the identification of these proteins and their human orthologues would certainly provide new insights about the transcription-independent p53 apoptotic activity;

d) The evidence herein provided about the regulation of p53 activities by PKCδ and ε indicate that coleon U could be used as a valuable pharmacological agent also in p53-related human diseases. The effect of this small molecule in human tumour cell lines with wt p53 could be therefore evaluated;

e) As a first-line screening tool, the activity of the small molecules identified as modulators of caspase-3 and -7 has to be validated in human cells. Additionally, the data provided by this study support the use of the developed yeast assays to evaluate the activity of new synthesized compounds on caspase-3 and -7;

f) Contrarily to p53, the regulation of other members of the p53 family, namely p63 and p73, by PKC isoforms is largely unknown. The use of the yeast cell system to study this issue would certainly provide new insights about the regulation of p63 and p73 by PKC isoforms. This would certainly provide new pharmacological strategies for the regulation of the activity of p63 and p73 by targeting these kinases.

7.6. REFERENCES

(1) Abbas T, White D, Hui L, Yoshida K, Foster DA, Bargonetti J. Inhibition of human p53 basal transcription by down-regulation of protein kinase Cdelta. J Biol Chem 2004 Mar; 279 (11): 9970-7.

(2) Yoshida K, Liu H, Miki Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. J Biol Chem 2006 Mar; 281 (9): 5734-40.

(3) Hadj Amor IY, Smaoui K, Chaabene I, Mabrouk I, Djemal L, Elleuch H, et al. Human p53 induces cell death and downregulates thioredoxin expression in Saccharomyces cerevisiae. FEMS Yeast Res 2008 Dec; 8 (8): 1254-62.

(4) Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature 2009 Apr; 458 (7242): 1127-30.

(5) Speidel D. Transcription-independent p53 apoptosis: an alternative route to death. Trends Cell Biol 2010 Jan; 20 (1): 14-24.

(6) Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochim Biophys Acta 2009 May; 1787 (5): 414-20.

(7) Silva RD, Manon S, Goncalves J, Saraiva L, Corte-Real M. The importance of humanized yeast to better understand the role of bcl-2 family in apoptosis: finding of novel therapeutic opportunities. Curr Pharm Des 2011; 17 (3): 246-55.

(8) Buttner S, Ruli D, Vogtle FN, Galluzzi L, Moitzi B, Eisenberg T, et al. A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. EMBO J 2011; 30 (14): 2779-92.

(9) Ahmad S, Mineta T, Martuza RL, Glazer RI. Antisense expression of protein kinase C alpha inhibits the growth and tumorigenicity of human glioblastoma cells. Neurosurgery 1994 Nov; 35 (5): 904-8; discussion 8-9.

(10) Dooley NP, Baltuch GH, Groome N, Villemure JG, Yong VW. Apoptosis is induced in glioma cells by antisense oligonucleotides to protein kinase C alpha and is enhanced by cycloheximide. Neuroreport 1998 Jun; 9 (8): 1727-33.

(11) Whelan RD, Parker PJ. Loss of protein kinase C function induces an apoptotic response. Oncogene 1998 Apr; 16 (15): 1939-44.

(12) Nowak G. Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells. J Biol Chem 2002 Nov; 277 (45): 43377-88.

(13) Shimizu T, Cao CX, Shao RG, Pommier Y. Lamin B phosphorylation by protein kinase calpha and proteolysis during apoptosis in human leukemia HL60 cells. J Biol Chem 1998 Apr; 273 (15): 8669-74.

(14) Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 2003 Sep; 278 (36): 33753-62.

(15) Saraiva L, Silva RD, Pereira G, Goncalves J, Corte-Real M. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. J Cell Sci 2006 Aug; 119 (Pt 15): 3171-81.

(16) Silva RD, Manon S, Goncalves J, Saraiva L, Corte-Real M. Modulation of Bax mitochondrial insertion and induced cell death in yeast by mammalian protein kinase Calpha. Exp Cell Res 2011 Apr; 317 (6): 781-90.

(17) Shirai Y, Saito N. Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. J Biochem 2002 Nov; 132 (5): 663-8.

(18) Wang QJ, Lu G, Schlapkohl WA, Goerke A, Larsson C, Mischak H, et al. The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-delta and -epsilon. Mol Cancer Res 2004 Feb; 2 (2): 129-40.

