U.PORTO

FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

Cláudia Sofia Santos Bessa

Targeted regulation of PKC signaling pathway: Searching for new therapeutic strategies against cancer

Março 2018

ACKNOWLEDGEMENTS

ABSTRACT

Cancer is a major public health concern with increasing incidence and mortality worldwide. The effectiveness of the current therapeutic options for cancer patients is still limited, mostly due to therapeutic resistance and severe toxic side effects. Additionally, the anticancer alternative drugs approved over the last years have been restricted. As such, the discovery of new therapeutic options against cancer is required.

Compelling evidence have established a key role of protein kinase C (PKC) isozymes in tumor growth and dissemination. However, despite being a promising target in cancer therapy, the lack of PKC isozyme-selective regulators has hampered the clinical translation of PKC-targeting agents.

With the present thesis, it was intended to identify PKC isozyme-selective agents, as well as new anticancer drug candidates by targeting PKC. For such purpose, the effect of a small chemical library of royleanone derivatives on the activity of classical (α and βI), novel (δ and ε) and atypical (ζ) PKC isozymes was evaluated using a previously developed yeast-based PKC assay. With this cell system, the compound 7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone (Roy-Bz) was identified as potential PKCδ-selective activator. Thereafter, *in vitro* PKC assays using recombinant PKC isozymes, molecular docking analysis, and studies in human cancer cells confirmed that Roy-Bz was a PKCδ-selective activator with the C1 domain as putative binding site. Additionally, in human colon cancer cells, Roy-Bz potently inhibited cell proliferation by prompting a mitochondria-mediated apoptotic cell death, and presented an anti-migratory activity, in a PKCδ-dependent manner. Notably, Roy-Bz showed PKCδ-dependent anticancer activity in xenograft mouse models with no apparent toxic side effects. Further supporting its potent anticancer activity, it was also shown that Roy-Bz potentially targets glucose metabolism by dual inhibition of glycolysis and oxidative phosphorylation (OXPHOS). Collectively, these results led to the identification of the first small-molecule PKCδ-selective activator with encouraging application in anticancer therapy, particularly in colon cancer.

In this thesis, it was also shown the growth inhibitory effect of the abietane diterpenoid, 6,7-dehydroroyleanone (DeRoy), as well as of two extracts obtained from *Plectranthus* species, on a panel of human cancer cells. These results reinforced the potential of the *Plectranthus* genus as a promising source of new anticancer drugs.

In conclusion, with the discovery of Roy-Bz, this thesis opens the way to a new era on PKC biology and pharmacology, contributing to the potential redefinition of the structural requirements of PKC isozyme-selective agents, and to the re-establishment of PKC

isozymes as feasible therapeutic targets in cancer. Additionally, it gives rise to new therapeutic opportunities in anticancer therapy.

RESUMO

O cancro representa uma das principais preocupações de saúde pública, com um aumento anual da sua incidência e mortalidade no mundo. A eficácia das opções terapêuticas existentes para os doentes oncológicos é ainda limitada, principalmente devido ao aumento da resistência à terapêutica e aos efeitos secundários indesejáveis. Além disso, o número de novos fármacos anticancerígenos aprovados, ao longo dos últimos anos, tem sido reduzido. Desta forma, a descoberta de novas oportunidades terapêuticas para o tratamento do cancro continua a ser uma prioridade.

Diversos estudos têm estabelecido um papel fundamental das isoformas da família da cínase C de proteínas (PKC) no crescimento e disseminação do cancro. No entanto, apesar de ser um alvo promissor na terapia do cancro, a falta de reguladores seletivos das isoformas da PKC tem dificultado a sua translação para a clínica.

A presente tese teve como objetivo identificar agentes seletivos das isoformas da PKC, assim como novos candidatos a fármacos anticancerígenos tendo como alvo a PKC. Para tal, avaliou-se o efeito de uma biblioteca de compostos derivados da roleanona na atividade das isoformas clássicas (α e βI), novel (δ e ε) e atípica (ζ) da PKC, utilizando um modelo de levedura previamente desenvolvido em estudos anteriores. Com este sistema celular, identificou-se o composto 7α-acetoxi-6β-benzoiloxi-12-O-benzoilroileanona (Roy-Bz) como um potencial ativador seletivo da PKCδ. Posteriormente, realizando-se ensaios *in vitro* com proteínas recombinantes das isoformas da PKC, estudos de 'docking' molecular e ensaios com células tumorais humanas, confirmou-se que o Roy-Bz era um ativador seletivo da PKCδ, tendo como possível local de ligação o domínio C1. Além disso, em células humanas do cancro do cólon, o Roy-Bz inibiu de forma marcada a proliferação celular, através da ativação de uma via apoptótica mediada pela mitocôndria, assim como a migração tumoral, de uma forma dependente da PKCδ. É ainda de realçar que, para além da marcada inibição do crescimento tumoral *in vitro*, o Roy-Bz apresentou atividade anti-tumoral *in vivo*, dependente da PKCδ, sem efeitos tóxicos secundários aparentes. O Roy-Bz demonstrou também atuar ao nível do metabolismo da glucose, através da inibição da glicólise e da fosforilação oxidativa. Tal facto reforçou a potente atividade anticancerígena do composto Roy-Bz. Os resultados obtidos conduziram à identificação da primeira pequena molécula ativadora seletiva da PKCδ com uma aplicação promissora na terapêutica anticancerígena, principalmente no tratamento do cancro do cólon.

Nesta tese, foram ainda identificados o diterpeno de abietano 6,7-dehidroroileanona (DeRoy) e dois extratos obtidos a partir de espécies do género *Plectranthus*, com atividade anti-proliferativa em linhas celulares de tumores humanos. Estes resultados corroboram a

relevância do género *Plectranthus* como uma fonte promissora de potenciais fármacos anticancerígenos.

Em conclusão, com a descoberta do composto Roy-Bz, esta tese iniciou uma nova era na biologia e farmacologia da PKC, contribuindo para uma potencial redefinição dos requisitos estruturais dos agentes seletivos das isoformas da PKC, assim como para o restabelecimento das isoformas da PKC como potenciais alvos terapêuticos no cancro.

Palavras-chave: Agente anticancerígeno, Cancro, Ativador isoforma seletivo, Género *Plectranthus*, PKC

INDEX

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

a.a.	Amino acids
ARA	Arachidonic acid
ATM	Aurothiomalate
BAK	Bcl-2-antagonist/killer
BAX	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
Ca^{2+}	Calcium
CF	Catalytic fragment
CKAR	C-kinase activity reporter
CP	Cyclophosphamide
CRC	Colorectal cancer
CSC	Cancer stem cell
Cyt *c*	Cytochrome *c*
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCA	Dichloroacetate
DGK	Diacylglycerol kinase
DiOC$_6$(3)	3,3'-dihexyloxacarbocyanine iodide
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ETC	Electron transfer chain
ETOP	Etoposide
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FDA	Food and Drug Administration
FRET	Förster/fluorescence resonance energy transfer
GFP	Green fluorescent protein
GOF	Gain-of-function
HDI	Human development index
HK2	Hexokinase 2

HRP	Horseradish-peroxidase
HSP90	Heat shock protein 90
H&E	Hematoxylin and eosin
IP$_3$	Inositol 1,4,5-trisphosphate
LOF	Loss-of-function
MCT4	Monocarboxylate transporter 4
MMP	Mitochondrial membrane potential
MMP-2/-9	Matrix metalloprotease-2/-9
MN	Micronucleus
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
NSCLC	Non-small cell lung cancer
OCR	Oxygen consumption rate
OD	Optical density
Par6	Partitioning-defective 6
PB1	Phox/Bem1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PI	Propidium iodide
PIP$_2$	Phosphatidylinositol-4,5-biphosphate
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PRB	13-acetylphorbol
PS	Pseudosubstrate
RACK	Receptor for activated C kinases
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
Ser	Serine
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TIGAR	*TP53*-induced glycolysis and apoptosis regulator
Thr	Threonine
VEGF	Vascular endothelial growth factor
Wt	Wild-type
YNB	Yeast nitrogen base

CHAPTER 1

Introduction

1.1. Cancer

1.1.1. Cancer epidemiology

Cancer is a major public health concern worldwide, causing even more deaths than all coronary heart disease or stroke according to World Health Organization (WHO) estimates (1). For 2012, the GLOBOCAN predicted approximately 14 million new cancer cases and 8.2 million deaths in the world (2). These data tend to increase over the next few years, being expected that 20 million people develop cancer annually and 11 million die by 2025. As indicated in Figure 1.1A, lung (1.82 million cases), breast (1.67 million cases) and colorectal (1.36 million cases) cancers exhibit the highest incidence rates, followed by prostate, stomach and liver cancers. However, lung (1.6 million deaths), liver (745,533 deaths) and stomach (723,073 deaths) cancers are the most deadly (Fig. 1.1A). The highest incidence of cancer is observed in Asian (6.76 million cases), with a mortality rate higher than 50% (4.5 million cases) (Fig. 1.1B). Europe and Northern America have the second highest incidence and mortality rates, in opposition to Oceania with the lowest rates (Fig. 1.1B). In Portugal, cancer is the second cause of death (https://www.ine.pt) and, following the worldwide trend, it is expected an increase in the new cancer cases, mainly due to the population ageing, human lifestyle, and infections. Contrary to global trends, in Portugal, colorectal cancer (CRC) has the highest incidence rate, followed by prostate and breast cancers, and it is also the most lethal (Fig. 1.1C).

As referred, CRC is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths in the world (Fig. 1.1A). CRC is characterized by a significant variation in incidence and mortality rates across the different human development index (HDI) levels of countries. Accordingly, a study from Arnold and co-workers (2016) concluded that in numerous medium-to-high HDI-indexed countries, such as Latvia, Estonia, Lithuania, Russia, China and Brazil, the CRC incidence and mortality rates tend to rapidly increase (3). Conversely, in some of the highest HDI-indexed countries, including USA, Japan and France, both CRC incidence and mortality have decreased. An intermediate pattern was also described for very high HDI-indexed countries, such as Canada, United Kingdom, Denmark and Singapore, with an increase of incidence and a decrease of mortality.

A

Incidence

Other: 4,969,280 (35.3%)
Lung: 1,824,701 (13.0%)
Breast: 1,671,149 (11.9%)
Colorectum: 1,360,602 (9.7%)
Prostate: 1,094,916 (7.8%)
Stomach: 951,594 (6.8%)
Liver: 782,451 (5.6%)
Cervix uteri: 527,624 (3.8%)
Oesophagus: 455,784 (3.2%)
Bladder: 429,793 (3.1%)

Mortality

Other: 2,788,798 (34.0%)
Lung: 1,589,925 (19.4%)
Breast: 521,907 (6.4%)
Colorectum: 693,933 (8.5%)
Prostate: 307,481 (3.7%)
Stomach: 723,073 (8.8%)
Liver: 745,533 (9.1%)
Cervix uteri: 265,672 (3.2%)
Oesophagus: 400,169 (4.9%)
Bladder: 165,084 (2.0%)

B

Asia: 6,763,000 (48.1%)
Europe: 3,420,000 (24.3%)
Oceania: 155,000 (1.1%)
Africa: 847,000 (6.0%)
Latin America and Carribbean: 1,096,000 (7.8%)
Northern America: 1,786,000 (12.7%)

Asia: 4,500,000 (54.9%)
Europe: 1,756,000 (21.4%)
Oceania: 60,000 (0.7%)
Africa: 591,000 (7.2%)
Latin America and Caribbean: 603,000 (7.4%)
Northern America: 692,000 (8.4%)

C

Other: 14,628 (29.7%)
Colorectum: 7,129 (14.5%)
Prostate: 6,662 (13.5%)
Breast: 6,088 (12.4%)
Lung: 4,192 (8.5%)
Stomach: 3,018 (6.1%)
Bladder: 2,876 (5.8%)
Non-Hodgkin lymphoma: 1,842 (3.7%)
Copus uteri: 1,485 (3.0%)
Lip, oral cavity: 1,294 (2.6%)

Other: 7,646 (31.7%)
Colorectum: 3,797 (15.7%)
Lung: 3,441 (14.3%)
Stomach: 2,285 (9.5%)
Prostate: 1,582 (6.6%)
Breast: 1,570 (6.5%)
Pancreas: 1,268 (5.3%)
Liver: 908 (3.8%)
Bladder: 854 (3.5%)
Leukaemia: 761 (3.2%)

Figure 1.1: Estimated incidence and mortality for: (A) different types of cancer; **(B)** cancer worldwide; **(C)** cancer in Portugal. [Graphs from GLOBOCAN 2012: Estimated cancer Incidence Mortality and Prevalence Worldwide in 2012 (http://globocan.iarc.fr/Default.aspx)].

1.1.2. Cancer hallmarks

Over the last years, the extensive research around cancer clearly indicated that tumorigenesis is a multistep process elicited by genome mutations that progressively drive the malignant transformation of normal cells into cancer cells. Currently, it is well-established that tumors are more than masses of proliferating cancer cells. Instead, they comprise heterogeneous subclonal populations involved in heterotypic interactions between them, thus forming a complex tissue (4). In addition, beyond tumor biology, the tumor microenvironment has proved to be crucial for tumor progression and maintenance. In fact, the role of microenvironment reinforces the idea that tumors are complex organs,

20

resulting from the process of human tumor pathogenesis, encompassing multiple cells, molecules and blood vessels that surround and feed the tumor (4).

In 2000, Hanahan and Weinberg described, for the first time, the major hallmarks (or physiologic alterations) of cancer cells involved in tumor growth and metastatic dissemination (5). The abilities acquired by cancer cells during tumor development included self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 1.2) (5). Contrary to normal cells that strictly depend on exogenous growth-promoting factors to proliferate, cancer cells are able to produce their own growth signals, and consequently sustaining a chronic proliferation. Besides of inducing pro-proliferative factors, the signaling pathways of cancer cells that negatively regulate proliferation, through activation of senescence or apoptosis, are also deregulated. Actually, several evidence indicate that numerous tumor suppressor genes, such as *Rb* (retinoblastoma-associated) and *TP53*, have mutations that lead to their inactivation. The resistance to cell death is another characteristic of tumors, where apoptosis is limited or circumvented, favoring the progression of tumors to states of high-grade malignancy and resistance to therapy (6, 7). However, the hallmarks mentioned above are not sufficient by themselves to generate macroscopic tumors. Indeed, contrasting to the behavior of normal cells that enter into senescence or crisis/apoptosis after a limited number of cell divisions, cancer cells have an unlimited replicative potential, being able to overcome these barriers. On the other hand, in order to supply all the needs both in terms of nutrients and oxygen during tumor growth, angiogenesis is permanently 'switch on', including in early development stages of invasive cancers (8, 9). Additionally, also invasion and metastasis have been intimately associated with highly aggressive carcinomas. This is a multistep process that begins with local invasion of cancer cells, intravasation into blood and lymphatic vessels, transport to distant tissues, and subsequent growth of tumor metastasis. These carcinomas have been mainly characterized by alterations in genes encoding cell-to-cell and cell-to-extracellular matrix adhesion molecules. In 2011, two other emerging hallmarks were described, namely the reprogramming of energy metabolism that ensures the neoplastic proliferation, and the evading immune destruction by tumor cells (Fig. 1.2) (4). At the same time, it was reported that all the functions acquired during the multistep process of tumorigenesis are dependent on two enabling characteristics of cancer cells, specifically the genome instability and mutation, and the tumor-promoting inflammation (Fig. 1.2) (4).
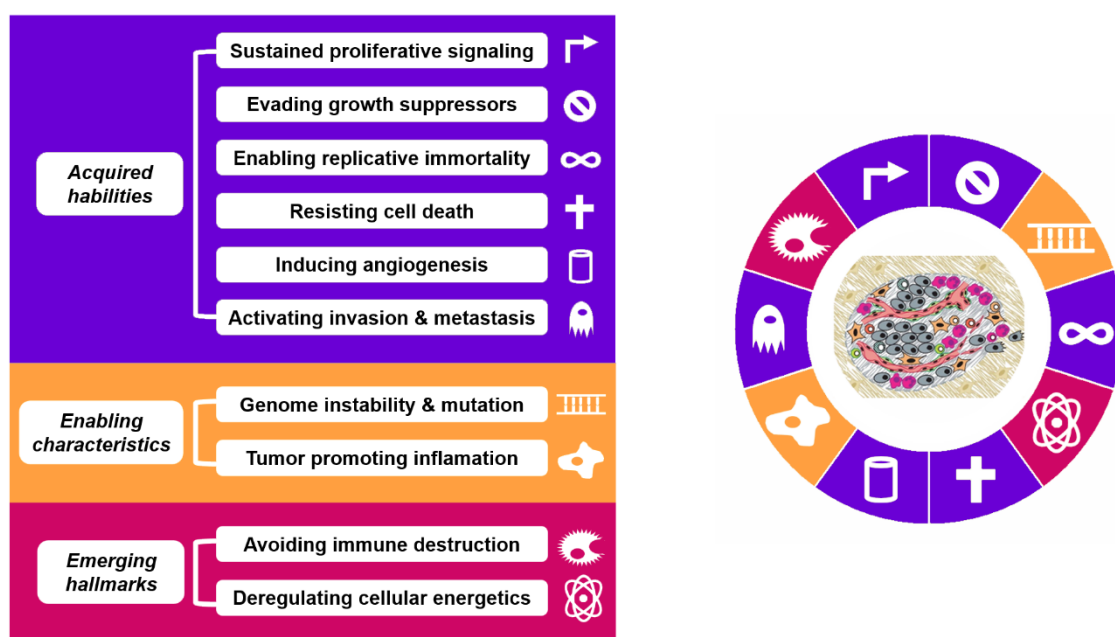
Figure 1.2: Cancer hallmarks. Schematic representation of the six hallmarks originally proposed in 2000, and of four additional hallmarks and/or characteristics later described for cancer cells. [Adapted from (4)].

1.1.3. Anticancer therapeutic strategies

Over the past few decades, surgical resection, radiotherapy and conventional chemotherapy have been the major approaches in cancer treatment. However, there are several inherent limitations in these therapeutic options, particularly secondary toxic side effects and therapeutic resistance, which have compromised their efficiency against cancer. Despite great advances in cancer research, with a deeper understanding of its pathobiology, the availability of effective anticancer agents remains unsatisfactory. Actually, even though anticancer drugs represent a substantial proportion of total new drug approvals, their success rates in clinical trials have been disappointing (7% in phase I and less than 50% in phase III) (10).

The conventional chemotherapy acts on rapidly replicating cells, being unable to discriminate between cancer and normal cells. Therefore, it is often associated with various undesirable toxic side effects, as fatigue, nausea, vomiting, diarrhea, weight changes, hair loss, anemia, easy bruising and bleeding, fertility problems, among others (www.cancer.org). In order to minimize this high toxicity, these drugs have been often administrated in regimens of single doses, or in short-term therapeutic cycles at higher concentrations (11).

22

In the last years, the interest of the pharmaceutical companies has moved from conventional non-specific cytotoxic agents to targeted therapy (12, 13). In fact, targeted therapies are currently the focus of anticancer drug development, being the basis of precision medicine, an emerging approach that takes into account individual genetic variability, environment, and lifestyle for each patient to prevent and treat disease. The major goal of targeted cancer therapy is to kill cancer cells without affecting the healthy ones, therefore minimizing undesirable toxic side effects and improving the quality of life of patients. Consistently, targeted therapies are designed to interfere with specific molecular targets involved in cancer growth and dissemination (14, 15). Currently, there are different types of targeted therapies approved for cancer treatment (either small-molecules or monoclonal antibodies), including hormones, immunotherapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, and angiogenesis inhibitors. However, although targeted therapies have proved to be a valuable alternative for cancer treatment, exhibiting less severe side effects and more effective results, they are closely associated with the acquisition of drug resistance, particularly due to the occurrence of mutations on the molecular target and/or to the recruitment of alternative compensatory growth pathways independent of the target (16).

Although the current cancer therapies can induce partial or even a complete cancer regression in some patients, the relapse is the most common scenario, with recurrent cancers becoming more aggressive and resistant to therapy (17). For long, the genetic and epigenetic alterations in malignant cells have been the main focus of study in cancer biology and in the development of new therapies (18). However, over the last years, it has become clear that tumor growth and progression are not only driven by cumulative gene mutations, but are highly affected by the heterogeneous tumor cell population and surrounded microenvironment. In fact, currently the development of novel anticancer therapeutic alternatives have been based on the emergent concept that tumors consist of heterogeneous cell populations mainly characterized by a high phenotypic diversity, which seems to be the major driver of cancer recurrence. Among these populations, the discovery of cancer stem cells (CSCs), also termed as tumor-initiating cells (TICs), have attracted increasing interest due to their capacity to self-renew and drive tumorigenesis (19, 20). Actually, it was demonstrated that only a small fraction of cells, namely CSCs, are able to form colonies *in vitro* or seed new tumors in mice (21). In addition, several works have shown that CSCs are involved in tumor initiation (22), maintenance (23, 24), metastasis (25), and recurrence (26-28). Accordingly, the limited efficacy of anticancer therapies may be attributed to the inability of targeting CSCs within the tumor. Indeed, cumulating data have shown that CSCs are more resistant than non-CSCs (29-31). Based on this, many

efforts have been made to better understand the epigenetic mechanisms that allow to differentiate between non-CSCs and CSCs, and to design novel therapeutic strategies able to target CSCs for complete cancer eradication. Moreover, the tumor microenvironment is a dynamic structure that, besides cancer cells, comprises the extracellular matrix and stromal, endothelial, and immune cells that are recruited from nearby tissues and bone marrow (32, 33). Accordingly, tumor microenvironment is continuously changing, both at molecular and cellular levels, as a result of the interaction between incipient cancer cells and non-transformed cells, leading to phenotypic alterations, and consequently to tumor growth and metastatic dissemination (34). The interplay between tumor cells and tumor microenvironment may occur through cell-to-cell and cell-to-extracellular matrix adhesion molecules interactions, or through regulation of the secretion of soluble molecules, such as growth factors, chemokines and cytokines, which are implicated in the activation/inhibition of the main tumor signaling pathways. As such, the identification and modulation of relevant targets in tumor microenvironment prove to be a valuable approach in the prevention and treatment of cancer. Indeed, a dramatic increase in the pre-clinical development and clinical testing of agents that target tumor microenvironment has been observed, being a large number of these drugs approved by the Food and Drug Administration (FDA). Currently, one of the most representative examples are the angiogenic inhibitors, that impact on vascular endothelial growth factor (VEGF) or other pro-angiogenic signaling pathways, which have been successfully tested in clinical trials and consequently approved for clinical use in a variety of cancers (35, 36).

Additionally, in response to genetic mutations and/or to the stressful and fluctuating microenvironment conditions, cancer cells are able to reprogram the entire metabolic pathway to sustain their high proliferation rates (4). Accordingly, almost a century ago, metabolic alterations, characterized by the enhancement of the glycolytic capacity, with increased glucose uptake and lactate production, and the absence of respiration even in aerobic conditions, were described in cancer cells, being termed as 'Warburg effect' (37). These alterations were in fact recognized as distinctive features of cancer cell metabolism, since in normal cells ATP is mainly produced through oxidative phosphorylation (OXPHOS) in mitochondria. Based on this, glucose metabolism has also received much attention in an attempt to better understand the cancer physiology, and to improve the pharmacology of anticancer agents. The extensive research around this issue has allowed establishing a close correlation between glucose metabolism and tumor heterogeneity, which in turn critically affect the resistance to anticancer therapy (38). Therefore, the use of glycolytic inhibitors was established as a promising therapeutic strategy against cancer. However, taking into account the tumor cell metabolic heterogeneity, the application of a single

metabolic therapeutic strategy, based on glycolysis inhibition, revealed to be ineffective, not leading to a complete tumor eradication. In fact, the combined use of glycolytic inhibitors, which act on highly proliferative cells eliminating the ATP produced via 'Warburg effect', with OXPHOS inhibitors that sensitize cancer cells with low proliferative rates and oxidative metabolism (including CSCs), has represented the most attractive approach to effectively kill heterogeneous cell populations, avoiding tumor cell relapse and therapeutic resistance (38, 39). One example is the widely used antidiabetic drug metformin, which is an inhibitor of glycolysis (by HK2 inhibition) and OXPHOS (by mitochondrial complex I inhibition) (40, 41). Based on this, metformin has been tested either alone or in combination therapy, namely with dichloroacetate (DCA; an inhibitor of pyruvate dehydrogenase kinase) (39). Nevertheless, its use in combination with other metabolic drugs has shown to be the most effective strategy against tumor relapse (42-44). The combination of metabolic modulators with other chemotherapeutic agents has also shown great potential against the ever-increasing resistance to conventional chemotherapy. Consistently, in paclitaxel-resistant human lung (45) and oral (46) cancer cells, with overexpression of pyruvate dehydrogenase kinase 1/2, the combination with DCA sensitized these cells to paclitaxel.

Collectively, cancer must be understood and treated as a whole. In fact, cancer medicine has changed drastically and currently encompasses a panel of integrative approaches ranging from personalized diagnostics to therapeutics (47). The combination of targeted therapies with other strategies that hit different parts of the cell signaling pathway, or with conventional chemotherapeutic drugs, has proven to be the most embracing approach, which has already shown successful results in clinical trials (15). However, the search for selective regulators of key molecular targets in cancer has been one of the major challenges in targeted therapy.

1.2. Protein kinase C

In 1977, Nishizuka and co-workers identified for the first time a new protein kinase, originally called PKM (48-50). Two years later, using a purified holoenzyme, it was found that this protein was a calcium (Ca^{2+})-activated and phospholipid-dependent kinase (51, 52). Based on this Ca^{2+}-dependent kinase activity, it was renamed protein kinase C (PKC). The continuous efforts to characterize PKC led to the identification of the lipid second messenger diacylglycerol (DAG) as an endogenous activator of this kinase (52, 53). Afterwards, several studies established PKC as a cellular receptor for the natural occurring

phorbol esters (54-56). Since then, an extensive research has been done in the PKC field, giving rise to over 60,000 citations in PubMed, making PKC one of the most studied kinases.

Early it was demonstrated the involvement of PKC isozymes in key cellular processes that regulate cell survival and cell death (57). This has supported the crucial role of PKC isozymes in numerous human pathologies, including diabetes (58, 59), heart failure (60, 61), Alzheimer and Parkinson (62, 63), autoimmune diseases (64-67), and cancer (57, 68). However, despite all the efforts to unravel the function of individual PKC isozymes in different cellular processes, discrepant outcomes have frequently been achieved. This has been mainly attributed to the high complexity of the PKC-signaling pathway, particularly the existence of several structurally-related isozymes in the same cellular environment, and the pronounced expression heterogeneity of PKCs in different cell types (57). The lack of isozyme-selective PKC modulators has also greatly contributed to the conflicting data. Nonetheless, despite this limitation, PKC has remained an appealing therapeutic target in the treatment of several human diseases.

1.2.1. PKC family

PKC belongs to the AGC family of kinases (also including PKA and PKG) (69), consisting of at least ten serine/threonine kinases encoded by nine different genes (70, 71). Briefly, PKC isozymes are grouped, according to their regulatory domain structure and co-factors required for activation, into three subfamilies: classical/conventional (cPKC; α, alternatively spliced βI and βII, and γ), novel (nPKC; δ, ϵ, η and θ), and atypical (aPKC; ζ and $\lambda\backslash\iota$). Besides the most representative members, it has also been described the brain-specific alternative transcript PKMζ, which only encodes the catalytic domain of PKCζ (72), and spliced PKCδ variants (73-75). Structurally, PKC isozymes share a highly conserved carboxy-terminal region (*C*-terminus), comprising an ATP-binding domain and a catalytic domain, linked through a flexible hinge region (V3) to the membrane-targeting amino-terminal regulatory domain (*N*-terminus) (Fig. 1.3) (76, 77). The regulatory domain encompasses two membrane targeting regulatory elements, C1 and C2 (or C2-like) regions, as well as the pseudosubstrate (PS) segment, which retains the kinase in an autoinhibited conformation, through engagement in the substrate-binding cleft of the catalytic domain. The C1 domain of cPKCs and nPKCs contains two tandem cysteine-rich zinc finger motifs (C1A and C1B) that bind to DAG or phorbol esters in membranes. The C2 domain differs from cPKCs to nPKCs. In fact, whereas in cPKCs the C2 domain binds anionic phospholipids in a Ca^{2+}-dependent manner, nPKCs do not bind Ca^{2+} due to the loss of functionality of the C2 domain (termed as C2-like) caused by the absence of key aspartate

26

residues required to coordinate Ca^{2+} (77). Contrary, aPKC isozymes bind neither DAG nor Ca^{2+}, but they contain a modified C1 domain that retains the binding ability to anionic phospholipids (although with lower affinity), and a Phox/Bem1 (PB1) domain that mediates protein–protein interactions on protein scaffolds. In particular, it was reported that PB1 domain of aPKCs specifically interact with the PB1 domain of scaffold proteins p62 and Partitioning-defective 6 (Par6) (78-82). Besides the PB1 domain of aPKCs, also the C2 domain of cPKCs and nPKCs is involved in protein-protein interactions, mediating the relocation of PKCs upon activation to specific subcellular compartments. Indeed, several data suggested that activated PKCs may bind to specific anchoring proteins, termed as receptors for activated C kinases (RACKs), localized at specific membrane microdomains in the cytoskeleton, through the C2 domain (83). This interaction of PKC with these proteins allows a higher proximity of its allosteric activators and substrates. Another PKC binding partner is the heat shock protein 90 (HSP90) chaperone that interacts with PKCε C2 domain, promoting the shuttling of PKCε into the mitochondria (84).
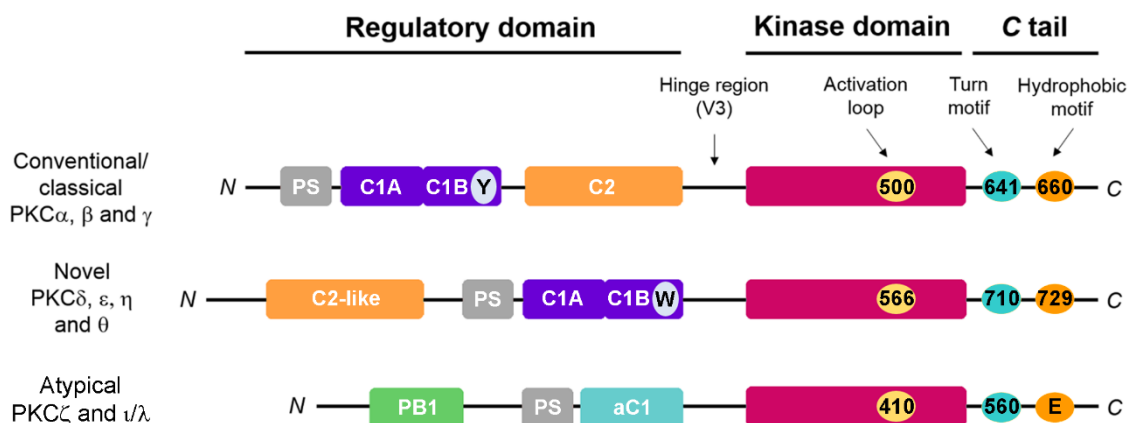
Figure 1.3: Schematic representation of the domain structure of PKC isozymes grouped by subfamilies. PKC isozymes share a common architecture, with a *N*-terminal regulatory moiety that regulates the catalytic activity of the *C*-terminal kinase domain. The regulatory domain comprises the autoinhibitory PS segment, C1, or atypical C1 (aC1; for atypical PKCs), C2 and PB1 domains. Concerning to C1 domain, cPKCs differs from nPKCs in a single amino acid, tyrosine (Y) in classical vs tryptophan (W) in novel isozymes in C1B domain, which increases the affinity of nPKCs to DAG in two order of magnitude compared to cPKCs. The *C*-terminal kinase moiety contains the catalytic domain (red rectangles) that is prime phosphorylated on the activation loop (yellow circle), and a *C*-terminal tail that is phosphorylated at the turn (cyan circle) and hydrophobic motifs (orange circles). Atypical isozymes have a glutamic acid (E) at the phosphoaceptor site on the hydrophobic motif.

1.2.1.1. *Regulation of PKC activity*

Newly synthesized PKCs are bind to plasma membrane in an open and degradation-sensitive conformation, in which its membrane targeting motifs and PS segment are exposed. Subsequently, PKCs are retained in an inhibitory conformation in the cytoplasm by a series of ordered, tightly coupled, and constitutive phosphorylations, designated as 'maturation' process, which ensures the stability and the catalytic competence of these enzymes (Fig. 1.4) (85, 86). The initiation of PKC processing is dependent on the binding of a conserved PXXP motif in the kinase domain to the molecular chaperone Hsp90 with Cdc37 (87). Thereafter, PKC is first phosphorylated on a conserved threonine in the activation loop (localized at the catalytic domain) by phosphoinositide-dependent kinase 1 (PDK1), in which it is also involved the kinase complex mTORC2 (Fig. 1.4) (85). The conformational change induced by this phosphorylation leads to the exposure of the turn and the hydrophobic motifs at the *C*-terminus, and to their subsequent autophosphorylation. These post-translational modifications render PKC resistant to proteases and phosphatases, preventing its degradation, as well as catalytically competent. The mature PKC is released into the cytosol in an autoinhibited conformation achieved by intramolecular interactions between the *N*-terminal PS region and the *C*-terminal catalytic domain, in which the substrate binding pocket is occupied by the PS sequence (Fig. 1.4) (57).

Under physiological conditions, the activation of PKC is regulated by extracellular agonists, which increase the intracellular levels of the second messengers DAG and Ca^{2+} in response to the stimulation of receptor tyrosine kinases (RTKs) or other cell surface receptors. In fact, the stimulation of these receptors induces the activation of effector molecules, including membrane-associated phospholipase C (PLC), which catalyze the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) into DAG and inositol 1,4,5-trisphosphate (IP_3). Additionally, the increase of cytoplasmic free Ca^{2+} results from the interaction of IP_3 with the inositol trisphosphate receptors (InsP3R), promoting the rapid release of Ca^{2+} from the endoplasmic reticulum (ER). In cPKCs, the activation occurs in two steps that begin with the induction of cytosol-to-membrane translocation, through binding of the C2 domain to membrane phospholipids in a Ca^{2+}-dependent manner, allowing the engagement of PKC to plasma membrane (88, 89). Thereafter, PKC binds its membrane-embedded ligand DAG, primarily via C1B domain, an event that prompts the release of the catalytic domain from the PS-binding cavity, enabling the substrate binding and phosphorylation, and the subsequent activation of downstream signaling cascades (Fig. 1.4) (77). Regarding nPKCs, these kinases are able to translocate to membranes in response to agonists, even in the absence of high intracellular Ca^{2+} levels. Actually, it was demonstrated that PKCδ compensates this gap on membrane recruitment since its C1

domain binds membranes with a two-fold higher affinity than the C1 domain of cPKCs (90, 91).

The inactivation of PKC may occur by two distinct processes, namely by the metabolism of DAG (92) and by a process termed as downregulation (93). The reduction in membrane DAG levels is catalyzed by diacylglycerol kinase (DGK), which phosphorylates DAG leading to its conversion into phosphatidic acid (PA). This event stimulates the redistribution of PKC to the cytosol, and the subsequent acquisition of an autoinhibited conformation (Fig. 1.4). On the other hand, when PKCs undergo prolonged activation, due to the exposition to extracellular ligands not readily metabolized like phorbol esters, they lose activity. Indeed, the long-term residency of PKC in an open and membrane-bound conformation leads to its dephosphorylation due to a higher sensitivity of PKC to phosphatases (94). This mechanism is initiated by the okadaic acid-insensitive PH domain Leucine-rich repeat Protein Phosphatase (PHLPP), which catalyzes the dephosphorylation of the hydrophobic motif of cPKCs and nPKCs (95) followed by dephosphorylation of the turn motif and activation loop by okadaic acid-sensitive PP2A phosphatases (96). Upon dephosphorylation, PKC is ubiquitinated and degraded by the proteasome (97).
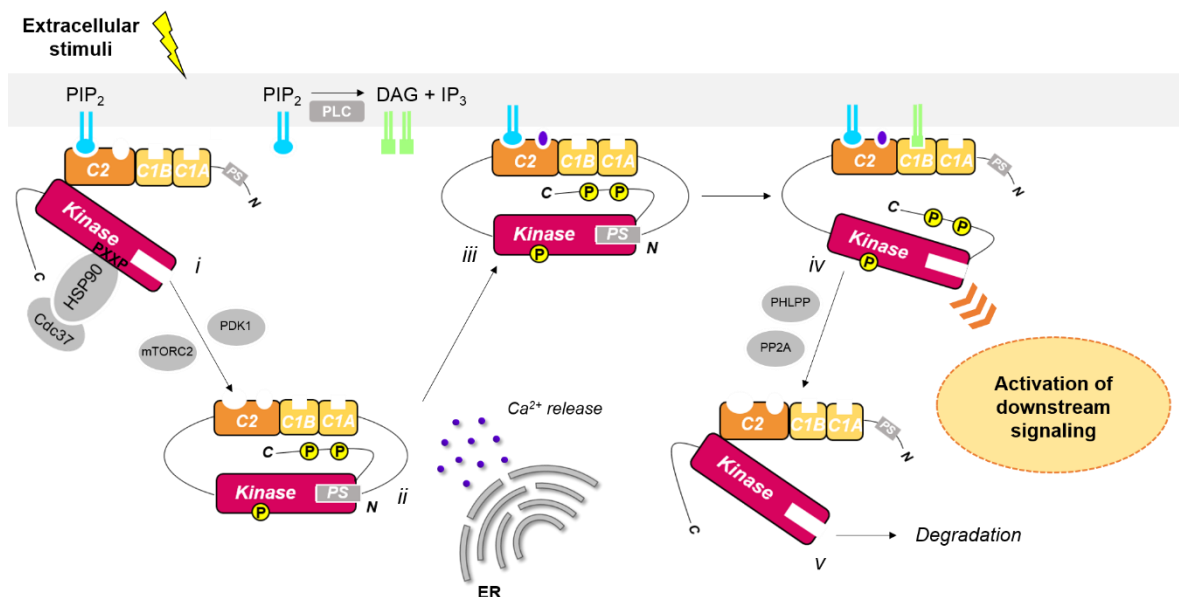
Figure 1.4: Proposed model for the regulation of cPKC isozymes. Following its biosynthesis, PKC exhibits an open and degradation-sensitive conformation, in which its regulatory modules are unmasked (*i*). Then, it is processed by a series of ordered phosphorylations at three priming sites, which leads to the acquisition of an autoinhibited conformation (*ii*). The hydrolysis of PIP_2 results in Ca^{2+}-dependent recruitment of PKC to the plasma membrane via engagement of the C2 domain (*iii*), and subsequently to DAG via C1B domain (*iv*). Once activated, PKC phosphorylates downstream

substrates, which in turn prompts the activation of signaling cascades. The membrane-bound conformation of PKC is sensitive to dephosphorylation, leading to its degradation by the proteosome (*v*).

1.2.2. Biological functions of PKC

The increasing interest in understanding the biology of PKC isozymes led to the development of alternative isozyme-specific tools, including dominant inhibitory kinases, transgenic and knock-out mouse models, antisense/small-interfering (si)RNA, and clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease (CRISPR/CAS9), besides the use of standard PKC modulators (98). The extensive work performed in this area has greatly contributed to our deeper knowledge about the role of PKCs in various cellular responses, including proliferation, survival, apoptosis, cell migration and metabolism (99, 100).

Amongst the numerous cellular processes regulated by PKC, cell death is one of the most studied, underlying the development of many pathologies, such as cancer (101). Particularly, apoptosis, also known as type I programmed cell death, is a genetically controlled mechanism, triggered by intra- or extracellular signaling, that ensures the removal of damaged cells from the organism (102). The apoptotic-signaling cascade is divided into two major pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, both pathways converge on the activation of cysteine aspartate proteases, also called caspases, the key executors of apoptosis (103). The extrinsic pathway is activated by binding of secreted ligands, such as Fas ligand (FasL) to the cell-surface death receptor of the tumor necrosis factor receptor (TNFR) family, Fas (also called CD95/Apo-1). The oligomerization of death receptor is induced upon ligand binding, which in turn leads to the recruitment of the adaptor protein Fas-associated death-domain protein (FADD) and of pro-caspase-8, forming the death-inducing signaling complex (DISC). Consequently, DISC cleaves pro-caspase-8, resulting in the activation of downstream effectors caspase-3 and caspase-7, amplifying the death signal that triggers apoptotic events (102). In contrast, the intrinsic mitochondrial pathway is initiated in response to intracellular stimuli, including oxidative stress, DNA damage, hypoxia or growth-factor deprivation, which prompt the mitochondrial outer membrane permeabilization (MOMP) (104). However, the mitochondrial integrity is tightly regulated by the interaction between pro- and anti-apoptotic proteins of the B cell lymphoma 2 (Bcl-2) family. Thus, under stress conditions, the pro-apoptotic Bcl-2 associated X protein (BAX) and Bcl-2-antagonist/killer (BAK) proteins undergo dimerization, and insert into the

mitochondrial outer membrane (MOM) forming pores that disrupt the membrane´s integrity (105). Following MOMP, cytochrome *c* (cyt *c*) is released into the cytosol, where it binds to the apoptotic protein activating factor-1 (Apaf-1) driving the formation of a caspase activation structure, termed apoptosome (105). The apoptosome recruits and activates the initiator caspase-9, which in turn cleaves the executioner caspase-3 and-7, thereby promoting apoptosis.

The first studies confirming the involvement of PKC isozymes in the regulation of apoptosis used phorbol esters as PKC activators (106, 107). Despite the attempt to clarify the role of specific PKC isozymes in the apoptotic process by several studies, this has proved to be a challenging task, since their impact on apoptosis showed to be highly dependent on cellular background and stimuli applied (108). In fact, distinct stimuli dictate the PKC translocation to distinct subcellular compartments, determining the phosphorylation of different substrates, and subsequently different cellular responses (108). PKCδ has been one of the most studied PKC isozymes, being intimately implicated in the apoptotic response to DNA damage and cytotoxic stress (99, 109-111). Actually, both *in vitro* and *in vivo* studies sustained the pro-apoptotic role of PKCδ. For instance, it was shown that cells isolated from PKCδ$^{-/-}$ mice were resistant to apoptosis, and the irradiation-induced damage was lower in PKCδ$^{-/-}$ mice (112, 113). Importantly, upon DNA damage, PKCδ is activated through phosphorylation on specific tyrosine residues, inducing its nuclear accumulation, which is considered a critical event for the execution of apoptosis by PKCδ in response to genotoxic stress (114). In the nucleus, activated caspase-3 proteolytically cleaves PKCδ in the hinge region, resulting in the release of a constitutively active PKCδ catalytic fragment (PKCδ-CF), recognized as a potent apoptotic inducer (115, 116). Accordingly, cumulating evidence have indicated that, during the apoptotic process, the majority of PKCδ substrates are nuclear proteins. Particularly, it was reported that PKCδ phosphorylates the DNA-dependent protein kinase catalytic subunit (DNA-PK), stimulating DNA-damage induced apoptosis (117). However, also mitochondria is described as a translocation site of PKCδ in response to oxidative stress, ischemia and phorbol esters (109, 118, 119). At mitochondria, it is suggested that PKCδ directly interferes with the regulation of the apoptotic machinery, namely through the induction of post-translational modifications that control the activity of Bcl-2 family proteins. For instance, it was demonstrated that PKCδ triggers apoptosis by suppressing the phosphorylation of the pro-apoptotic protein, Bcl-2-associated death promoter (BAD), or by stimulating BAX and BAK activity (119, 120). The regulation of p53 activity by PKCδ , both at transcriptional and post-translational levels, has also been suggested (121, 122). Nonetheless, despite numerous studies have corroborated

its pro-apoptotic activity, there are also reports describing a pro-survival function of PKCδ, which is generally preceded by its translocation to the ER (111).

Despite the great advances in the knowledge of PKC cellular functions, the full understanding of the role of individual PKC isozymes in human diseases, including cancer, is still far from being achieved.

1.2.2.1. *Dual role of PKC isozymes in cancer*

Phorbol esters, a family of natural occurring diterpenoids isolated from the seed oil of the plant *Croton tiglium* of the *Euphorbiaceae* family (123), established the first connection between PKC and cancer. The carcinogenic activity of the croton oil was first described in the early 40´s, based on the development of papillomas in mouse skin after exposure to a single application of a sub-effective dose of a carcinogen followed by chronical application of phorbol 12-myristate 13-acetate (PMA) (124, 125). Later, it was reported that phorbol esters bind with high affinity to PKC, competing with the physiological ligand DAG for the same binding site, with subsequent PKC activation (54, 56, 126). Particularly, like with DAG, phorbol esters retain the C1 domain of PKC on membranes through a contiguous hydrophobic surface, which is assembled by their insertion into the hydrophilic cleft on C1 domain (127, 128). Collectively, these findings allowed to recognize PKC as a major kinase in tumorigenesis, and contributed to the dogma that PKC acts as an oncoprotein (57). However, besides the induction of an acute activation of PKC, phorbol esters also trigger a PKC downregulation process. As such, currently, it is still controversial if the tumor-promoting properties of phorbol esters are due to PKC activation or to its loss by downregulation. Nevertheless, phorbol esters have represented crucial tools in PKC research field, greatly contributing to the elucidation of PKC cellular functions in cancer.

PKCα has been frequently associated with increased proliferation and survival, being commonly recognized as tumor promoter (129-132). Yet, several evidence have indicated that PKCα has tissue-specific functions, acting either as tumor promoter or tumor repressor depending on cell type. Consistently, it is known that the levels of PKCα differ with the type of cancer, being overexpressed in bladder, endometrial and breast cancers, and downregulated in colorectal and malignant renal cell carcinomas (68). Accordingly with its pro-survival role, the overexpression of PKCα in U87 glioblastoma cells stimulated proliferation, and increased the resistance to apoptosis in response to radiation and chemotherapy (129, 133, 134). Moreover, the ectopic expression of PKCα in MCF-7 breast cancer cells increased proliferation, anchorage-independent growth, and tumorigenic potential in nude mice (135). Nevertheless, in LNCaP prostate cells, PKCα behaved as

tumor suppressor, inducing apoptosis upon treatment with PMA and radiation, an effect abrogated by the expression of a PKCα kinase-dead mutant (136). Additionally, activation of PKCα in non-small cell lung cancer (NSCLC) inhibited cell growth and senescence with p21 upregulation (137). PKCα was also implicated in the regulation of invasion and metastasis. Actually, in breast cancer cells, cell invasion was promoted by ErbB2-dependent activation of PKCα (138). Additionally, the overexpression of PKCα in MCF-7 cells increased motility, which was associated with a decrease in the expression levels of E-cadherin and β-catenin, and an increase of matrix metalloprotease-2 and -9 (MMP-2 and -9) (139, 140). Consistently, the number of metastasis in lung was significantly reduced by the treatment with a PKCα antagonist peptide (141).