(19) Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 2009; 14: 2386-99.

(20) Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ 2006 Jun; 13 (6): 941-50.

(21) Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Karino T, et al. Roles of protein kinase C delta in the accumulation of P53 and the induction of apoptosis in H2O2-treated bovine endothelial cells. Free Radic Res 2002 Nov; 36 (11): 1147-53.

(22) Pospisilova S, Brazda V, Kucharikova K, Luciani MG, Hupp TR, Skladal P, et al. Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. Biochem J 2004 Mar; 378 (Pt 3): 939-47.

(23) Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal 2007 Aug; 19 (8): 1633-42.

(24) Zhang Y, Venugopal SK, He S, Liu P, Wu J, Zern MA. Ethanol induces apoptosis in hepatocytes by a pathway involving novel protein kinase C isoforms. Cell Signal 2007 Nov; 19 (11): 2339-50.

(25) Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? Nat Rev Cancer 2007 Jul; 7 (7): 554-62.

(26) Irie K, Nakagawa Y, Ohigashi H. Toward the development of new medicinal leads with selectivity for protein kinase C isozymes. Chem Rec 2005; 5 (4): 185-95.

(27) Cerqueira F, Cordeiro-Da-Silva A, Gaspar-Marques C, Simoes F, Pinto MM, Nascimento MS**.** Effect of abietane diterpenes from Plectranthus grandidentatus on T- and B-lymphocyte proliferation. Bioorg Med Chem 2004 Jan; 12 (1): 217-23.
(28) Maghzal N, Vogt E, Reintsch W, Fraser JS, Fagotto F**.** The tumor-associated EpCAM regulates morphogenetic movements through intracellular signaling. J Cell Biol 2010 Nov; 191 (3): 645-59.
(29) Ohmori S, Shirai Y, Sakai N, Fujii M, Konishi H, Kikkawa U, et al. Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C. Mol Cell Biol 1998 Sep; 18 (9): 5263-71.
(30) Yoshida K**.** Nuclear trafficking of pro-apoptotic kinases in response to DNA damage. Trends Mol Med 2008 Jul; 14 (7): 305-13.
(31) DeVries-Seimon TA, Ohm AM, Humphries MJ, Reyland ME**.** Induction of apoptosis is driven by nuclear retention of protein kinase C delta. J Biol Chem 2007 Aug; 282 (31): 22307-14.
(32) Lavrik IN, Golks A, Krammer PH**.** Caspases: pharmacological manipulation of cell death. J Clin Invest 2005 Oct; 115 (10): 2665-72.
(33) Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland MS, Kwon JT, et al. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nat Chem Biol 2006 Oct; 2 (10): 543-50.
(34) Wolan DW, Zorn JA, Gray DC, Wells JA**.** Small-molecule activators of a proenzyme. Science 2009 Nov; 326 (5954): 853-8.
(35) Callus BA, Vaux DL**.** Caspase inhibitors: viral, cellular and chemical. Cell Death Differ 2007 Jan; 14 (1): 73-8.
(36) Cheok CF, Verma CS, Baselga J, Lane DP**.** Translating p53 into the clinic. Nat Rev Clin Oncol 2011 Jan; 8 (1): 25-37.
(37) Amaral JD, Xavier JM, Steer CJ, Rodrigues CM**.** Targeting the p53 pathway of apoptosis. Curr Pharm Des 2010; 16 (22): 2493-503.
(38) Santos MM, Moreira R**.** Michael acceptors as cysteine protease inhibitors. Mini Rev Med Chem 2007 Oct; 7 (10): 1040-50.
(39) Neves MP, Cidade H, Pinto M, Silva AM, Gales L, Damas AM, et al. Prenylated derivatives of baicalein and 3,7-dihydroxyflavone: synthesis and study of their effects on tumor cell lines growth, cell cycle and apoptosis. Eur J Med Chem 2011 Jun; 46 (6): 2562-74.
(40) Pedro M, Ferreira MM, Cidade H, Kijjoa A, Bronze-da-Rocha E, Nascimento MS**.** Artelastin is a cytotoxic prenylated flavone that disturbs microtubules and interferes with DNA replication in MCF-7 human breast cancer cells. Life Sci 2005 Jun; 77 (3): 293-311.