Regarding PKCδ, compelling evidence have shown that it mostly acts as an anti-proliferative and pro-apoptotic kinase. Accordingly, the overexpression of PKCδ in ovary (CHO) cells induced a G2/M-phase arrest, with inhibition of cell division upon PMA treatment (142). PKCδ also caused a G1-phase arrest, with upregulation of p21, in lung adenocarcinoma cell lines (143). In addition, PKCδ mediated apoptosis in response to genotoxic agents in C5 rat parotid salivary acinar cells, with translocation from cytosol to nucleus, through proteolytic activation by caspase-3 cleavage (114). Of note that, PKCδ-mediated apoptosis may only require an allosteric activation, without the generation of the PKCδ-CF, as demonstrated in androgen-dependent prostate cancer cells (144). In an additional work, it was shown that the tumor promoting effect of phorbol esters was abrogated by the transgenic overexpression of PKCδ in mouse skin (145). Moreover, clinical data have revealed reduced protein levels of PKCδ in colon cancer tissues compared to normal ones (146). Consistently, overexpression of PKCδ in Caco-2 colorectal adenocarcinoma cells inhibited anchorage-dependent and independent growth, increasing apoptosis and inducing differentiation (146). Similarly, its overexpression also inhibited the transformed phenotype of *src*-overexpressing rat colonic epithelial cells (147). In fact, several studies are consistent with a tumor-suppressive function of PKCδ in colon cancer (146-150). On the other hand, it was reported that the ectopic expression of PKCδ promoted anchorage-independent growth and developed resistance to apoptotic stimuli in mammary cells (151). Also in PC-3 human prostate xenograft model, the activation of PKCδ stimulated tumor growth and angiogenesis (152). Concerning to migration and invasiveness, there are many contradictory results. For example, it was shown that overexpression of PKCδ in highly motile BT-549 breast cancer cells reduced migration, while its downregulation enhanced motility and MMP-9 secretion in MCF-7 cells (153). Conversely, downregulation of PKCδ in murine MTLn3 mammary breast cancer model suppressed the metastatic

process to lung (154). More recently, it was reported that PKCδ phosphorylates BAG3, which in turn regulates epithelial-mesenchymal transition (EMT) in thyroid cancer cells (155).

Conversely, PKCε has been widely recognized by its oncogenic properties (156-158). In fact, in mouse NIH3T3 embryonic fibroblasts, the overexpression of PKCε induced a transforming phenotype, increasing cell proliferation and anchorage-independent growth (156). Additionally, PKCε-overexpressing NIH3T3 cells were able to growth and form solid tumors into nude mice, an effect not observed with non-transfected cells (156). Interestingly, some reports have established a correlation between the proliferative and pro-survival role of PKCε and its ability to regulate caspases and Bcl-2 family proteins. In particular, it was shown that PKCε inhibited apoptosis in breast cancer cells by preventing BAX activation through inhibition of its translocation to mitochondria (159). Several works have also demonstrated that overexpression of PKCε induced tumor cell invasion and metastasis in different cell models, such as human breast, glioma and renal cell carcinoma (160-162). On the other hand, inoculation of PKCε-depleted cells into nude mice resulted in tumor growth inhibition and decreased incidence of lung metastases (160). Also corroborating its oncogenic role, overexpression of PKCε has been observed in numerous cancers, including in majority of primary tumors from invasive ductal breast cancer and NSCLC patients (160, 163). A correlation between increased PKCε levels and enhanced cancer recurrence, as well as decreased overall survival, was also found in head and neck squamous cell carcinoma (HNSCC) patients (164).

Concerning to atypical PKCs, it is interesting to note that while PKCι is overexpressed in the majority of cancers, PKCζ can be either up- or downregulated in different human cancers (68). Despite conflicting results, it is widely accepted that PKCζ mainly exerts tumor suppressor functions, whereas PKCι promotes tumor growth (68). Actually, a pro-apoptotic role of PKCζ has been described in various cancer models, including colon (149) and ovarian cancer (165). Additionally, PKCζ-deficient mice exhibited increased Ras-induced lung carcinogenesis, arguing for a tumor suppressor function of PKCζ. Interestingly, although little is known about the involvement of PKCs in metabolism, PKCζ was also reported as a critical regulator of tumor metabolic pathways. Particularly, under starvation conditions, PKCζ-deficient cancer cells are able to reprogram their metabolism, to consume glutamine through the serine biosynthetic pathway, in order to supply the energetic cellular needs, which further reinforces the a tumor suppressor function of PKCζ (166). However, there are also some reports highlighting a pro-survival role of PKCζ (167, 168). Regarding PKCι, it was shown that it is an essential mediator of oncogenic K-Ras, being essential for

34

the maintenance of the tumor-initiating properties (169, 170). Moreover, in ovarian cancer, disruption of PKCι inhibited the clonal expansion, anchorage-independent growth, and tumorigenic potential of tumor initiating cells (171, 172).

1.2.3. PKC mutation in cancer

To date, in diverse cancer types, over 1,000 cancer-associated somatic mutations were identified in the structural domains of PKC isozymes (173). The results of the analysis of approximately 50 of these mutations showed that about two-thirds of them led to inactivation/loss-of-function (LOF), without the identification of any gain-of-function (GOF) mutation (100). Additionally, it is possible to predict the existence of many other LOF mutations in PKC family members localized in highly conserved motifs required for catalysis (174). These inactivating mutations may interfere with crucial processes involved in PKC activation, namely by preventing their processing phosphorylation and the binding of second messengers, or even their catalytic activity (77). Indeed, it was reported that mutant PKC can impair the processing phosphorylation of PKC isozymes through inhibition of effectors of this mechanism, thus avoiding the accumulation of other PKC isozymes (175). They can also disrupt normal PKC signaling, redirecting PKC to other substrates, and potentially activating other signaling pathways involved in tumor development (176).

The majority of PKC LOF mutations are heterozygous, acting in a dominant negative manner with respect to the wild-type (wt) counterpart, and even to other PKC isozymes, which contributes to their malignancy (77). Consistently, the correction of the mutant allele (A509T) of *PRKCB* to wt by genome editing in DLD1 colon cancer cells, not only led to the suppression of anchorage-independent growth, but also reduced the tumor growth in xenograft models (100). Contrary, its deletion only partially decreased anchorage-independent growth, revealing that PKCβ is haploinsufficient with respect to this outcome (100).

Accumulated data have also supported that PKC prevents oncogenesis, through inhibitory phosphorylation of oncoproteins. Particularly, phosphorylation of epidermal growth factor receptor (EGFR), which is one of the main PKC substrates, on threonine (Thr)654 leads to the reduction of EGFR tyrosine kinase activity, decreasing ligand binding capacity, and promoting its internalization (177-180). Besides transmembrane receptors, other PKC substrates were also identified, including K-Ras oncogene. Actually, it was recently reported that PKC phosphorylates K-Ras on serine (Ser)181, preventing tumor growth in mouse models by inhibiting its interaction with calmodulin, an effect promoted by the PKC activator, prostratin (181). Therefore, it is expected that LOF PKC mutations

stimulate tumor cell growth and survival by abolishing the inhibitory activity of PKC in these signaling pathways.

As a whole, based on the evidence that PKC activity is often compromised in cancer due to LOF mutations, it is currently claimed that PKC isozymes function as tumor suppressors rather than tumor promoters.

1.2.4. Targeting PKC activity

As promising targets in cancer, amazing efforts have been made in an attempt to develop PKC isozymes-targeting agents for anticancer therapy. However, this has proved to be a challenging task, mainly due to the high degree of sequence homology and structural resemble among PKC isozymes. This is particularly evident for the catalytic domain, since the similarity extends to other kinases, (76). The complexity of this targeting process is increased by the co-expression of multiple PKC isozymes in cells, some of which exhibiting antagonistic functions (182). Therefore, for long, the modulation of PKC isozymes has been associated with off-target effects, and subsequently with unwanted toxic side effects.

In general, approaches to target PKC can be classified according to their specific site of action. Agents that interfere with the PKC catalytic activity can competitively bind either to the ATP- or substrate-binding site. The most common and potent kinase inhibitors are the ATP-competitive small-molecules, although in most cases they have little or no selectivity for PKC isozymes. Among these, staurosporine, a natural indolocarbazole produced by *Streptomyces staurosporeus*, inhibits all PKC isozymes, as well as other kinases, including PDK1, with high binding affinity (183-186). The synthesis of semi-synthetic derivatives of staurosporine led to the identification of *N*-benzoyl-staurosporine, also known as midostaurin or PKC412, as a potent inhibitor of all PKCs (187, 188). This compound has been tested in clinical trials of patients with different cancers (https://clinicaltrials.gov/ct2/results?term=Midostaurin), either as monotherapy or in combination therapy, and recent data revealed improved results in terms of patients survival (189, 190). Enzastaurin (also known as LY317615) is another synthetic compound initially reported as selective for PKCβ (191). However, later studies demonstrated that it also targets other PKC isozymes, as well as other kinases. Despite the promising results obtained in phase I and II clinical trials (https://clinicaltrials.gov/ct2/results?term=LY317615), the use of enzastaurin hydrochloride alone in patients with diffuse large B-cell lymphoma (DLBCL) did not improve patient survival (192). Currently, enzastaurin is under clinical trials as monotherapy and in combination with conventional chemotherapies for pediatric brain tumors and malignant

36

gliomas (193, 194). Rottlerin (also termed mallotoxin), a natural product isolated from the Asian tree *Mallotus philippensis*, was originally described as PKCδ-selective inhibitor (195). Nevertheless, the selectivity of rottlerin for PKCδ has also been questioned by several studies. In fact, it has been demonstrated that rottlerin could also inhibit multiple kinases, including a potassium channel opener regulator of mitochondrial membrane potential (MMP) (196). Interestingly, using computational pharmacophore modeling, novel PKCδ-selective inhibitors were developed, particularly the chimeric hybrid BJE106, which targets PKCδ with high selectivity (IC_{50} of 50 nM), inhibiting the growth of NRAS-mutant melanoma cells and inducing cell death in BRAF-mutant melanomas (197).

Bryostatin-1 is a macrocyclic lactone isolated from the marine bryozoan *Bugula neritina* (198), which binds with high affinity to the C1 domain. However, in contrast to phorbol esters, it has low pro-tumorigenic activity, antagonizing the tumor-promoting effects of phorbol esters (199, 200). Additionally, bryostatin-1 inhibits tumor growth both *in vitro* and *in vivo*. Based on these results, bryostatin-1 was tested in several clinical trials (https://clinicaltrials.gov/ct2/results?term=bryostatin), and despite the promising anticancer activity obtained in phase I, the results of phase II trials as monotherapy were disappointing (201-203). However, in combination therapy with other cytotoxic agents, it has shown improved efficacy, particularly in advanced esophageal, gastric, and gastroesophageal cancers (203-205), and in B-cell non-Hodgkin lymphoma (205). Yet, the complexity of its synthesis have made unfeasible the large-scale production of bryostatin-1, restricting its use in other studies. As such, several efforts have been made to synthesize structural-related analogs with similar activity for clinical use (206). Picolog is an example of a synthetic bryostatin analogue with picomolar affinity for PKC, which induces apoptosis in mantle lymphoma cells with inhibition of tumor growth in xenograft mouse models (207, 208). However, Picolog and other bryostatin derivatives, such as Merle 23 and neristatin-1, have still not been tested in patients. Moreover, following the rational design of C1 domain ligands, a set of five-membered ring DAG lactones were generated based on the 3D structure of the PKCδ C1B domain in complex with PMA. These novel synthetic DAG analogues bind to PKC C1 domain with an affinity three- to four-fold higher than DAG. Among these compounds, HK434 and HK654 induced apoptosis in human prostate LNCaP cancer cells (209). However, the high degree of identity in the C1 domain of cPKC and nPKC, particularly in the DAG binding motif, continue to be the major obstacle to the development of isozyme-selective compounds.

Ingenols are another group of C1 domain ligands with clinical application, which bind to PKC isozymes with similar *in vitro* affinity, but differentially activate PKCs in distinct cell models (210). Particularly, ingenol mebutate (Picato^R) is in the market since 2012 for the

treatment of actinic keratosis, and seems to be effective for the treatment of basal cell carcinomas (211).

Another strategy to target specific PKC isozymes has been focused on intra- and intermolecular interactions established by PKCs. Actually, the C2 domain (in cPKCs and nPKCs) and the PB1 domain (in aPKCs) are involved in the recruitment of activated PKC isozymes to distinct subcellular compartments, through interaction with unique isozyme-specific anchoring proteins (83). As such, peptides have been generated to selectively inhibit PKC isozymes by preventing the PKC interaction with anchoring proteins, as RACKs (212). In fact, several peptides derived from different regions of PKC, as C2 domain, have been developed, which can prevent scaffolding interactions with subsequent inhibition of PKC activity (213, 214). Although these peptides have been initially designed to elucidate the cellular function of these protein-protein interactions, they quickly reached therapeutic relevance. Actually, the εV1-2 peptide, which targets PKCε, showed anticancer activity in xenograft models of NSCLC and impaired lung cancer cell motility signaling (215, 216). Beyond the peptides, the small-molecule PKC inhibitor aurothiomalate (ATM) was also identified as disruptor of the PB1-PB1 domain-mediated binding of PKCι to Par6, inhibiting the transformed growth of NSCLC models (217, 218).

The antisense therapy, based on the inhibition of expression of PKC isozymes using oligonucleotides with complementary sequences to mRNA of genes of interest, has been also used. Aprinocarsen (ISIS-3521) is a 20-base antisense oligonucleotide that induces PKCα downregulation, markedly reduces cancer cell proliferation and invasion, and inhibits xenograft tumor growth (219, 220). Although it has been tested in clinical trials for patients with prostate, breast, ovarian, colon, lung, glioblastoma, melanoma, and other cancers (221), the results obtained were not encouraging.

The PKC modulators under clinical trials as anticancer agents, and their clinical status, are presented in Table 1.1.

Table 1.1: PKC modulators under clinical trials as anticancer agents.

Agents	Clinical status	References
ATP*-competitive *inhibitors		
UCN-01 *Non-selective*	Phase II in metastatic TNBC (NCT00031681)	(222)
	Phase II in metastatic melanoma (NCT00072189)	(223)
	Phase II in ovarian cancer (NCT00072267)	(224)
Midostaurin (PKC412) *Non-selective*	Phase III in AML (NCT03379727)	(225)
	Phase IIa in metastatic melanoma	(226)
	Phase I in NSCLC	(188)
Sotrastaurin (AEB071) *Selectivity for cPKCs and nPKCs*	Phase II in CLL (NCT02285244)	(227)
Enzastaurin (LY317615) *Some selectivity for PKCβ*	Phase III in diffuse large B-cell lymphoma (NCT03263026)	(192)
	Phase II in pancreatic cancer (NCT00267020)	(228)
	Phase II in lung cancer (NCT00414960)	(229)
	Phase I in pediatric brain tumor (NCT00503724)	(193)
	Phase II in recurrent malignant gliomas (NCT00586508)	(194)
C1 domain ligands		
Bryostatin-1 *Selectivity for cPKCs and nPKCs*	Phase II in ovarian cancer (NCT00006942)	(230)
	Phase II in pancreatic cancer (NCT00031694)	(231)
	Phase II in esophageal cancer (NCT00005599)	(204)
	Phase II in renal cell carcinoma (NCT00003968)	(232)
Ingenol mebutate (PEP005) *Non-selective*	Phase IIa, IIb and III in actinic keratosis (NCT0010796; NCT00742391; NCT00916006; NCT00915551; NCT00942604; NCT01541553)	(233-236)
	Phase I/II in Non-melanoma skin cancer	(237)
Inhibitors of the protein-protein interactions		
Aurothiomalate (ATM) *Selectivity for PKCι*	Phase I in NSCLC (NCT00575393)	(238)
	Phase I in ovarian cancer	(238)
	Phase I in pancreatic cancer	(238)
Antisense oligonucleotides inhibitors		
Aprinocarsen (ISIS-3521) *Selectivity for PKCα*	Phase II in ovarian cancer	(239)
	Phase II in high-grade gliomas	(240)
	Phase II and III in NSCLC (NCT00042679; NCT00034268)	(241-243)
	Phase II in Non-Hodgkin's lymphoma	(244)
	Phase II in colorectal cancer	(245, 246)

TNBC, triple negative breast cancer; *AML*, acute myeloide leukemia; *NSCLC,* non-small cell lung cancer; CLL chronic lymphocytic leukemia.

1.2.5. Screening strategies

1.2.5.1. *Genetically encoded fluorescent reporters*

Traditionally, the most used strategy to demonstrate the phosphorylation of residues that activate protein kinases has been the immunoblotting, using phospho-specific antibodies (247). Nonetheless, this can be an ineffective measure of prior cell activation since PKCs are constitutively phosphorylated during the maturation process. To overcome this limitation, the analysis of PKC translocation to membranes, by immunoblotting or immunofluorescence using PKC-specific antibodies, became the most reliable activation measure, as a result of allosteric alterations (248-250). Additionally, the cellular activity of PKC has also been studied by immunoblotting using phospho-specific antibodies against PKC substrates. However, although *in vitro* kinase activity assays are still the basis for the analysis of PKC activity, they do not provide a complete spatiotemporal picture of all signaling events (251). In turn, the use of fluorescently-tagged PKC in live cells has allowed to monitor membrane translocation as a readout of activation. Actually, this approach has been used to study the translocation kinetics of PKC over time in response to natural agonists (252, 253), or phorbol esters (254). On the other hand, a live-cell imaging system with genetically encoded biosensors based on Förster/fluorescence resonance energy transfer (FRET) has also been extensively used. Particularly, this technique allows to visualize dynamic conformational changes of macro-biomolecules (255-257), the binding between two molecules (258, 259), and it is also applied to the high-throughput screening (260, 261). Regarding PKC, C-kinase activity reporter (CKAR) is the most used tool to directly measure PKC activity, and employs the modular architecture of activity reporter originally developed for tyrosine kinases (262) and protein kinase A (PKA) (263). Briefly, once activated, PKC phosphorylates the substrate, resulting in a conformational change in the reporter that produces a FRET change, thus enabling the readout of PKC activity in real time. Besides the preservation of the physiological cellular context and spatiotemporal information, FRET reporters also demonstrated great sensitivity for low affinity or transient interactions.

1.2.5.2. *Yeast-based assays*

In 1993, a yeast model system was established by Riedel and colleagues to separately analyse the function of each PKC isozyme (264). Particularly, they demonstrated that the expression of mammalian cPKCα and βI in yeast induced biological responses similar to those obtained in mammalian cells. In fact, the activation of these isozymes by phorbol esters triggered a specific phenotype characterized by a delay in yeast growth,

which was proportional to the degree of enzymatic activation (Fig. 1.5) (264, 265). On the other hand, PKC inhibitors, such as chelerythrine, reverted the growth inhibitory effect of PKC activators (266). Based on this, the yeast PKC phenotypic assay became a valuable tool to screen for PKC isozyme-specific modulators (267). It was also used to establish a PKC structure-activity relationship, namely using PKCα mutants lacking certain amino acid sequences within the regulatory and catalytic domains (265). Additionally, using a PKCαβI chimera, in which the *C*-terminal of PKCα was replaced by the corresponding PKCβI sequence, it was demonstrated that these two isozymes were complementary, exhibiting a similar phenotype (268). Furthermore, with deletions in PKCα, it was identified a novel regulatory segment in C2 domain that retains the enzyme in an inactive conformation and simultaneously regulates the PKC catalytic activity (269). This finding led other authors to identify other regions in the regulatory domain that, like PS segment, were also implicated in the PKCα auto-inhibition (270). In fact, with these studies, it was demonstrated the existence of intramolecular interactions between the regulatory and catalytic regions of PKCα, which contribute to the maintenance of the enzyme in an inactive conformation. Altogether, these works validated yeast as a promising cell system to study individual mammalian PKCs, in a simplified eukaryotic cellular environment without the interference of other isozymes.

In particular, the yeast PKC phenotypic assay was extensively used in the characterization of the potency and isozyme-selectivity of well-known PKC activators and inhibitors. Notably, it has also contributed to the discovery of new PKC isozyme-selective activators (271, 272) and inhibitors (273, 274) from the screening of a chemical library of xanthones. A new selective activator of nPKCδ and ε, the diterpene compound 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U), with potent antiproliferative effects against several human tumors cells was also discovered using the yeast approach (275). In that work, it was verified that while PMA activated nPKCs, inducing their translocation from the cytosol to the plasma membrane and a G2/M-phase cell cycle arrest, coleon U induced the translocation of nPKCs to the nucleus and a metacaspase- and mitochondria-dependent apoptosis. Interestingly, these results were in accordance with those described for mammalian cells (276), confirming that also in yeast distinct stimuli prompted the translocation of PKCs to different cellular compartments with the subsequent induction of distinct cellular responses.

Collectively, with these works, it was shown that several functional features of mammalian PKC isozymes are conserved in yeast. Most importantly, yeast proved to be a valuable and reliable drug screening tool, providing relevant clues about the molecular mechanism of action of several drugs. Moreover, due to its easy manipulation and reduced

doubling time, yeast allows simple and fast screenings well-adaptable to HTS methodologies in a cost-effective manner. As a whole, the yeast-cell model revealed to be useful as a first-line targeted screening strategy for a preliminary selection of compounds to be tested in more complex cell systems (277, 278).
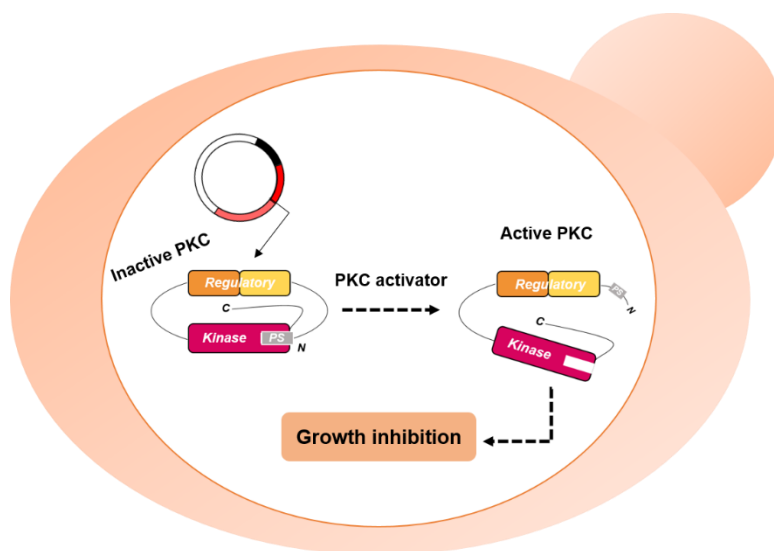
Figure 1.5: Yeast PKC phenotypic assay developed to screen PKC isozyme-selective activators. This system is based on the heterologous expression of individual mammalian PKC isozymes, and on the subsequent evaluation of the impact of small-molecules on yeast cell growth. The activation of mammalian PKC induces yeast growth inhibition, not observed in control yeast (yeast transformed with the empty vector), which is proportional to the degree of PKC activation.

1.3. Natural products

Over the years, compounds derived from natural sources such as plants, have been the most profitable source of new therapeutic agents. Although the pharmaceutical industry has tried to redirect its focus to the production of synthetic compounds, this strategy was not well-succeeded, with an evident decrease of the new drugs that reached the market (279). Indeed, recent data showed that from 1,135 new drugs approved by FDA, from 1981 to 2010, over 50% were from natural sources (280). This led the research groups to reconsider the natural products as a major source of promising therapeutic agents. Natural compounds from plants are derived from the secondary metabolism, and are themselves, in the unmodified state, a source of new bioactive agents. In addition, they can be used as

scaffolds for the development of semi-synthetic analogues with improved pharmacological properties. The selection of species for the isolation of bioactive compounds has been supported by references from traditional medicine and by chemotaxonomic studies (281). Particularly, chemotaxonomic studies with the Lamiaceae family led to an increasing interest for *Plectranthus* L'Her genus, which comprises more than 300 species mainly distributed by Africa, Asia and Australia (282). Actually, these species have been used for many years in traditional medicine, and are recognized by the wide range of biological activities of their extracts or isolated compounds, including anti-infectious, antioxidant, anti-inflammatory and anticancer (283-286). The *Plectranthus* genus is chemotaxonomically characterized by the presence of caffeic acid esters, rosmarinic acid and nepetoidins A and B (287). However, the most commonly studied compounds from this genus are diterpenoids, a structurally diverse class of compounds consisting of four isoprene units forming a 20-carbon backbone (288). Particularly, abietanes such as royleanones, which have been widely associated with antimicrobial, antimycobacterial and anticancer activities, are among the most frequently reported diterpenic structures (283, 284, 286, 289).

Concerning to anticancer properties, over half of the compounds with this biological activity are natural products or their derivatives (290). Several studies have validated the cytotoxic potential of both extracts and isolated compounds from *Plectranthus* genus against cancer cells. This is the case of the nPKCδ and ϵ selective activator coleon U, and of 7α-acetoxy-6β-hydroxyroyleanone, obtained from *P. grandidentatus* (283), and their semi-synthetic derivatives (283, 284). These works have reinforced the potential of abietane diterpenes, as promising anticancer drug candidates particularly by targeting PKC.

1.4. Scope of the thesis

Compelling data have established PKC isozymes as major regulators of tumor growth and dissemination. However, we are still far from a complete understanding of the function of each PKC isozyme in cancer (76). The lack of isozyme-selective modulators has hampered the achievement of such a goal, as well as the translation of PKC modulators into the clinic. Therefore, for long, it became imperative the development of PKC isozyme-selective targeting agents. These compounds would greatly contribute to the advance of the biology and pharmacology of PKCs and would have promising applications in anticancer therapy. With the present thesis, we aimed to identify:

i) PKC isozyme-selective agents;
ii) New anticancer drug candidates by targeting PKC.

To this end, it was screened a small chemical library of royleanone derivatives from the *Plectranthus* genus. The drug discovery strategy used, in this thesis, encompassed the identification of potential PKC isozyme-selective agents using a yeast-based PKC assay, followed by validation of their molecular mechanism of action and anticancer activity in human cancer cells and animal models (Chapter 3.1 and 3.2). Additionally, other species of *Plectranthus* genus were studied as potential sources of new anticancer agents (Chapter 3.3).

The major outcomes achieved in this thesis, particularly its contribution to anticancer drug development by targeting PKC, are discussed in Chapter 4.

CHAPTER 2

Material and Methods

2.1. Compounds

PMA and arachidonic acid (ARA) were purchased from Enzo Life Science (Grupo Taper SA, Sintra, Portugal); etoposide (ETOP) from Calbiochem (VWR, Carnaxide, Portugal); hydroxyurea and cyclophosphamide (CP) from Sigma-Aldrich (Sintra, Portugal). All natural and semi-synthetic compounds, as well as plant extracts were provided by Prof Doutora Patrícia Rijo (CBIOS, Universidade Lusófona, Lisboa, Portugal). Natural diterpene 7α-acetoxy-6β–hydroxyroyleanone (Roy) was extracted from *Plectranthus grandidentatus* as described (286). Subsequently, the semi-synthetic derivatives 7α-acetoxy-6β-propionyloxy-12-*O*-propionylroyleanone (Roy-Pr2) and 7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone (Roy-Bz) were synthesized following procedures formerly described (289). Natural diterpene 6,7-dehydroroyleanone (DeRoy) was isolated from essential oil of *P. madagascariensis* plant as described (291). Their synthetic derivates, 6,7-dehydro-12-*O*-pro-2-yn-1-yloxy-royleanone (FRP19) and 6,7-dehydro-12-*O*-($1R$,$2S$,$5R$)-2-isopropyl-5-methylcyclohexyl)oxy)-royleanone (FRP20), were prepared according to procedures described (292). The extracts were prepared from sixteen different *Plectranthus* spp. plants cultivated in Instituto Superior de Agronomia do Campus da Universidade de Lisboa, Portugal. Briefly, the extractions were prepared with the organic solvent, acetone, in a concentration of approximately 10% (w/v), according to the procedure described in (293). All tested compounds and extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), except hydroxyurea and CP that were dissolved in water.

2.2. Construction of pESC-(*LEU2*)-PKCδΔC1 plasmid

The wt *PRKCD* gene was amplified from the original clone provided by Dr. Nigel Goode (The Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK) using the gene specific primers Xho1 PKCδ forward (F) (5'-CCGCTCGAGATGGCCCCGTTCCTGCGCATC-3') and NheI PKCδ reverse (R) (5'-CTAGCTAGCCTATTCCAGGAATTGCTC-3'). After verifying the fragment by sequencing, it was cloned into the XhoI /NheI sites of pESC-(*LEU2*) plasmid (Agilent Technologies, Chandigarh, India) under control of the *GAL1* promoter. According to manufacturer's instructions, open reading frame (ORF) cloned in this vector will be expressed as a tagged protein with an *N*-terminal Myc-tag. Positive clones (pESC-(*LEU2*)-PKCδ) were further confirmed by sequencing and maintained in *Escherichia coli* strain NovaBlue cells. The deletion of C1 domain in the wt *PRKCD* gene was carried out by standard protocol of overlap primer extension using polymerase chain reaction (PCR) as described (294), and

with the help of the outer gene specific primers Xho1 PKCδ F and NheI PKCδ R, and the inner primers PKCδ C1 del F (5'-AACATCGACATGCCTCAAAAGCTCTTAGCTG-3') and PKCδ C1 del R (5'-CAGCTAAGAGCTTTTGAGGCATGTCGATGTT-3'). First, the region upstream and downstream to the C1 domain was amplified using outer and inner primer pair followed by overlapping PCR to obtain the final product lacking C1 domain. The final product was digested with XhoI and NheI enzymes and ligated with similarly digested pESC-(*LEU2*). Positive clones were sequenced in their entirety to confirm the desired modification at the corresponding position and maintained in *E. coli* NovaBlue cells.

2.3. Yeast transformation

The pESC-(*LEU2*) plasmid (empty vector) and the plasmid obtained in 2.2 (pESC-(*LEU2*)-PKCδΔC1) used in the yeast transformation process were firstly amplified in *E. coli* DH5α strain (Lucigen, Frilabo, Porto, Portugal), and thereafter extracted using the *GenEluteTM HP Plasmid Miniprep Kit* (Sigma-Aldrich). After extraction, *Saccharomyces cerevisiae* CG379 strain was transformed using the lithium acetate/single-stranded carrier/polyethylene glycol (DNA LiAc/SS Carrier DNA/PEG) method as described (295). For selection of transformed yeast, cells were routinely grown in selective minimal medium with 2% (w/w) glucose (Sigma-Aldrich), 0.7% (w/w) yeast nitrogen base (YNB) without amino acids (a.a.) (Difco, Quilaban, Sintra, Portugal), and all the a.a. required for yeast growth (50 µg/mL), except leucine, and incubated at 30ºC, under continuous orbital shaking (200 rpm).

2.4. Yeast-based screening assay

Yeast cells expressing PKCα, βI, δ, ε, ζ and control yeast (transformed with empty vector) obtained in previous works (275), and PKCδΔC1 obtained in 2.3, were used. The expression of PKCδΔC1 in yeast was previously confirmed by immnubotting (see Appendix; Fig. 1). For expression of mammalian proteins, cells (routinely grown in minimal selective medium) were diluted to an optical density (OD) at 600 nm (OD$_{600}$) of 0.05 in induction selective medium containing 2% (w/w) galactose (Sigma-Aldrich), 1% (w/w) raffinose (Acros Organics, VWR), 0.7% (w/w) YNB without a.a., and a mixture of all the a.a. required for yeast growth (50 µg/mL), except leucine. Yeast cells were incubated at 30ºC under continuous orbital shaking (200 rpm), in the presence of 0.1 - 30 µM of compounds (or 0.1% DMSO) only for approximately 42 h (time required by control yeast, incubated with DMSO

only, to achieve 0.4 OD$_{600}$). Yeast growth was analyzed by counting the number of colony-forming units (CFU), after 2 days incubation at 30ºC, on Sabouraud Dextrose Agar (Liofilchem, Frilabo).

2.5. Yeast cell cycle analysis

Yeast cell cycle progression was analyzed basically as described (275). Briefly, about 1×10^7 cells (incubated in galactose selective medium with 10 μM PMA, 10 μM Roy-Bz (or DMSO only) for 42 h) were fixed in 70% (v/v) ethanol overnight at 4ºC, followed by incubation with 250 μg/mL RNase A (DNase-free; Sigma-Aldrich) for 3 h at 50ºC, and thereafter with 1 mg/mL Proteinase K (Sigma-Aldrich) for 3 h at 37ºC. Afterwards, cells were stained with 10 μM Sytox Green Nucleic Acid (Invitrogen, Alfagene, Carcavelos, Portugal) overnight at 4ºC, followed by flow cytometry analysis. Fluorescence from at least 30,000 cells was analyzed.

2.6. Analysis of yeast plasma membrane integrity and DNA fragmentation

Propidium iodide (PI) and TUNEL staining, to monitor plasma membrane integrity and DNA fragmentation respectively, were carried out as described (275). Briefly, about 1×10^7 cells (incubated in galactose selective medium with 10 μM PMA, 10 μM Roy-Bz, or DMSO only, for 42 h) were collected and incubated with 5 μg/mL PI (Sigma-Aldrich) for 10 min at room temperature. TUNEL was performed using the *In Situ* Cell Death Detection Kit Fluorescein (Roche Diagnostics, Sigma-Aldrich) and according to the manufacturer's instructions. Approximately 500 cells were counted in five random microscope fields using an Eclipse E400 fluorescence microscope (Nikon).

2.7. *In vitro* PKC assay

To measure *in vitro* PKC activation, the non-radioactive PKC kinase activity kit (ADI-EKS-420A; Enzo Life Sciences), and purified recombinant human PKC proteins, cPKCs (mix of PKCα, β and γ), PKCδ, PKCε, and PKCζ (Merck Millipore, Grupo Taper), were used according to the manufacturer's instructions. Briefly, 10 ng of recombinant PKC was incubated with 10^{-7} - 10^{-3} μM PMA/ARA or Roy-Bz (or DMSO only) for 1 h, and then transferred to a 96-well plate pre-coated with a synthetic peptide. A phosphospecific substrate antibody that recognizes the phosphorylated form of the substrate was added and

detected using a peroxidase-conjugated antibody. The degree of PKC activation was directly proportional to the amount of phosphorylated substrate determined by measuring the OD_{450} using a microplate reader (Biotek Instruments Inc., Synergy MX, USA). The EC_{50} (concentration required to induce 50% of the maximal response achieved with the positive control (constitutively activated PKC) of the kit) values were then calculated.

2.8. Molecular docking

The crystallographic structure of PKCδ with protein data bank (PDB) code 1PTR having 13-acetylphorbol (PRB) as co-crystallized ligand as described (127) was used to test the binding mode of Roy-Bz. First, the co-crystallized PRB molecule was re-docked to test the efficiency of the docking procedure in reproducing the experimental binding pose. The binding pose was reproduced with autodock 4.2 with a root-mean-square deviation (RMSD) of 0.47Å. The exact same conformation was reproduced with the molecule being slightly less inserted into the protein. All autodock docking parameters were produced with Autodock Tools and were kept at the default. The docking box was centered on the crystallographic position of the PRB molecule having 42, 62 and 36 grid points in x, y and z with a grid spacing of 0.375.

2.9. Human cancer cell lines and culture conditions

Human colon (HCT116 and HT-29) and colorectal (SW-837) adenocarcinoma, human breast adenocarcinoma (MCF-7), and human NSCLC (NCI-H460) cell lines were purchased from ATCC (LGC Standards S.L.U., Barcelona, Spain, 2013). Cell lines were frozen down at early passages (<5), routinely tested for mycoplasma contamination using *MycoAlert Plus Mycoplasma Detection Kit* (Lonza, VWR), and used between 2 to 8 passages after thawing. All tumor cells were routinely cultured in RPMI-1640 medium with ultraglutamine (Lonza), and supplemented with 10% fetal bovine serum (FBS; Gibco, Alfagene). For metabolism assays, HCT116 cells were routinely cultured in Dulbecco′s Modified Eagle′s Medium (DMEM) glucose-free (D5030; Sigma-Aldrich), with 5 mM glucose (Sigma-Aldrich), 4 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 21 mM sodium bicarbonate (Sigma-Aldrich), and supplemented with 10% FBS (pH 7.4). Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.10. Immunofluorescence and confocal microscopy

For localization studies of green fluorescent protein (GFP)-fused PKCs, HCT116 cells (at a final density of 1×10^5 cells/well) were transfected with 2 μg of pEGFP-N1-PKCα, pEGFP-N1-PKCδ or pEGFP-N1-PKCϵ using lipofectamine 3000 (Invitrogen), plated on coverslides in 12-well plates, and incubated for 48 h. Thereafter, cells were treated with 0.01 - 100 μM Roy-Bz, 100 nM PMA (positive control) (or DMSO only) for 1 h, washed with phosphate buffer saline (PBS; Sigma-Aldrich), and fixed with ice-cold methanol. Samples were stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) for 10 min at 4ºC, mounted on a glass slide, and the cellular localization of GFP-fused PKCs was visualized using a Nikon TE2000-U microscope.

2.11. Sulforhodamine B (SRB) assay

For analysis of *in vitro* proliferation, human cell lines were seeded in 96-well plates at a final density of 5.0×10^3 (for HCT116, HT-29, MCF-7 and NCI-H460) and 7.5×10^3 (for SW-837) cells/well, and incubated for 24 h. Cells were thereafter treated with 0.1 - 10 μM PMA, 0.11 - 2.84 μM Roy-Bz, 1.56 - 50 μM 6,7-dehydroroyleanone and derivatives, or 1.56 - 50 μg/mL plant extracts, for 48 h. For the SRB assay, following fixation with 10% trichloroacetic acid (TCA), plates were stained with 0.4% SRB (Sigma-Aldrich) and washed with 1% acetic acid (VWR). The bound dye was solubilized in 10 mM Tris Base (Sigma-Aldrich), and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc.). The maximum concentration of solvent used in this assay (0.25% DMSO) was included as control, and the growth obtained was set as 100%. The IC_{50} (concentration that causes 50% of growth inhibition) values were determined from the concentration-response curves.

2.12. Trypan blue assay

For analysis of cell viability, HCT116 cells were seeded in 24-well plates at a final density of 6×10^4 cells/well for 24 h, followed by treatment with 0.1 - 6 μM Roy-Bz for 24 h. For trypan blue exclusion assay, cells (non-adherent and adherent) were harvested and resuspend in PBS. An equal volume (1:1) of cell suspension was mixed with 0.4% Trypan Blue (Sigma-Aldrich), and then the number of viable/dead cells was determined on a hemocytometer and normalized to a drug-free control (DMSO; correspondent to the maximum concentration used). Cell viability was calculated as the number of viable cells

divided by the total number of cells. The IC_{50} values were determined from the concentration-response curves.

2.13. Colony formation assay

Human cell lines were seeded in 6-well plates at a final density of 5.0×10^2 (for HCT116 and SW-837) and 1.0×10^3 (for HT-29) cells/well, and incubated with 0.07 - 0.3 μM Roy-Bz (or DMSO only, correspondent to the maximum concentration used) for 11 days. Thereafter, colonies were fixed using 10% methanol and 10% acetic acid for 10 min and stained with 0.5% crystal violet (Sigma-Aldrich) in methanol/H_2O (1:1) for 15 min. Colonies containing more than 20 cells were counted.

2.14. Analysis of cell cycle and apoptosis in human cell lines

Human cell lines were seeded in 6-well plates at a final density of 1.5×10^5 (for HCT116 and HT-29) or 2.25×10^5 (for SW-837) cells/well, and incubated for 24 h. Cells were thereafter treated with 8 μM PMA, 1 μM Roy-Bz, or DMSO only for 24 h. For cell cycle analysis, cells were fixed in ice-cold 70% ethanol, incubated with RNase A at a final concentration of 20 μg/mL for 15 min at 37°C, and further incubated with 50 μg/mL PI for 30 min at room temperature, followed by flow cytometry analysis. Apoptosis analysis was performed by flow cytometry using the *Annexin V-FITC Apoptosis Detection Kit I* (BD Biosciences, Enzifarma, Porto, Portugal), according to the manufacturer's instructions.

2.15. Western blot analysis

For analysis of protein expression in human tumor cell lines, cells were seeded in 6-well plates at a final density of 1.5×10^5 (for HCT116 and HT-29) or 2.25×10^5 (for SW-837) cells/well, and incubated for 24 h, and thereafter treated with 0.5 - 3.5 μM Roy-Bz (or DMSO only) between 0 - 96 h. Whole cell lysates were prepared by lysing the cells with RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris, pH 8.0; Sigma-Aldrich) in the presence of ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Sigma-Aldrich) for 1 h at 4°C. Soluble fractions of cell lysates were then isolated by centrifugation at 8000 rpm for 10 min. For mitochondrial and cytosolic fractions of human tumor cells, the *Mitochondrial Fractionation Kit* (Active Motif, Frilabo) was used according to the manufacturer's

instructions. For analysis of yeast protein expression, samples were lysed with CellyticTM Y Cell Lysis Reagent (Sigma-Aldrich) in the presence of EDTA-free protease inhibitor cocktail (Sigma-Aldrich). Protein extracts were quantified using the Coomassie staining, Bradford (Sigma-Aldrich).

Proteins (40 μg) were denatured by boiling in Laemmli Sample Buffer (4×; Bio-Rad, Life Science, Amadora, Portugal) containing 10% β-mercaptoethanol (National Diagnostics, Frilabo) for 5 min, electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a Whatman nitrocellulose membrane (Protan, VWR). Membranes were blocked with 5% milk or 5% bovine serum albumin (BSA; Sigma-Aldrich) in Tris Buffered Saline with Tween 20 (TBST; Sigma-Aldrich), and probed overnight with a primary antibody (see Appendix; Table 1) followed by 2 h incubation with a horseradish-peroxidase (HRP)-conjugated secondary antibody (see Appendix; Table 1). For analysis of cytosolic and mitochondrial fractions, membranes were stripped (Abcam protocol), and reprobed with the primary antibodies for the loading controls GAPDH and COX IV, respectively. The signal was detected using the *ECL Amersham Kit* (GE Healthcare, VWR) and the Kodak GBX developer and fixer (Sigma-Aldrich). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat, Marne La Vallée, France), and were normalized against the control sample (DMSO; set as 1). Western blots presented in this thesis are representative of three independent experiments.

2.16. Analysis of reactive oxygen species (ROS) generation

HCT116 cells were seeded in 6-well plates, at a final density of 1.5×10^5 cells/well for 24 h, and then treated with 0.5 μM Roy-Bz (or DMSO only) for 24 h. Afterwards, cells were harvested and stained with 3 µM MitoSOX (Invitrogen) for 30 min at 37°C, followed by flow cytometry analysis.

2.17. Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

HCT116 cells were seeded in 6-well plates, at a final density of 1.5×10^5 cells/well for 24 h, and then treated with 0.5 μM Roy-Bz or DMSO only for 16 h. Thereafter, cells were harvested and incubated with 1 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$(3)) (Alfagene) for 30 min, at 37ºC, followed by flow cytometry analysis.

2.18. Generation of colon cancer spheroids (colonosphere assay)

HCT116 cells were resuspended in serum-free stem cell culture media consisting of DMEM (Lonza) supplemented with 10 ng/mL bFGF and 20ng/mL EGF (Bio-techne, Citomed Lda, Lisboa, Portugal), 1× B27 (Life Technologies, Porto, Portugal), and 5 μg/mL insulin (Sigma-Aldrich). Cells were plated in 24-well ultra-low attachment plates (Corning Inc.; one spheroid/well) at 1×10^3 cells/well. Treatments with 2 - 3.5 μM Roy-Bz (or DMSO only) were performed 3 days after spheroid formation for up to 96 h, or with 0.25 - 1 μM Roy-Bz (or DMSO only) were performed at the seeding time for 48 h. Spheroid formation was monitored using an inverted Nikon TE 2000-U microscope at 100× magnification with a DXM1200F digital camera and using Nikon ACT-1 software. Spheroid diameters were quantified using the ImageJ software.

2.19. Extraction and purification of plasmid DNA

E. coli bacterial cell cultures transformed with control pSuper plasmid or pSuperPKCδ.RNAi [Addgene plasmid #10819; (296)] were inoculated in Luria-Bertani (LB) medium (Liofilchem), containing 1 μg/mL ampicillin (Sigma-Aldrich), and grown overnight at 37 ºC. Bacterial cells were pelleted by centrifugation, and highly purified plasmid DNA was obtained using the *PureYield™ Plasmid Midiprep System* (Promega, VWR) according to the manufacturer's instructions. The amount of plasmid DNA was quantified using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific).

2.20. Generation of stable PKCδ-knockdown cell line

HCT116 cells were seeded in 24-well plates at a final density of 6×10^4 cells/well for 24 h. For the generation of stable cell lines, cells were co-transfected with 1 μg of control pSuper plasmid (control; mock) or 900 ng of pSuperPKCδ.RNAi [Addgene plasmid #10819;(296)] and 100 ng of pSUPERpuro plasmids, and 2 μg/mL Lipofectamine 2000 (Invitrogen) for 72 h. Stable transfectants were selected with 3 μg/mL puromycin (Sigma-Aldrich) for 4 weeks. Clones were screened for PKCδ expression silencing by Western blot using an anti-PKCδ antibody (see Appendix; Fig. 2).

2.21. *In vitro* migration assays

Cell migration was analyzed using the Wound Healing Scratch assay as described (297), and the *QCM 24-Well Fluorimetric Chemotaxis Cell Migration Kit* (8 μm; Merck Millipore). In the Wound Healing Scratch assay, PKCδ-knockdown and control HCT116 cells were seeded at a density of about $5{\times}10^5$ cells/well and grown to confluence in 6-well plates. After reaching the confluence, a fixed-width wound was created in the cell monolayer using a sterile 200 μL tip, and then cells were treated with 0.25 μM Roy-Bz (or DMSO only) for 48 h. Cells were thereafter photographed, using the Moticam 5.0MP camera with Motic's AE2000 inverted microscope (VWR) with 100× magnification, at different time-points of treatment until complete closure of the wound. Wound closure was calculated by subtracting the 'wound' area (measured using Image J software) at the indicated time-point of treatment from the 'wound' area at the starting point. In the *QCM 24-Well Fluorimetric Chemotaxis Cell Migration Kit*, about $5{\times}10^5$ cells/mL of PKCδ-knockdown and control HCT116 cells were prepared in serum free RPMI 1640 medium and treated with 0.25 μM Roy-Bz or DSMO only, followed by incubation for up to 24 h. Cell suspensions were distributed into 24-well plates (300 μL/insert), followed by addition of 500 μL medium containing 10% FBS to the lower chamber. Cells that migrated through the 8 μm pore membranes were eluted, lysed and stained with a green-fluorescence dye that binds to nucleic acids. The number of migrated cells is proportional to the fluorescence signal measured using a microplate reader (Biotek Instruments Inc.) at 480/520 nm (ex/em).

2.22. Comet assay

HCT116 cells were seeded in 6-well plates at a final density of $1.5{\times}10^5$ cells/well for 24 h, followed by treatment with ETOP, Roy-Bz (or DMSO only) for 48 h. To evaluate DNA damage, *OxiSelect Comet Assay Kit* (Cell Biolabs, MEDITECNO, Carcavelos, Portugal) was used according to the manufacturer's instructions, with Tris/borate/EDTA (TBE) for electrophoresis. Tail DNA quantification considers the percentage of cells with more than 5% of DNA in the tail (assessed by Open Comet/ImageJ); tail moment corresponds to the product of the tail length and the % of DNA in the tail. Cells were photographed using a Nikon DS-5Mc camera and a Nikon Eclipse E400 fluorescence microscope, and images processed using a Nikon ACT-2U software (Izasa).

2.23. Micronucleus assay

Genotoxicity was analyzed by cytokinesis-block micronucleus assay in lymphocytes as described (298). Fresh peripheral blood samples were collected from healthy volunteers into heparinized vacutainers. Blood samples, suspended in RPMI 1640 medium and supplemented with 10% FBS, were treated with 0.5 - 1.5 μM Roy-Bz, 1μg/mL CP (positive control; Sigma-Aldrich) (or DMSO only) for 44 h. Cells were thereafter treated with 3 μg/mL cytochalasin B (Sigma-Aldrich) for 28 h. Lymphocytes were isolated by density gradient separation (Histopaque-1077 and -1119; Sigma-Aldrich), fixed in methanol/glacial acetic acid (3:1), and stained with Wright stain (Sigma-Aldrich). For each sample, 1000 binucleated lymphocytes were blindly scored using a Nikon AlphaPhotoF2 YS2 light optical microscope; the number of micronuclei per 1000 binucleated lymphocytes was recorded.

2.24. *In vivo* antitumor and toxicity assays

Animal experiments were conducted according to the EU Directive 2010/63/EU and the National Authorities. Female BALB/c nude mice with about 14 weeks of age and 20 g of body weight, and male Wistar rats with about 12 weeks of age and 300 g of body weight were purchased from Charles-River Laboratories. All mice were randomly housed (3 mice per cage) under pathogen free conditions in individual ventilated cages (standard 12 h light/12 h dark cycle at 22ºC). Mice were given ad libitum water and food for 7 days prior to experimentation. For the toxicity assays, Wistar rats were treated with 10 mg/kg Roy-Bz, vehicle, or saline solution (control) by intraperitoneal injection, twice a week during two weeks. Samples of blood and organs (heart, liver, kidney and spleen) were then collected for toxicological analysis. Each group was composed of four animals. Xenograft tumor assays were performed with HCT116 cells stably transfected with control pSUPER plasmid or pSuperPKCδ-RNAi. Briefly, 1×10^6 cells (in PBS) were inoculated subcutaneously in the dorsal flank of mice. Tumor dimensions were assessed by caliper measurement and their volumes were calculated [tumor volume=(L×W2)/2; L and W represent the longest and shortest axis of the tumor, respectively]. Treatment was started when tumors reached a volume of about 100 mm^3 (which occurred 14 days after the grafts). Mice were treated twice a week for two weeks with 10 mg/kg Roy-Bz or vehicle by intraperitoneal injection. Tumor volumes and body weights were monitored twice a week until the end of the treatment. Animals were sacrificed by cervical dislocation at the end of the study, when tumors reached about 1500 mm^3, or the animals present any signs of morbidity. Each group was composed of six animals.

2.25. Immunohistochemistry assay

Tumor tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E) or antibodies, following standard methodologies. Briefly, after deparaffination and rehydration, antigen retrieval was performed by boiling the sections, for 20 min, in 10 mM citrate buffer (pH 6.0) for all antibody's staining, with the exception of anti-VEGF, for which the tissue was treated with 10 mM EDTA buffer (pH 8.0). Slices were then held for 20 min at room temperature. After dewaxing, and blocking the endogenous peroxidases with UltraVision Hydrogen Peroxide Block, sections were treated with UltraVision Protein Block solution, both from Lab Vision Thermo Scientific (Grupo Taper SA). Incubation with primary antibodies for Ki-67, VEGF, and GLUT1 (see Appendix, Table 2) was performed for 2 h at room temperature, and for BAX, caspase-3, HK2, MCT4 and TIGAR (see Appendix, Table 2) overnight at 4°C. Immunostaining was carried out using the *UltraVision Quanto Detection System HRP DAB Kit* (Lab Vision Thermo Scientific), according to the manufacturer's instructions. Tissue sections were counterstained with Gill's heamatoxylin (Lab Vision Thermo Scientific), dehydrated, clarified and mounted. The primary antibody was replaced by 10% non-immune serum for negative controls. Images were obtained using an Eclipse E400 fluorescence microscope (Nikon) with 200× magnification, with Digital Sight camera system (Nikon DS-5Mc) and software Nikon ACT-2U.

2.26. TUNEL assay

The analysis of DNA fragmentation was performed using the *In Situ Cell Death Detection Kit Fluorescein* (Roche Diagnostics), according to the manufacturer's instructions. Briefly, after deparaffination and rehydration, tissues sections were treated for 20 min in 10 mM citrate buffer (pH 6.0), and incubated with TUNEL Reaction Mixture for 60 min at 37ºC in the dark. Tissues were counterstained with 0.1 μg/mL DAPI. Images were obtained using an Eclipse E400 fluorescence microscope (Nikon) with 200× magnification, with Digital Sight camera system (Nikon DS-5Mc) and software Nikon ACT-2U.

2.27. Tandem resazurin/SRB assay

HCT116 cells were seeded in 96-well plates, at a final density of 4.5×10^3 cells/well, for 24 h. Cells were thereafter treated with 1.5 - 4 μM Roy-Bz (or DMSO only) for 48 h, in normoxic conditions (99% O$_2$). Briefly, after treatments, the medium was removed and cells

were incubated with 1 μg/mL resazurin solution for 30 min as described (299). Metabolic activity was determined based on the reduction of resazurin to resorufin by the dehydrogenases present in viable cells, and subsequent resofurin fluorescence measurement at 540/590 nm (ex/em) in a microplate reader at 30, 60, 240 and 360 min, and expressed in relative fluorescence units (rfu's). After resazurin assay, the solution was removed, cells were fixed with 10% TCA, and the cell mass was determined by the SRB assay as described in 2.11. The maximum concentration of solvent used in this assay (0.25% DMSO) was included as control, and the values obtained were set as 100%. Data were expressed as percentage of vehicle.

2.28. Cellular oxygen consumption measurements

The oxygen consumption rate (OCR) was analysed by the Agilent Seahorse XF Cell Mito Stress Test (Agilent Technologies, Soquímica, Lisboa, Portugal) using a Seahorse XF^{e}96 Extracellular Flux Analyzer (Seahorse Bioscience, Germany), and according to the manufacturer's instructions . Briefly, HCT116 cells were seeded in Seahorse XF cell culture microplate, at a final density of 2.5×10^4 cells/well in D5030 medium with 5 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 21 mM sodium bicarbonate, and supplemented with 10% FBS (pH 7.4), for 24 h. Thereafter, cells were treated with 1.5 - 4 μM Roy-Bz, for 48 h in normoxic conditions. XF^{e}96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 μL/well calibration buffer and left to hydrate overnight at 37ºC. After treatments, the culture medium was removed, replaced by pre-warmed XF Cell Mito Stress Test Assay medium, consisting of D5030 with 5 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, and without FBS (adjusted pH to 7.4 with 1N NaOH), and incubated at 37ºC for 1h. The compounds (oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and the mix of rotenone and antimycin A) provided by the Seahorse XF Cell Mito Stress Test Kit were reconstituted with the previously prepared assay medium. For OCR measurements, XF^{e}96 sensor cartridge was pre-loaded with 1 µM oligomycin into reagent delivery port A, 0.5 µM FCPP into port B, and 1 µM rotenone/1 µM antimycin A mix into reagent delivery port C. Oligomycin, FCCP, and the mix of rotenone and antimycin A, were then pneumatically injected by the XFe96 Analyzer into each well, with measurement of OCR using a 3 min mix, 5 min measure cycle. Results were analyzed using the Software Version Wave Desktop 2.2.

2.29. Flow cytometric data acquisition and analysis

The Accuri™ C6 flow cytometer and the CellQuest software from BD Biosciences (Enzifarma, Porto, Portugal) were used. The FlowJo software was used to identify and quantify cell cycle phases.

2.30. Statistical analysis

Data are presented as mean ± SEM of 'n' samples, where 'n' refers to independent experiments, not replicates. Data analyses were carried out using GraphPad Prism Software version 6.0. For comparison of two groups, unpaired student's t-test was used; statistical significance was set as *p<0.05; **p<0.01; ***p<0.001. For comparison of multiple groups statistical analysis relative to controls was performed using the two-way analysis of variance (ANOVA).

CHAPTER 3

Results

Identification of PKC isozyme-selective agents and anticancer drugs

CHAPTER 3.1

Discovery of a small-molecule protein kinase Cδ-selective activator with promising application in colon cancer therapy

Bessa, C., Soares, J., Raimundo, L., Loureiro, J.B., Gomes, C., Reis, F., Soares, M.L., Santos, D., Dureja, C., Chaudhuri, S.R., Lopez-Haber, C., Kazanietz, M.G., Gonçalves, J., Simões, M.F., Rijo, P., Saraiva, L.

Based on the previous identification of coleon U (an abietane diterpene compound isolated from *P. grandidentatus*), described as selective activator of nPKCδ and ε (275), it was obtained a small library of royleanone derivatives. This library was composed by the natural diterpenoid, 7α-acetoxy-6β-hydroxyroyleanone (Roy; Fig. 3.1.1A), and the semi-synthetic derivatives, 7α-acetoxy-6β-propionyloxy-12-*O*-propionylroyleanone (Roy-Pr2; Fig. 3.1.1B), and 7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone (Roy-Bz; Fig. 3.1.1C). The ability of these compounds to activate PKC isozymes from classical (α and βI), novel (δ and ε) and atypical (ζ) subfamilies was studied using a previously developed yeast PKC screening assay (275). In this assay, PKC activators induce a significant growth inhibition in mammalian PKC-expressing yeast, which is proportional to the degree of PKC activation, having no effect on control yeast (empty vector) (275).

A   **B**   **C**
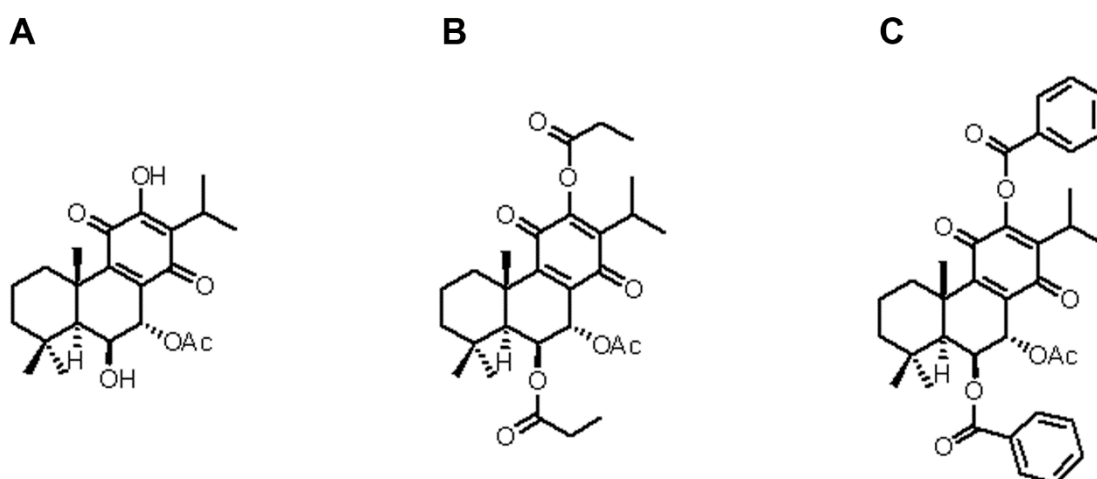
Figure 3.1.1: Schematic representation of royleanone structures. (A) 7α-acetoxy-6β-hydroxyroyleanone; **(B)** 7α-acetoxy-6β-propionyloxy-12-*O*-propionylroyleanone; **(C)** 7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone.

3.1.1. Effect of compounds on yeast growth

Using the yeast PKC assay, the inhibitory effect of 0.1 - 30 μM PMA (an established activator of cPKCs and nPKCs), ARA (activator of cPKCs, nPKCs and aPKCζ), Roy, Roy-Pr2 or Roy-Bz on the growth of yeast expressing individual PKC isozymes was analyzed. From the dose-response curves obtained, they were determined the EC$_{50}$ values for the tested compounds (Table 3.1.1) and their selectivity for the different PKC isozymes was evaluated. The results obtained showed that the natural diterpene Roy activated all PKC

isozymes with the exception of PKCδ, the derivative Roy-Pr2 activated cPKCs and nPKCs with a similar pharmacological profile to PMA, and the compound Roy-Bz selectively activated PKCδ with a higher potency than the standard activator, PMA (Table 3.1.1). Altogether, these results indicate that promising modulators of PKC with high potency and isozyme-selectivity may emerge from the exploitation of this family of compounds.

Table 3.1.1. EC_{50} values of compounds tested on individual PKC isozymes.

Compounds	EC_{50} (nM)				
	PKCα	PKCβI	PKCδ	PKCε	PKCζ
PMA	111,6 ± 18,4	243,2 ± 69,1	573,8 ± 36,7	1678 ± 46,48	-
ARA	-	-	-	-	205,4 ± 32.6
Roy	350 ± 42	423 ± 67	*ND*	994 ± 63	4113 ± 159
Roy-Pr2	195 ± 16	229 ± 21	325 ± 49	770 ± 46	*ND*
Roy-Bz	*ND*	*ND*	107 ± 53	*ND*	*ND*

EC_{50} values were considered the concentration of compound that caused 50% of the maximal growth inhibition caused by the positive controls (PMA, for cPKCs and nPKCs; ARA, for PKCζ), which was set as 100%. Data are mean ± SEM of four independent experiments. -, not tested; *ND*, non-determinable (when the maximal response achieved was lower than 50% growth inhibition).

Based on the results obtained in yeast, the compound Roy-Bz was selected for further studies in order to confirm its selectivity for PKCδ and to study its anticancer activity.

3.1.2. Roy-Bz is a selective activator of PKCδ that binds to the C1-domain

As previously referred, using the yeast PKC assay, it was observed that whereas 0.1 - 30 µM PMA inhibited the growth of yeast expressing cPKCs (α and βI) and nPKCs (δ and ε), 0.1 - 30 µM Roy-Bz only inhibited the growth of PKCδ-expressing yeast without significant effects on yeast expressing other PKCs (Fig. 3.1.2A). Interestingly, in PKCδ-expressing yeast, while PMA-induced growth inhibition was associated with G2/M-phase cell cycle arrest, Roy-Bz-induced growth inhibition was mediated by apoptotic cell death, as demonstrated by the increase in DNA fragmentation with preservation of plasma membrane integrity (Fig. 3.1.2C, D). Additionally, Roy-Bz-induced growth inhibition was completely

abolished in yeast expressing a PKCδ in which the C1-domain has been deleted (ΔC1-PKCδ; Fig. 3.1.2B) (Fig. 3.1.2A). In fact, a similar result was obtained with PMA, which is known to bind PKCs at the C1-domain (Fig. 3.1.2A). These results indicate that Roy-Bz is a PKCδ-selective activator and, like PMA, it should have the C1-domain as the predicted binding site.
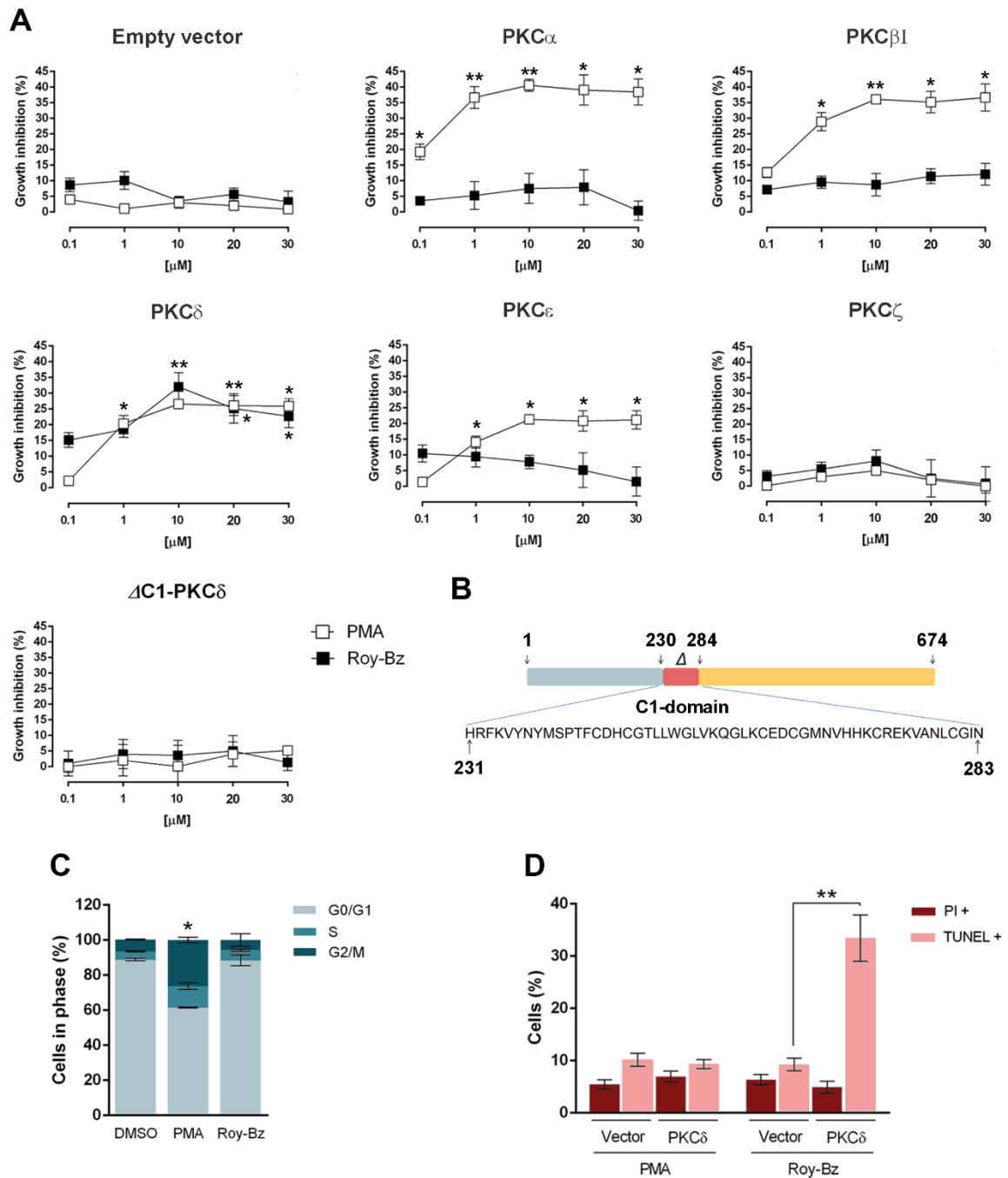

Figure 3.1.2: Roy-Bz selectively activates PKCδ in yeast and induces an apoptotic cell death.
(A) Dose-response curves for the inhibitory effect of PMA and Roy-Bz on the growth of yeast

67

expressing mammalian PKC isozymes and control yeast (empty vector), for 42 h treatment; growth of yeast incubated with vehicle was set as 100%. **(B)** Schematic representation of deletion of C1-domain at amino acids 231-283 in the *PRKCD* gene. **(C,D)** Effect of 10 μM PMA and Roy-Bz on **(C)** cell cycle progression, and **(D)** DNA fragmentation (TUNEL+)/loss of plasma membrane integrity (PI+) of PKCδ-expressing yeast (and control yeast in **D**), for 42 h treatment. In **A**, **C** and **D**, data are mean ± SEM of five independent experiments; values significantly different from control yeast **(A,D)** or vehicle **(C)**: *$p<0.05$, **$p<0.01$, unpaired Student's *t*-test.

To confirm the PKC isozyme selectivity observed in the yeast assay, we next performed an *in vitro* kinase assay using recombinant human cPKCs (a mix of PKCα, β and γ), nPKCδ, nPKCε, and aPKCζ. All recombinant PKCs were effectively activated at 10^{-4} μM PMA/ARA (positive controls: PMA for cPKCs and nPKCs; ARA for aPKCζ) (Fig. 3.1.3A). Remarkably, 10^{-7} - 10^{-3} μM Roy-Bz only increased the activity of PKCδ (Fig. 3.1.3B). Roy-Bz activated PKCδ with an EC$_{50}$ (concentration required to induce 50% effect) value of 58.8 ± 3.6 nM (*n*=5), similar to that of PMA (61.9 ± 4.3 nM, *n*=5). Thus, the *in vitro* kinase assays, in accordance with the yeast assays, revealed a remarkable selectivity of Roy-Bz for PKCδ. Besides, these results show that Roy-Bz is as effective as PMA to activate PKCδ.

Next, the potential binding mode of Roy-Bz to PKCδ was explored by molecular docking studies (Fig. 3.1.3C, D). Like PRB (positive control), which establishes direct strong hydrogen bond interactions with *Gly253* and *Thr242* (hydrogen bond donors), and *Leu251* (hydrogen bond acceptor) in the C1-domain of PKCδ, Roy-Bz also interacted with *Gly253* and *Thr242*, but exchanged *Leu251* with *Gln257*, all acting as hydrogen donors to carbonyl oxygen functional groups. It is interesting to note that like in the case of PRB, these interactions also stabilized the molecule from both sides of the cleft. The estimated autodock free energy of binding for PRB was -7.54 kcal/mol and for Roy-Bz was -7.87 kcal/mol, and a clustering analysis of the molecular docking results showed other three poses similar to the top-ranked (the most populated one; in a total of 10 poses and 6 different clusters). These predicted binding models support the Roy-Bz binding to the PKCδ-C1-domain.
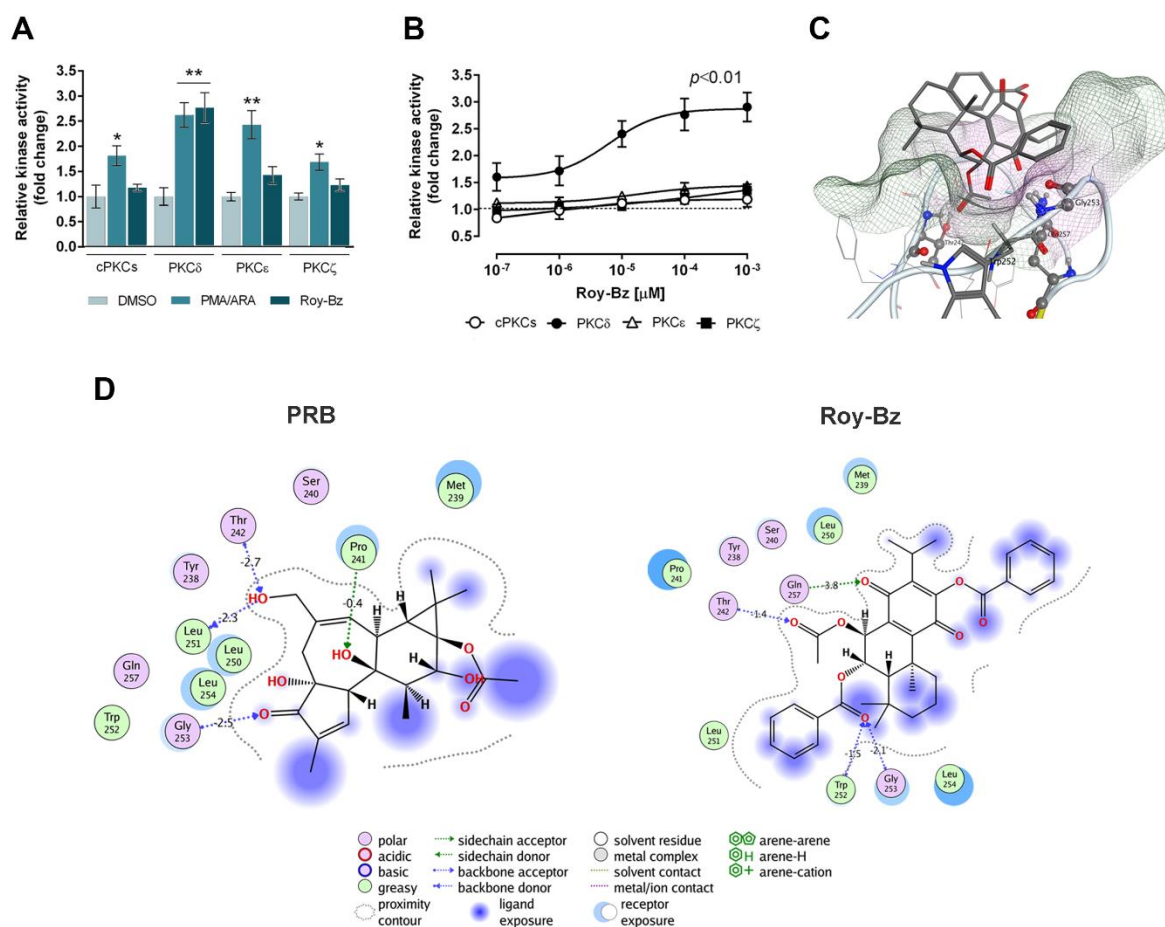
Figure 3.1.3: Roy-Bz selectively activates PKCδ through binding to the C1-domain. (A,B) *In vitro* kinase assay with recombinant PKCs; **(A)** increase of PKC activity by 10^{-4} μM PMA/ARA and 10^{-4} μM Roy-Bz; **(B)** dose-response curves for the increase of PKC activity by Roy-Bz; kinase activity of endogenous PKC activator phosphatidylserine was set as 1. In **A**, data are mean ± SEM of five independent experiments; values significantly different from vehicle: *$p<0.05$, **$p<0.01$, unpaired Student's *t*-test; in **B** two-way analysis of variance (ANOVA) ($p<0.01$). **(C,D)** Molecular docking studies for Roy-Bz interaction with the C1-domain of PKCδ. **(C)** Top ranked docking pose of Roy-Bz with *Gly253*, *Thr242* and *Gln257* highlighted through a CPK representation. The hydrogen bonds between Roy-Bz and these residues are also displayed through a doted blue line. The interaction between the alpha-hydrogen of *Trp252* and the carbonyl (CH•••O) is also represented and the residue is highlighted in licorice. All residues within 4.5 Å of Roy-Bz are represented and the molecular surface of the pocket is displayed through a mesh that changes from green in lipophilic regions to pink (hydrophilic). **(D)** Scheme of PRB and Roy-Bz interactions. The pocket proximity contour is represented by grey doted lines, the ligand exposure is depicted in violet and the receptor exposure in blue. Direct interactions are represented by a doted or green line along with the interaction energy (kcal/mol) estimated by MOE.

The translocation of PKC from the cytosol to membranes, either plasma membrane or internal membranes, is a well-known hallmark of PKC activation (83, 212). As such, we evaluated the impact of Roy-Bz on the translocation of PKCα, PKCδ and PKCε in human HCT116 colon cancer cells using GFP-fused PKCs (Fig. 3.1.4A, B). As previously reported in LNCaP prostate cancer cells (300), also in HCT116 cells the treatment with PMA triggered the subcellular redistribution of the three PKC isozymes. There was a clear translocation to the plasma membrane for PKCα, PKCδ and PKCε, and as reported in other models (300), PKCδ also translocated to a perinuclear compartment. When we assessed the ability of Roy-Bz to translocate PKCs, there were obvious differences with regard to PMA. Indeed, Roy-Bz was unable to translocate PKCα or PKCε, while it selectively translocated PKCδ to the perinuclear region (Fig. 3.1.4A, B). A dose-dependent analysis of PKCδ translocation in HCT116 cells revealed an EC_{50} value of 0.19 ± 0.07 µM (n=3). Thus, these results reinforce those previously obtained and further demonstrate the selectivity of Roy-Bz for PKCδ.
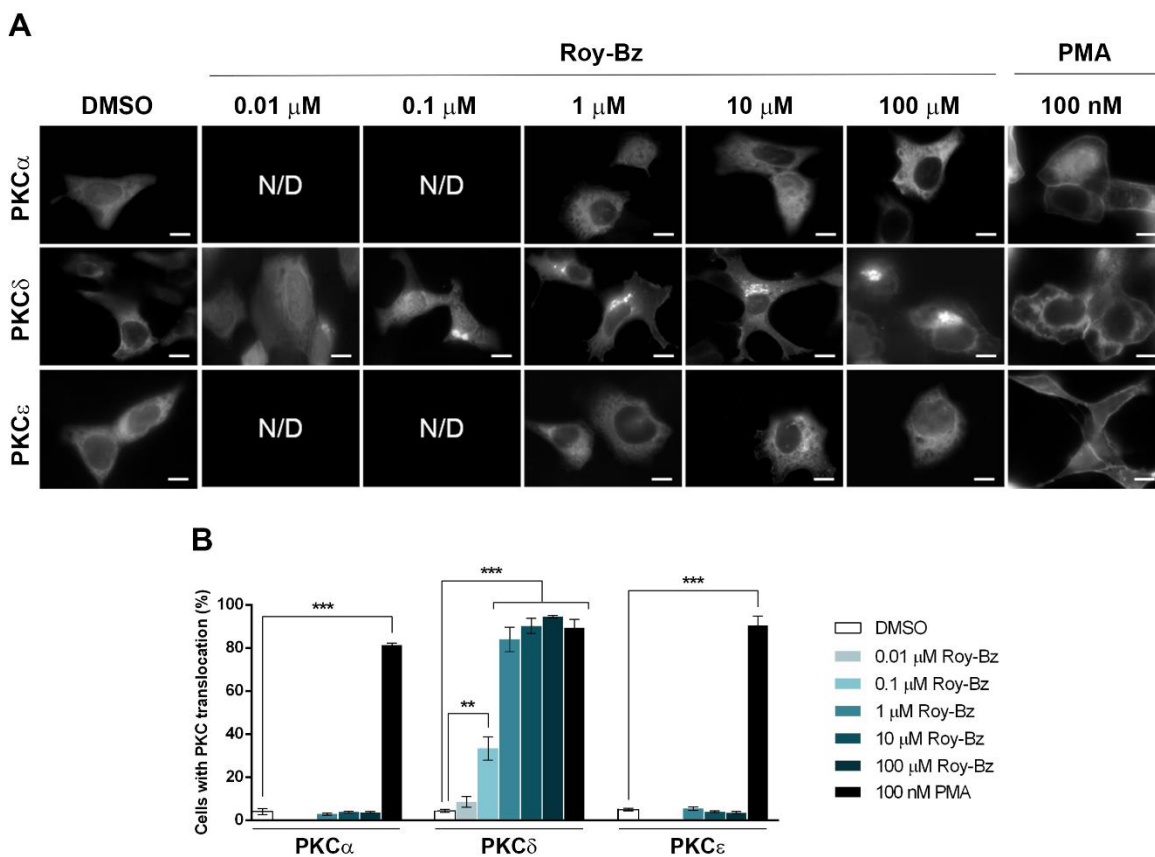

Figure 3.1.4: Roy-Bz selectively translocates PKCδ to the perinuclear region in human tumor cells. HCT116 cells transfected with pEGFP-N1-PKCα, pEGFP-N1-PKCδ, or pEGFP-N1-PKCε were

treated with PMA, Roy-Bz, or vehicle for 1 h; PKC localization was determined by confocal microscopy. **(A)** Images are representative of three independent experiments; scale bar=10 μm and magnification=100×. **(B)** Quantification of cells with PKC translocation; data are mean ± SEM of three independent experiments; values significantly different are indicated *(***p<0.001)*, unpaired Student's *t*-test.

3.1.3. Roy-Bz inhibits the proliferation of colon cancer cells

The effect of Roy-Bz on proliferation of human colorectal cancer cells was evaluated using the SRB assay (Fig. 3.1.5A, B). Treatment of HCT116, HT-29 and SW-837 cells (with similar PKCδ expression levels; see Appendix, Fig. 3) with Roy-Bz for 48 h resulted in a dose-dependent inhibition of cell growth (IC_{50} values of 0.58 ± 0.05 μM for HCT116, 1.50 ± 0.06 μM for HT-29, and 1.08 ± 0.03 μM for SW-837; *n*=5). The pronounced inhibitory effect of Roy-Bz on cell proliferation/viability of colorectal cancer cells was further demonstrated by assessing the colony forming ability (Fig. 3.1.5C). In fact, even in the most resistant SW-837 cells, 0.3 μM Roy-Bz reduced the colony forming ability ~75% relative to vehicle.

Interestingly, while in HT-29 and SW-837 cells the Roy-Bz growth inhibitory effect was associated with G2/M-phase cell cycle arrest (Fig. 3.1.5D; particularly pronounced in HT-29) and apoptosis (Fig. 3.1.5E), in HCT116 cells the Roy-Bz-induced growth inhibition was only mediated by apoptosis. In fact, Roy-Bz had no effect on cell cycle progression (Fig. 3.1.5D; similar results were obtained for 8, 16 and 48 h treatment, data not shown) and markedly increased Annexin V-positive cells (Fig. 3.1.5E). Conversely, in HCT116 cells, PMA, with an IC_{50} value about 14-fold higher than that of Roy-Bz (7.83 ± 1.67 μM, *n*=5; Fig. 3.1.5A), inhibited cell growth through induction of S-phase cell cycle arrest and apoptosis (see Appendix; Fig. 4). Additionally, in HCT116 cells, the induction of apoptosis by Roy-Bz was further reinforced by the occurrence of caspase-3 and PARP cleavage, an increase in pro-apoptotic p53 and BAX levels, and a reduction in the levels of anti-apoptotic proteins Bcl-2 and survivin (Fig. 3.1.5F, G).
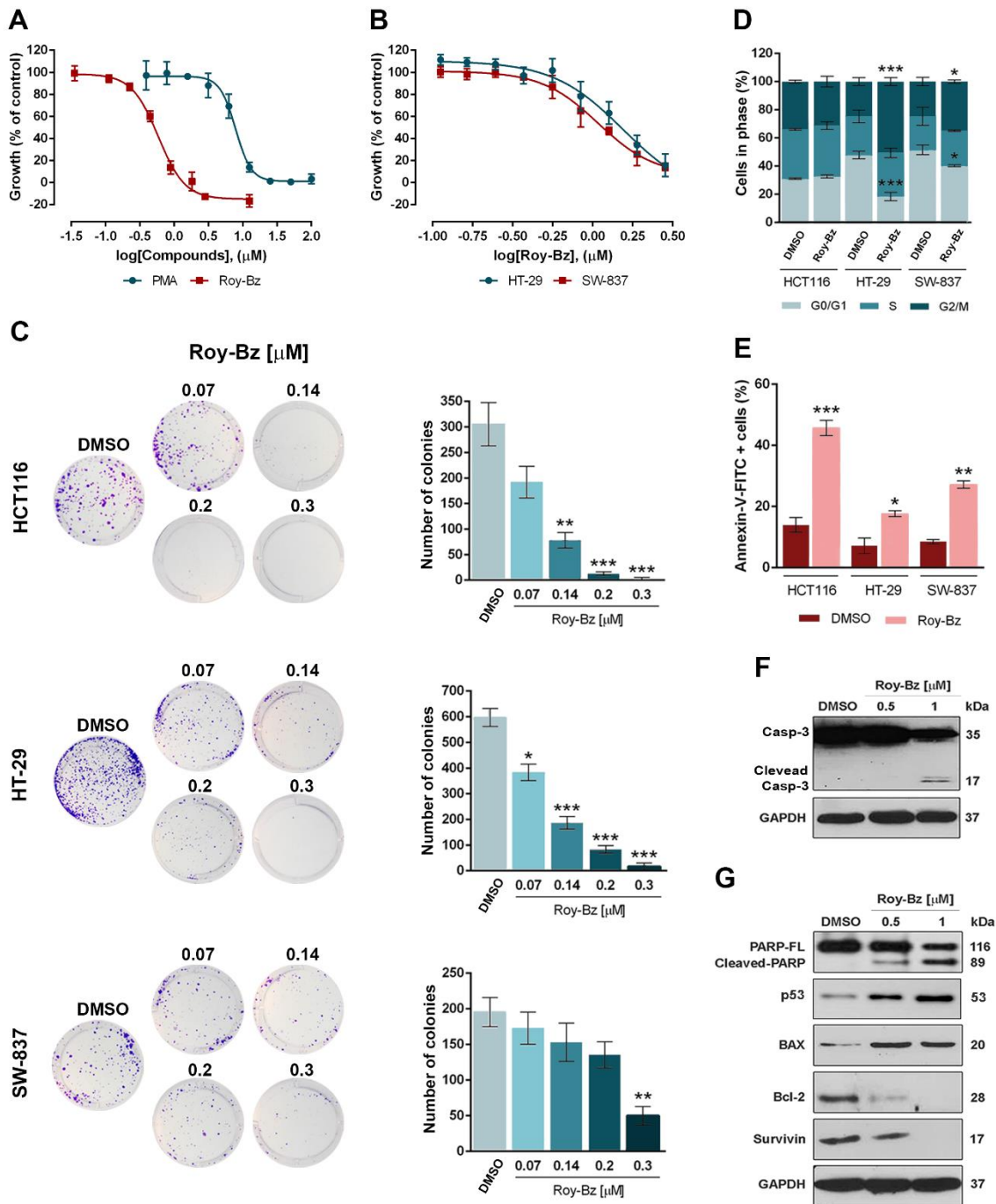
Figure 3.1.5: Roy-Bz inhibits the growth of colon cancer cells. (A,B) Dose-response curves for the growth of **(A)** HCT116, **(B)** HT-29 and SW-837 cells treated with 0.11 - 10 μM Roy-Bz or PMA for 48 h; data are mean ± SEM of five independent experiments; growth obtained with vehicle was set as 100%. **(C)** Colony formation assay for HCT116, HT-29 and SW-837 cells treated with Roy-Bz; images correspond to a representative experiment of four; graphs represent mean ± SEM of four independent experiments. **(D)** Cell cycle arrest, and **(E)** Annexin V-positive cells were determined for 24 h treatment with 1 μM Roy-Bz; data are mean ± SEM of four independent experiments. **(F,G)** Western blot analysis of **(F)** caspase-3 and **(G)** PARP cleavage, BAX, p53, Bcl-2, and survivin expression levels, for 24 h treatment with Roy-Bz in HCT116 cells. In **F** and **G** immunoblots represent

one of three independent experiments; GAPDH was used as loading control. In **C**, **D** and **E**, values significantly different from vehicle (*$p<0.05$, **$p<0.01$, ***$p<0.001$), unpaired Student's *t*-test.

In an attempt to explore the antitumor activity of Roy-Bz in a system that more closely resembles the *in vivo* features of the tumor microenvironment and is highly enriched in a small population of CSCs, a colonosphere culture model was generated from HCT116 cells. In fact, the spheroid-formation (colonosphere) assay is recognized as a valuable tool for assessment and expansion of stem cells in colon cancer (301). In addition, according to several studies, colon CSCs can mainly be identified by the expression of cell biomarkers, such as CD44 and ALDH (302-305). Actually, a positive correlation between the CD44 decrease and the therapeutic response of patients with colorectal cancer has been recently identified (304, 305). To test the effect of Roy-Bz in colonosphere growth, 3-day-old spheroids were treated with Roy-Bz for up to 96 h (Fig. 3.1.6A). A significant reduction in colonosphere diameter by Roy-Bz relative to vehicle was observed both at 72 h (at 3 and 3.5 μM) and 96 h (at 2 - 3.5 μM). Accordingly, 3.5 μM Roy-Bz markedly reduced the expression levels of the stemness markers CD44 and ALDH2 after 96 h treatment (Fig. 3.1.6B). Of note, a pronounced reduction of matrix metalloproteinase-9 (MMP-9) and increase of E-cadherin, prominent players in extracellular matrix homeostasis and metastasis (73), were also observed under these treatment conditions (Fig. 3.1.6B). The effectiveness of Roy-Bz in colonosphere formation was also checked by evaluating the colonosphere diameter after 48 h treatment with Roy-Bz added at the seeding time of HCT116 cell suspension (Fig. 3.1.6C). The results showed a notable dose-dependent reduction in colonosphere formation ability by Roy-Bz, with an abolishment of colonosphere formation at 1 μM Roy-Bz. Altogether, these results evidence a potent inhibitory activity of Roy-Bz in the growth of colon cancer cells, including in CSCs, suggesting a potential effect of the drug against cancer chemoresistance, dissemination and recurrence.

3.1.4. Roy-Bz pro-apoptotic and anti-migratory activity in HCT116 cancer cells is mediated by PKCδ-selective activation

To evaluate the involvement of PKCδ in Roy-Bz-induced apoptosis, a PKCδ stable knockdown model was generated upon transfection of the metastatic colon cancer HCT116 cells with a PKCδ RNAi plasmid. As control, we used HCT116 cells transfected with non-target control RNAi. PKCδ silencing was confirmed by Western blot (see Appendix, Fig. 2). The impact of 0.1 - 6 μM Roy-Bz on the viability of PKCδ-depleted and control HCT116 cells

was thereafter evaluated by a trypan blue assay. As shown in Fig. 3.1.7A, silencing PKCδ decreased Roy-Bz-induced cell death compared to control HCT116 cells. Accordingly, the depletion of PKCδ in HCT116 cells abolished Roy-Bz-induced Annexin V-positive cells (Fig. 3.1.7B), PARP cleavage, and increase of BAX and p53 expression levels (Fig. 3.1.7C).
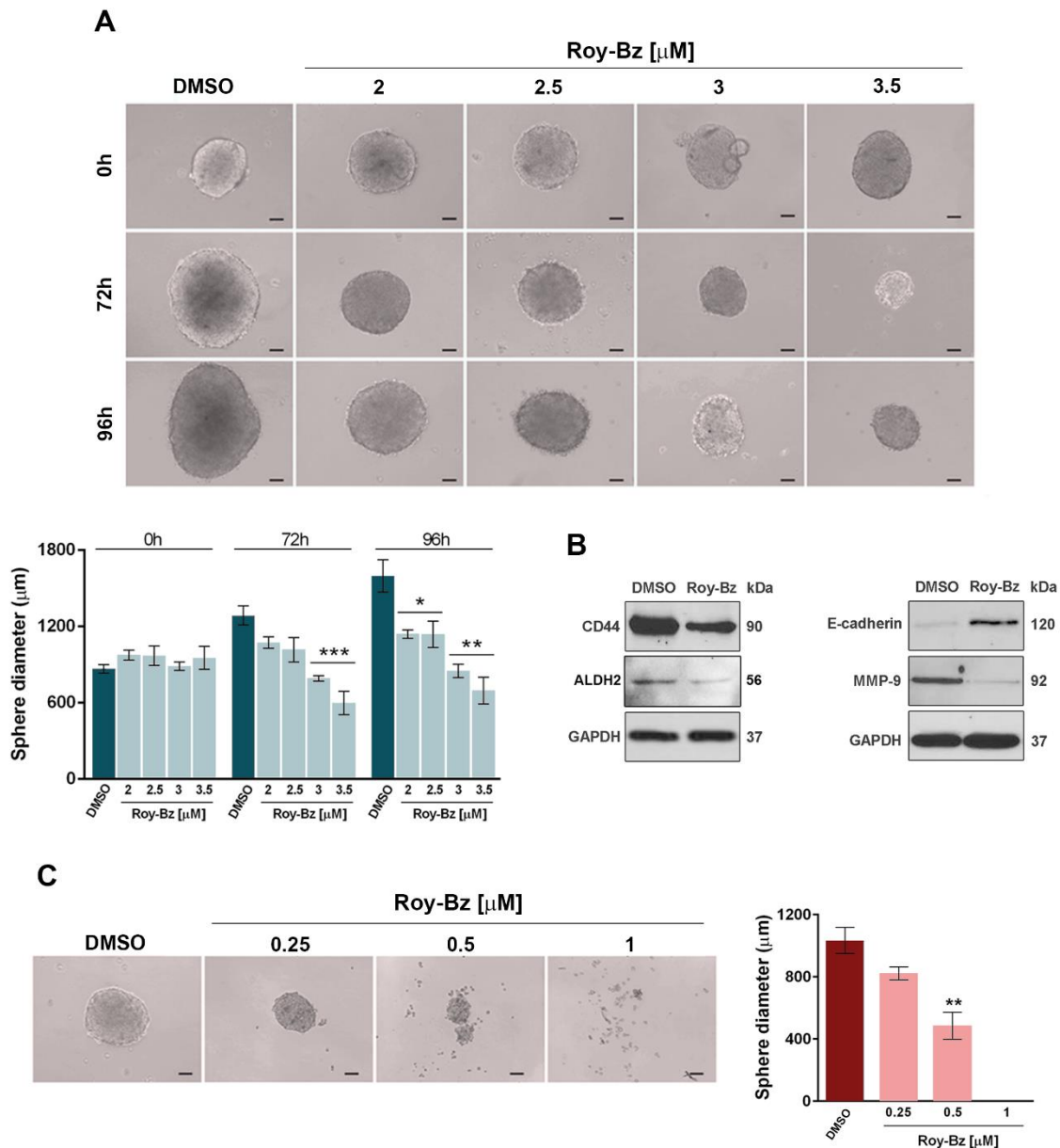
Figure 3.1.6. Roy-Bz inhibits the growth of colonospheres. (A) Evaluation of 3-day-old HCT116 spheroids diameter for 72 and 96 h treatment with Roy-Bz or vehicle. **(B)** CD44, ALDH2, E-cadherin and MMP-9 expression levels for 96 h treatment with 3.5 μM Roy-Bz. **(C)** Evaluation of spheroids diameter formed after 48 h treatment of seeded HCT116 cells with Roy-Bz or vehicle. In **B** immunoblots represent one of three independent experiments; GAPDH was used as loading control. In **A** and **C**, brightfield imaging of spheroids (scale bar=50 μm, magnification=100x); graphs

represent mean ± SEM of four independent experiments; values significantly different from vehicle (*p<0.05, **p<0.01, ***p<0.001), unpaired Student's *t*-test.

It has been previously established in certain cellular models that PKCδ undergoes a proteolytic cleavage by caspases that generates a catalytically active fragment, termed PKCδ-CF (116, 306). Interestingly, we found that in HCT116 cells, Roy-Bz caused a time-dependent increase in PKCδ-CF levels, without a visible interference in total PKCδ expression levels (Fig. 3.1.7D). Additionally, since it was previously reported that activation of PKCδ is a required factor for histone H3 phosphorylation on Ser-10 (pSer-10 histone H3), which is a crucial event in apoptosis (307), we next assessed the effect of Roy-Bz on pSer-10 histone H3 levels in HCT116 cells. To this end, HCT116 cells were synchronized in G1-phase by hydroxyurea, in order to exclude mitotic pSer-10 histone H3, followed by treatment with 1 µM Roy-Bz for up to 8 h. We observed that 1 µM Roy-Bz caused a pronounced increase of pSer-10 histone H3 levels that was noticeable at 8 h. This effect was abolished by PKCδ-knockdown (Fig. 3.1.7E, F), again reinforcing the concept that Roy-Bz is a potent inducer of apoptotic cancer cell death via activation of PKCδ.

Figure 3.1.7: Roy-Bz pro-apoptotic activity is mediated by PKCδ-selective activation in HCT116 cancer cells. (A) Cell viability dose-response curves were determined by trypan blue assay

for control (Mock) and PKCδ-knockdown (PKCδ RNAi) HCT116 cells treated with 0.1-6 μM Roy-Bz for 24 h; cell viability of vehicle was set as 100%. **(B)** Annexin V-positive cells were determined in control and PKCδ-knockdown HCT116 cells after 24 h treatment with 1 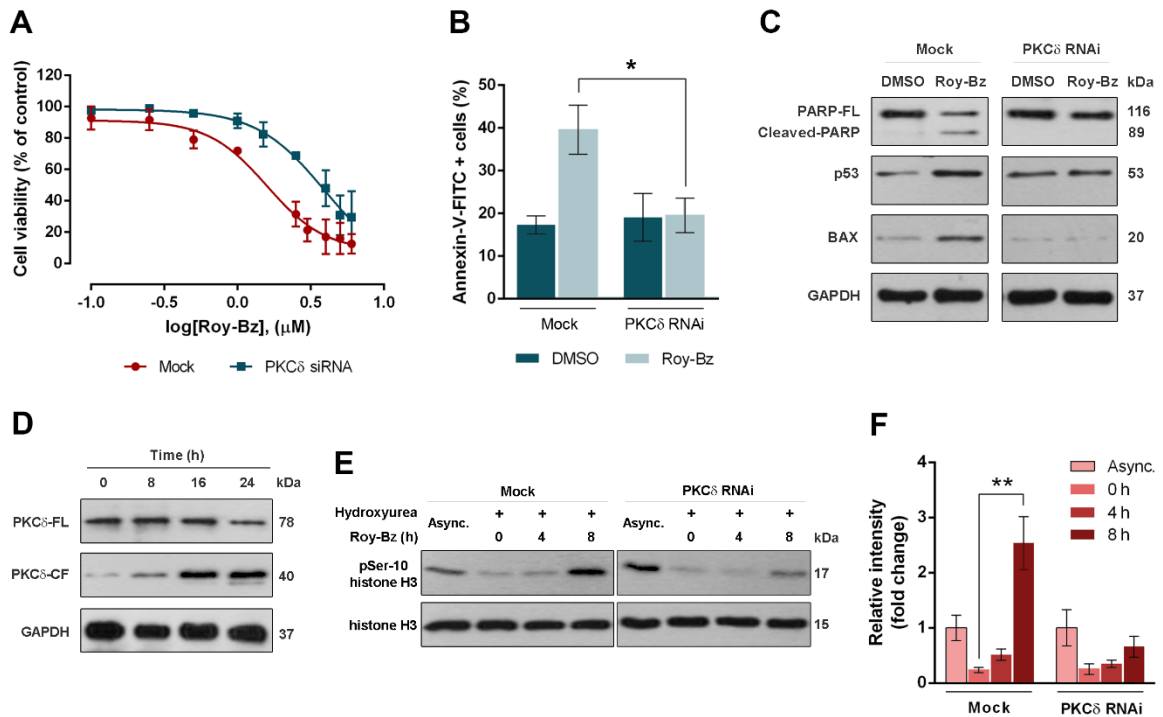μM Roy-Bz or vehicle. **(C)** Western blot analysis of PARP cleavage, p53 and BAX expression levels for 16 h treatment with 1 μM Roy-Bz or vehicle in control and PKCδ-knockdown HCT116 cells; GAPDH (loading control). **(D)** Western blot analysis of full-length PKCδ (PKCδ-FL) and cleaved PKCδ-CF in HCT116 cells treated with 1 μM Roy-Bz for up to 24 h; GAPDH (loading control). **(E,F)** Levels of histone H3 phosphorylation on Ser-10 (pSer-10) in control and PKCδ-knockdown HCT116 cells treated with 1 μM Roy-Bz for up to 8 h; histone H3 (loading control); in **F**, quantification of pSer-10 levels normalized to histone H3, values of asynchronized cells were set as 1. In **C**, **D** and **E**, immunoblots represent one of three independent experiments; in **A**, **B** and **F**, data are mean ± SEM of three independent experiments; values significantly different are indicated (*p<0.05, **p<0.01), unpaired Student's t-test.

The impact of Roy-Bz on MMP-9 and E-cadherin expression levels in colonosphere cultures led us to further investigate the effect of Roy-Bz on the migration of HCT116 cells and its dependence on PKCδ. Using a wound healing assay, we found that Roy-Bz caused a pronounced inhibition of migration. Notably, the inhibitory effect of Roy-Bz was not observed in HCT116 cells subject to PKCδ-knockdown (Fig. 3.1.8A, B). It is important to note that at the concentration of Roy-Bz used in these assays (0.25 μM), there were no significant effects on cell viability. These results were also recapitulated using a chemotaxis cell migration assay, in which the anti-migratory effect of Roy-Bz observed in control HCT116 cells was significantly prevented by RNAi silencing of PKCδ expression (Fig. 3.1.8C). Altogether, these results showed that Roy-Bz is a potent pro-apoptotic and anti-migratory agent in human colon cancer cells through PKCδ-selective activation.

3.1.5. Roy-Bz is non-genotoxic in human cancer and normal cells and has *in vivo* PKCδ-dependent antitumor activity with no apparent toxic side effects

The genotoxicity of Roy-Bz in human cancer cells was evaluated by checking comet-positive cells and histone H2AX phosphorylation on Serine-139 (γH2AX) as markers of DNA damage (single/double-strand breaks). Our results showed that, conversely to 25 μM ETOP (positive control), 48 h treatment with Roy-Bz failed to induce DNA damage in HCT116 cells, as demonstrated by the negligible number of cells with more than 5% of DNA in tail (Fig. 3.1.9A, B), by the low tail moment (Fig. 3.1.9A, C), as well as by the absence of increased γH2AX levels compared to vehicle (Fig. 3.1.9D). Additionally, Roy-Bz failed to

increase the number of micronuclei in peripheral lymphocytes of normal individuals, compared to vehicle (Fig. 3.1.9E).
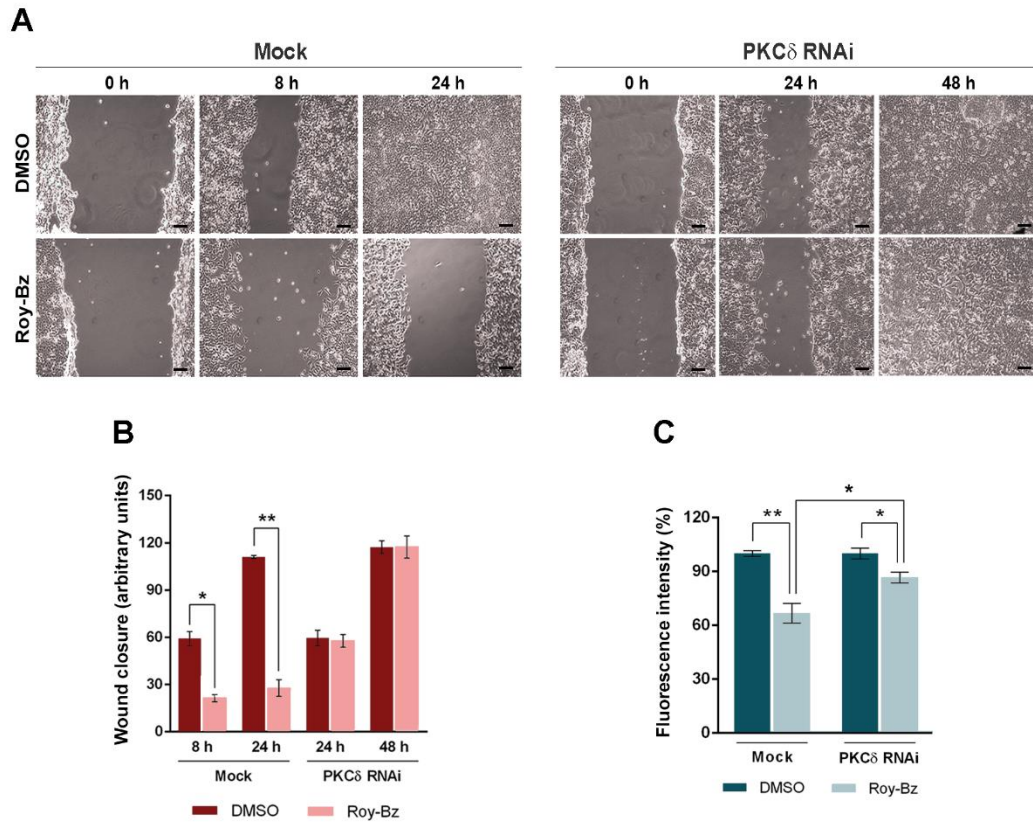
Figure 3.1.8: Roy-Bz anti-migratory activity is mediated by PKCδ-selective activation in HCT116 cancer cells. (A,B) Wound healing assay for control and PKCδ-knockdown HCT116 confluent cells treated with 0.25 μM Roy-Bz or vehicle, at different time-points; in **A**, scale bar=50 μm and magnification=100×; in **B**, quantification of wound closure in five randomly selected microscopic fields. **(C)** Chemotaxis migration assay for control and PKCδ-knockdown HCT116 cells for 24 h treatment with 0.25 μM Roy-Bz; migratory cells were quantified by fluorescence intensity, which was set as 100% for untreated cells. In **B** and **C**, data are mean ± SEM of three independent experiments; values significantly different are indicated (*$p<0.05$, **$p<0.01$), unpaired Student's t-test.
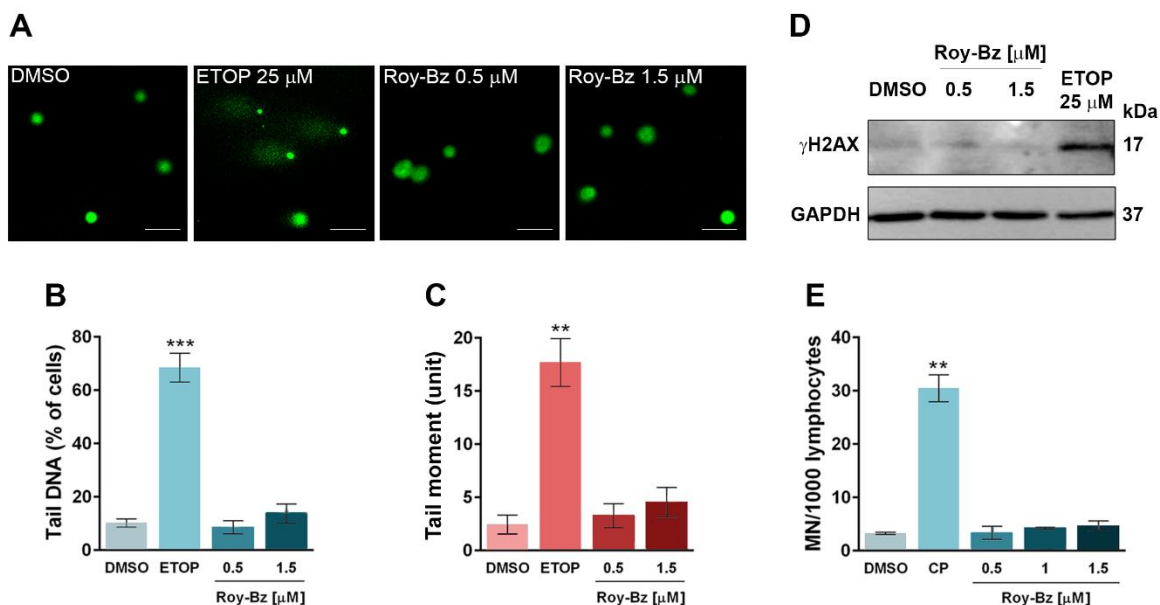
Figure 3.1.9: Roy-Bz is non-genotoxic in human cancer and normal cells. (A-D) Analysis of DNA damage was performed for 48 h treatment with ETOP, Roy-Bz or vehicle; in **(A-C)**, comet assay in HCT116 cells; in **A**, scale bar=20 μm; magnification=200×; in **B** and **C**, one hundred cells were analyzed in each group; in **D**, western blot analysis of γH2AX levels; immunoblots are representative of three independent experiments; GAPDH (loading control). **(E)** Genotoxicity of Roy-Bz evaluated by cytokinesis-block micronucleus (MN) assay for 72 h treatment, in human lymphocyte cells; 5μg/mL CP (positive control); the number of MN per 1000 binucleated lymphocytes was recorded. In **B**, **C**, and **E**, data are mean ± SEM of three independent experiments, values significantly different from vehicle (**$p<0.01$, ***$p<0.001$), unpaired Student's t-test.

We next examined potential primary toxicity signs induced by Roy-Bz treatment *in vivo*. To this end, Wistar rats were treated with 10 mg/kg Roy-Bz, vehicle, or saline solution (control) by intraperitoneal injection, twice a week during two weeks, followed by analysis of organs relative weight, biochemical and hematological data (Table 3.1.2). No differences in the relative weight of liver, kidneys, heart and spleen were observed among the three groups. Regarding biochemical data, despite the slight increase in uric acid and total proteins, compared to control groups, caused by Roy-Bz, the values of both parameters are in accordance with reference database (308, 309). These results indicated no apparent liver or kidney toxicity. Additionally, no differences in hematological data were observed between the three groups. Overall, no apparent toxic side effects were observed for Roy-Bz on the tissues most commonly affected by conventional chemotherapeutics.

Table 3.1.2. Toxicity studies of Roy-Bz in Wistar rats.

	Saline	Vehicle	Treated
Body weight and relative tissue weight (trophism)			
BW (g)	340.30 ± 13.45	361.80 ± 0.01	308.25 ± 10.51
Heart/BW (g/kg)	3.03 ± 0.03	3.01 ± 0.09	3.33 ± 0.21
Liver/BW (g/kg)	38.71 ± 2,36	38.52 ± 0.89	42.06 ± 0.98
Kidney/BW (g/kg)	7.08 ± 0.40	6.81 ± 0.27	8.07 ± 0.31
Spleen (g/kg)	2.10 ± 0.20	2.15 ± 0.12	2.93 ± 0.14
Biochemical data			
Blood glucose (mg/dL)	188.00 ± 7.51	207.20 ± 7.00	155.00 ± 22.67
Urea (mg/dL)	20.67 ± 0.77	18.34 ± 0.24*	17.95 ± 0.59
Uric acid (mg/dL)	1.07 ± 0.07	0.82 ± 0.10	2.08 ± 0.45#
Creatinine (mg/dL)	0.30 ± 0.00	0.30 ± 0.01	0.29 ± 0.01
Total Proteins (g/dL)	5.50 ± 0.00	5.35 ± 0.17	6.30 ± 0.11#
Albumin (g/dL)	3.03 ± 0.03	2.90 ± 0.05	3.13 ± 0.10
ALT (U/L)	36.00 ± 2.89	30.75 ± 3.30	45.75 ± 10.29
AST (U/L)	95.33 ± 25.36	56.25 ± 4.60	125.67 ± 41.79
Total Chol (mg/dL)	44.33 ± 1.76	52.40 ± 3.54	67.00 ± 6.10
Triglycerides (mg/dL)	107.00 ± 16.29	161.40 ± 17.55	137.00 ± 0.07
Hematological data			
RBC count (x10^6/μL)	7.10 ± 0.15	7.41 ± 0.28	7.84 ± 0.30
HGB (g/dL)	13.97 ± 0.18	13.95 ± 0.46	13.48 ± 0.54
HCT (%)	40.50 ± 0.82	42.28 ± 2.03	43.33 ± 1.89
WBC counts (x10^3/μL)	1.93 ± 0.68	2.15 ± 0.88	1.55 ± 0.49
PLT counts (x10^3/μL)	811.00 ± 29.61	793.80 ± 28.35	913.25 ± 81.96
RET counts (%)	2.80 ± 0.12	3.56 ± 0.23	4.65 ± 0.33

Data were analyzed for saline, vehicle and 10 mg/kg Roy-Bz (treated) rat groups, after four intraperitoneal administrations (twice a week). Results are mean ± SEM of four independent experiments; *$p<0.05$ (comparison between saline and vehicle groups; control groups); #$p<0.05$ (comparison between Roy-Bz and control groups), and between vehicle and treated groups). *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *BW*, body weight; *CK*, Creatine Kinase; *HCT*, hematocrit; *HGB*, Hemoglobin concentration; *PCT*, plateletcrit; *PLT*, platelet; *RBC*, red blood cell count; *RET*, reticulocytes; *WBC*, white blood cells.

The *in vivo* antitumor potential of Roy-Bz was thereafter evaluated using human tumor xenograft mouse models of control and PKCδ-knockdown HCT116 cells, following the same administration procedure conducted in the toxicological studies. A significant inhibition in the growth of control HCT116 tumors was observed after administration of Roy-Bz (10 mg/kg) compared to vehicle (Fig. 3.1.10A, left panel). Notably, the antitumor activity of Roy-Bz was lost when mice where inoculated with PKCδ-depleted HCT116 cells (Fig. 3.1.10A, right panel), further reinforcing the concept that the antitumor activity of Roy-Bz was mediated by PKCδ. Of note, no significant body weight loss or morbidity signs were observed in Roy-Bz-treated mice compared to vehicle (Fig. 3.1.10B).
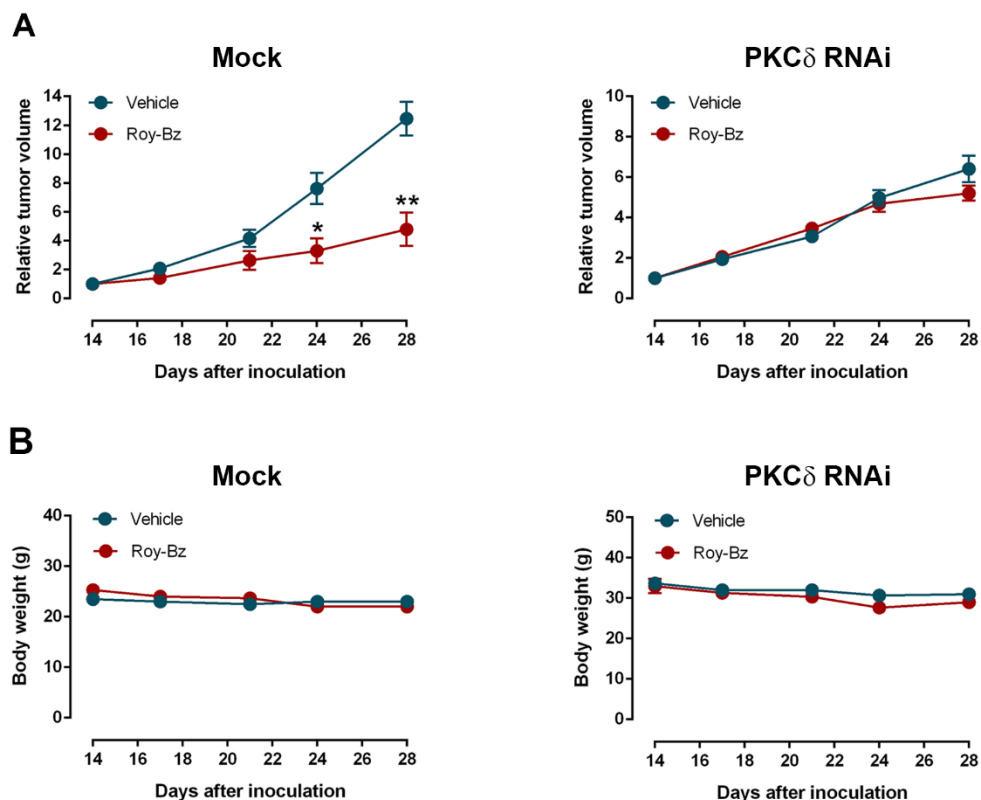
Figure 3.1.10: Roy-Bz has *in vivo* PKCδ-dependent antitumor activity with no apparent toxic side effects. (A) Growth curves for relative tumor volume of BALB/c nude mice carrying control (Mock) and PKCδ-knockdown (PKCδ RNAi) HCT116 xenografts treated with vehicle or 10 mg/kg Roy-Bz; data are mean ± SEM of tumor volume fold change to the start of treatment, values significantly different from vehicle (*$p<0.05$, **$p<0.01$), unpaired Student's *t*-test. **(B)** BALB/c nude mice body weight during vehicle or Roy-Bz treatment; no significant differences between vehicle- and Roy-Bz-treated mice: $p>0.05$, unpaired Student's *t*-test.

Lastly, proliferation, apoptosis and angiogenesis markers were checked in tumor samples from control and PKCδ-knockdown HCT116 xenografts obtained at the end of *in vivo* antitumor assays (Fig. 3.1.11A-D). In PKCδ-expressing tumor samples, Roy-Bz reduced proliferation (decrease in Ki-67-positive staining, Fig. 3.1.11A, B) and stimulated apoptosis (increase in BAX expression, caspase-3 cleavage and DNA fragmentation as demonstrated by TUNEL-positive staining, Fig. 3.1.11A, C-D), when compared to vehicle. The expression levels of VEGF were also analyzed as a readout of angiogenesis. Data showed a marked reduction in VEGF expression in samples from tumors treated with Roy-Bz relative to vehicle (Fig. 3.1.11A, C). On the other hand, no apparent differences in these markers were observed between Roy-Bz and vehicle in PKCδ-knockdown tumor samples (Fig. 3.1.11A-D). Altogether, these results demonstrated that Roy-Bz has strong *in vivo* PKCδ-dependent antitumor activity, through inhibition of proliferation and angiogenesis, and stimulation of apoptosis.
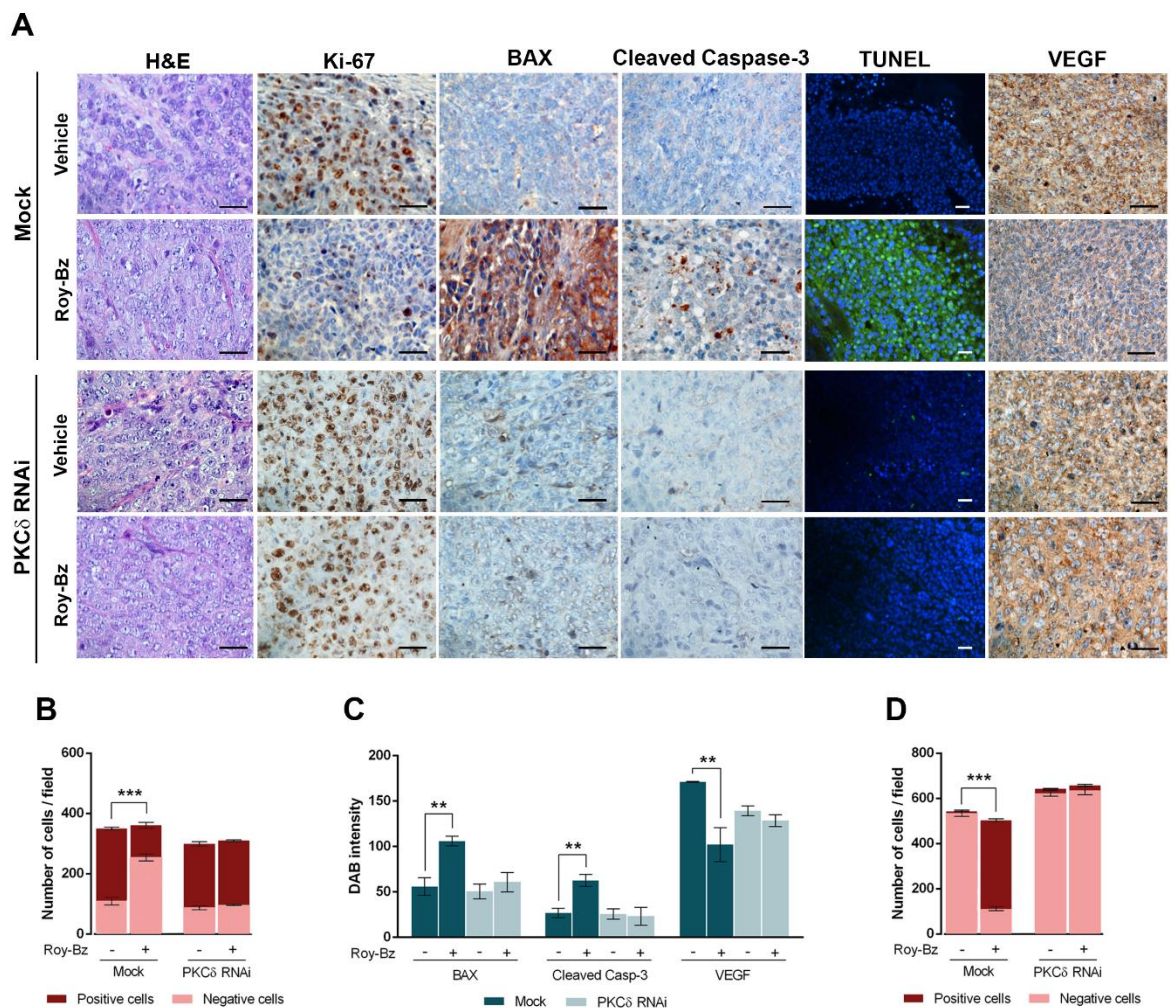
Figure 3.1.11: Roy-Bz inhibits proliferation and angiogenesis, and induces apoptosis in tumor tissues of human HCT116 xenograft mouse models. (A) Representative images of Ki-67, BAX, cleaved caspase-3, DNA fragmentation (TUNEL) and VEGF detection in tumor tissues of control and PKCδ-knockdown HCT116 xenografts treated with Roy-Bz or vehicle, collected at the end of treatment (scale bar=5 μm; magnification=400×, 200× for TUNEL). **(B-D)** Quantification of immunohistochemistry of HCT116 xenograft tumor tissues of Mock and PKCδ RNAi treated with Roy-Bz or vehicle; in **B**, quantification of the number of positive and negative Ki-67 cells; in **C**, BAX, cleaved caspase-3 and VEGF staining quantification by evaluation of 3,3'-diaminobenzidine (DAB) intensity; in **D**, quantification of the number of positive and negative TUNEL cells. In **B, C** and **D**, data are mean ± SEM of three independent experiments, values significantly different from vehicle (*p<0.05, **p<0.01, ***p<0.001), unpaired Student's t-test.

3.1.6. Discussion

Despite the critical role of PKCs in numerous human diseases, the understanding of the function of specific isozymes in a given disease remains under intense controversy due to the lack of PKC isozyme-selective modulators (76, 212). Herein, the compound Roy-Bz was identified as a potent and selective activator of PKCδ that, like phorbol esters, has the C1-domain as binding site. Additionally, it was shown that Roy-Bz has potent anticancer properties against colon cancer. Accordingly, it strongly inhibited the proliferation of colon cancer cells by triggering a PKCδ-dependent apoptotic pathway involving caspase-3 activation. The selectivity of Roy-Bz towards PKCδ in human colon cancer cells is further reinforced by the observation of the specific translocation of PKCδ, without any noticeable effect on the relocalization of other phorbol ester responsive PKC isozymes. Activation of PKCδ was also associated with the generation of a constitutive active PKCδ catalytic fragment, an effect observed in other models (116, 306), and the phosphorylation of histone H3 on Ser-10, a PKCδ-dependent event crucial in chromatin condensation during apoptosis (307).

Despite the positive role of PKCδ in migration and invasiveness in some cellular models (310, 311), it was also disclosed that PKCδ suppressed migration and the secretion of MMP-9 in highly motility breast cancer cells (153, 154). Supporting this anti-migratory activity of PKCδ, we found that Roy-Bz inhibited the migration of colon cancer cells in a PKCδ-dependent manner, with a reduction of MMP-9 and up-regulation of E-cadherin expression levels in colonospheres.

Recent studies have also demonstrated the relevant role of PKC isozymes in controlling cellular signaling of CSCs, which are critical in drug resistance, metastasis and

relapse of cancer (312). However, further studies are required since scarce data are available on this subject. Our results support a negative regulation of stemness of colon cancer cells by PKCδ. Additionally, they indicate that Roy-Bz may also target drug resistant CSCs, preventing tumor dissemination and recurrence, as evidenced by the pronounced reduction of colonosphere growth and formation, and by the depletion of the CD44 stemness marker. Interestingly, CD44 is a known downstream target of the Wnt/β-catenin pathway (313, 314), which is intricately involved in the growth and maintenance of colonospheres (315). Accordingly, our results are in line with the recently reported inhibition of the Wnt/β-catenin pathway by PKCδ to suppress proliferation of colon cancer cells (150).

Notably, a PKCδ-dependent antitumor effect of Roy-Bz, associated with anti-proliferative, pro-apoptotic, and anti-angiogenic activities, was demonstrated in xenograft mouse models. The great potential of Roy-Bz as an anticancer agent in colon cancer treatment was reinforced by the absence of genotoxicity in normal and tumor cells, as well as the lack of *in vivo* apparent toxic side effects.

PKCδ has been generally assumed as an anti-proliferative and pro-apoptotic kinase, and subsequently as a crucial death mediator of chemotherapeutic agents and radiotherapy (76, 116, 306, 316). Our experimental results with Roy-Bz in colon cancer cells are consistent with the notion that PKCδ preferentially acts as a tumor suppressor in intestinal carcinogenesis (146-150). In fact, numerous studies have shown that PKCδ inhibits cell proliferation as well as anchorage-dependent and -independent growth, and in addition it enhances differentiation of colon cancer cells (146-150). PKCδ has also been implicated in growth inhibition and apoptosis of several other cancer types, including prostate and glioma (133, 144). However, anti-apoptotic and pro-survival roles of PKCδ have also been reported in other cancer types, particularly in breast cancer (317, 318), suggesting notable differences depending on the cellular context. It has been also reported that the tumor promoting properties of PKCδ after PMA treatment may result from the loss of PKC (down-regulation) due to its chronic application, rather than to its acute activation (319). Interestingly, as observed with the PKC activator prostratin (320), Roy-Bz did not significantly affect the PKCδ stability, what may explain the tumor suppression rather than tumor promoting activity of PKCδ associated with Roy-Bz.

In conclusion, in this work Roy-Bz was identified as the first small-molecule PKCδ-selective activator, with encouraging clinical application in colon cancer therapy. Moreover, the elucidation of the structural requirements underlying its selectivity for PKCδ will be crucial to the structure-based design of other PKC isozyme-selective agents. In turn, these new agents will help in the elucidation of the specific functions of PKC isozymes in human diseases.

CHAPTER 3.2

Roy-Bz: a potential dual inhibitor of glycolysis and oxidative phosphorylation in colon cancer

In the present chapter, it was studied the impact of Roy-Bz on mitochondrial respiration and glycolysis of colon cancer.

3.2.1. Roy-Bz induces mitochondrial dysfunction with inhibition of cellular respiration in colon cancer cells

The cytotoxic effect of Roy-Bz was evaluated both in terms of cell mass and metabolic activity, using the SRB and resazurin reduction assays, respectively, as described (321). The results showed that the treatment of HCT116 cells with increasing doses of Roy-Bz induced a concomitant decrease of cell mass (Fig. 3.2.1A) and metabolic activity (Fig. 3.2.1B), for all concentrations tested when compared to vehicle.
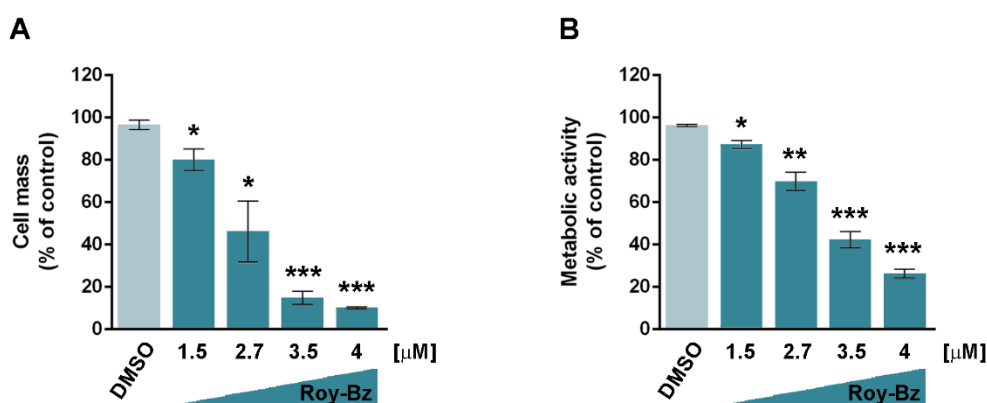
Figure 3.2.1: Roy-Bz has cytotoxic effect in colon cancer cells. (A, B) HCT116 cells were treated with 1.5 to 4 μM Roy-Bz for 48 h. In **A,** cell mass was determined using SRB assay. In **B,** metabolic activity was determined by resorufin fluorescence measurement after 6 h incubation using resazurin assay. In **A** and **B**, data are expressed as percentage of vehicle and are mean ± SEM of five independent experiments; values significantly different from vehicle: *$p<0.05$, **$p<0.01$, ***$p<0.001$, unpaired Student's t-test.

Afterwards, the involvement of the mitochondrial pathway in Roy-Bz-induced growth inihibition was studied in HCT116 cells. In these cells, Roy-Bz increased the production of mitochondrial ROS and caused $\Delta\psi_m$ dissipation (Fig. 3.2.2A). Additionally, it increased the release of cyt c to cytosol (Fig. 3.2.2B).
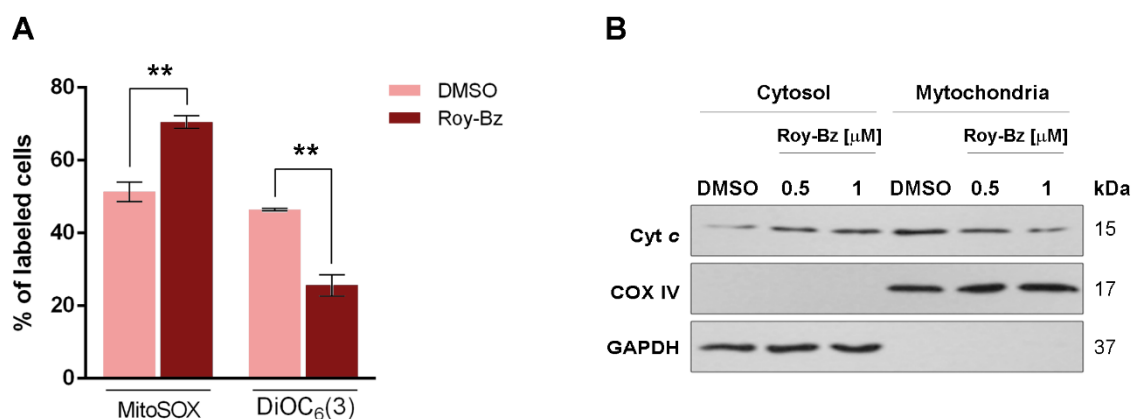
Figure 3.2.2: Roy-Bz induces mitochondrial dysfunction in colon cancer cells. (A) Mitochondrial ROS generation (MitoSOX labeled cells) and $\Delta\psi_m$ dissipation (DiOC$_6$(3) labeled cells), after 24 h and 16 h, respectively, treatment with 0.5 µM Roy-Bz, in HCT116 cells; data are mean ± SEM of four independent experiments. **(B)** Mitochondrial cyt c release in HCT116 cells after 24 h treatment with Roy-Bz; immunoblots represent one of three independent experiments; loading control of cytosolic (GAPDH) and mitochondrial (COX IV) fractions. In **A**, values significantly different from vehicle (**$p<0.01$), unpaired Student's t-test.

Based on these data, it was hypothesized that Roy-Bz may directly interfere with mitochondrial activity. As such, we next investigated the effect of Roy-Bz on mitochondrial respiration using a Seahorse Bioanalyzer to measure the OCR, a surrogate readout of mitochondrial function. As shown in Fig. 3.2.3A, increasing doses of Roy-Bz (from 1.5 to 4 µM) resulted in a dose-dependent decrease in OCR. Thereafter, different parameters associated with mitochondrial respiration were real-time assessed in cells treated with Roy-Bz, through sequential administration of complex inhibitors (Fig. 3.2.3B). Particularly, it was used oligomycin (complex V inhibitor) to determine the amount of ATP production linked to respiration, FCCP (mitochondrial uncoupler) to measure the maximal respiratory capacity, and a combination of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) to determine the reserved respiratory capacity. Impressively, with exception of non-mitochondrial respiration, Roy-Bz impaired all the parameters evaluated, including maximal respiration, spare respiratory capacity and ATP production, when compared to vehicle (Fig. 3.2.3C).
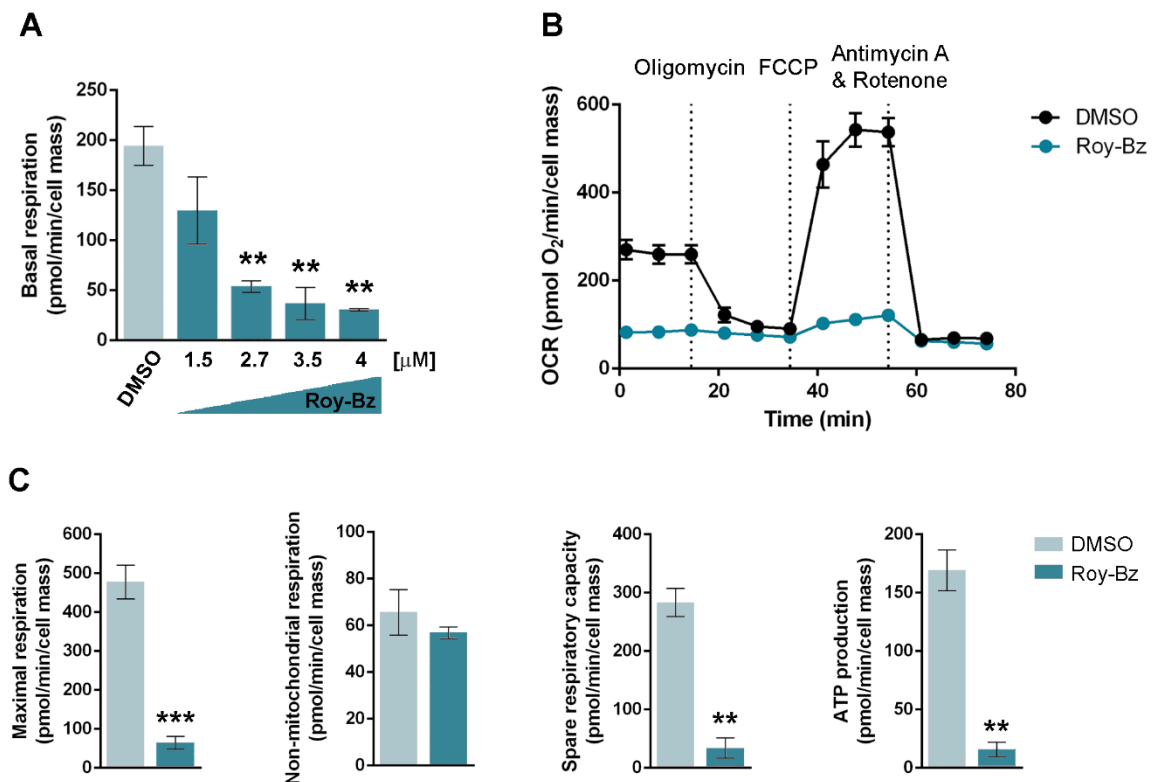
Figure 3.2.3: Roy-Bz inhibits cellular respiration in colon cancer cells. (A-C) The Seahorse XFe96 Extracellular Flux Analyzer was used to measure OCR. In **A**, measurement of basal cell respiration (prior to oligomycin addition minus non-mitochondria-derived OCR) after treatment with 1.5 to 4 μM Roy-Bz (or DMSO only) for 48 h. In **B**, baseline measurements of OCRs prior to sequential administration of oligomycin, FCCP, and a combination of rotenone and antimycin A; data correspond to treatments with 4 μM Roy-Bz (or DMSO only) for 48 h. In **C**, measurement of several OCR parameters: maximal respiration (maximal measurement after FCCP addition minus non-mitochondria-derived OCR), non-mitochondrial respiration (minimal measurement after rotenone and antimycin A addition), spare respiratory capacity (maximal respiration minus basal respiration), and ATP production (basal respiration minus proton leak); data correspond to treatments with 4 μM Roy-Bz (or DMSO only) for 48 h. In **A** and **C**, data are mean ± SEM of three independent experiments; values significantly different from vehicle: *$p<0.05$, **$p<0.01$, ***$p<0.001$, unpaired Student's t-test.

3.2.2. Roy-Bz inhibits glycolysis in tumor tissues of human HCT116 xenografts

The anti-glycolytic potential of Roy-Bz was studied in tumor tissues of HCT116 xenografts, obtained at the end of the *in vivo* antitumor assays described in Chapter 3.1. To this end, the expression of some glycolytic markers was assessed by immunohistochemistry (Fig. 3.2.4). Consistently with an inhibition of glycolysis, Roy-Bz significantly reduced the expression levels of GLUT1, hexokinase 2 (HK2) and

monocarboxylate transporter 4 (MCT4), and increased *TP53*-induced glycolysis and apoptosis regulator (TIGAR) levels, when compared to vehicle (Fig. 3.2.4A and B). The results obtained showed that Roy-Bz had *in vivo* anti-glycolytic activity.
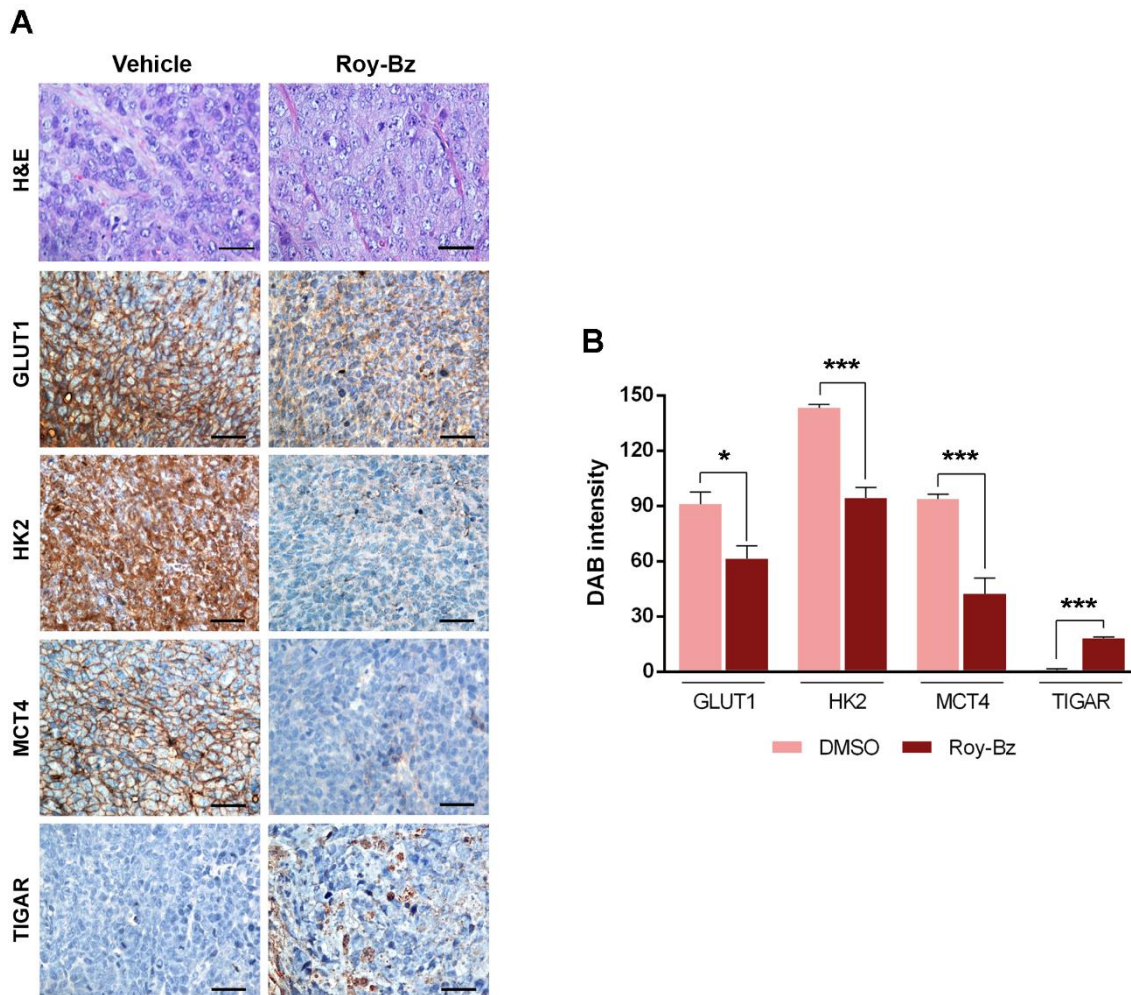
Figure 3.2.4: Roy-Bz has anti-glycolytic activity in tumor tissues of human HCT116 xenograft mouse models. (A) Representative images of GLUT1, HK2, MCT4 and TIGAR detection in tumor tissues of HCT116 xenografts treated with 10 mg/kg Roy-Bz or vehicle, collected at the end of the treatment (scale bar=5 μm; magnification=400×). **(B)** Quantification of immunohistochemistry of HCT116 xenograft tumor tissues by evaluation of DAB intensity; data are mean ± SEM of three independent experiments, values significantly different from vehicle ($*p < 0.01$, $***p < 0.001$), unpaired Student's *t*-test.

3.2.3. Discussion

In this chapter, it was shown that Roy-Bz stimulated a mitochondrial pathway in colon cancer cells, increasing mitochondrial ROS production, depolarization of $\Delta\psi_m$, and release of cyt c to cytosol. Moreover, Roy-Bz greatly decreased the OCR of colon cancer cells, with simultaneous reduction of ATP synthesis and maximal respiration. These results strongly supported an inhibition of mitochondrial respiration by Roy-Bz. It is possible to hypothesize that the mechanism of action of Roy-Bz may involve the inhibition of the ETC, particularly complexes I/III and/or V, which are directly related with alterations in mitochondrial parameters measured. Being Roy-Bz a PKCδ-selective activator, these results also supported an involvement of PKCδ in the impairment of mitochondrial activity. This is in accordance with a previous work, which reported the PKCδ interaction with mitochondria, thereby regulating mitochondrial metabolism (322).

Interestingly, *in vivo*, Roy-Bz also inhibited glycolysis, inducing the downregulation of enzymes directly implicated in glucose metabolism, including GLUT1 (involved in the uptake of glucose into the cell), HK2 (that catalyzes the first committed step of the glycolytic flux with phosphorylation of glucose to glucose-6-phosphate), and MCT4 (that mediates the export of lactate). Additionally, Roy-Bz upregulated TIGAR, which is a negative regulator of the glycolytic flux through inhibition of phosphofructose kinase 1 (PFK1) and subsequent accumulation of glucose-6-phosphate, a substrate of glucose-6-phosphate dehydrogenase (G6PDH), thereby redirecting the glucose to nucleic acid synthesis.

Although further studies are still required to validate the results obtained in the present chapter, they strongly support the ability of Roy-Bz to target both ATP producing pathways of glucose metabolism. In fact, this dual-inhibitory effect on glucose metabolism was already described for metformin (as described in introductory section).

As a whole, with this work, the anticancer potential of Roy-Bz was further reinforced by showing its ability to also target the glucose metabolism of cancer cells. The exploitation of its potential as dual inhibitor of glycolysis and OXPHOS in combination therapy with other metabolic drugs or chemotherapeutic agents holds great promise in cancer therapy.

CHAPTER 3.3

Exploring the anticancer potential of *Plectranthus* genus

Garcia, C., Roque, L., Rebelo, A., Borozan, A., Ntungwe, E., Andrade, J.M., **Bessa, C.**, Saraiva, L., Lanza, A.D., Reis, C.P., Stankovic, T., Dinic, J., Roberto, A., Pesic, M., Duarte, N., Rijo, P.

Abietane diterpenoids are the major constituents of some species of the *Plectranthus* genus, and the main responsible for the therapeutic value of these plants (323, 324). In fact, these natural occurring compounds exhibit a plethora of biological activities, including cytotoxic and anti-proliferative effects in human cancer cells (323, 324). This has encouraged the exploitation of *Plectranthus* species as a source of potential anticancer agents. In addition, the discovery of the semi-synthetic derivative Roy-Bz, obtained from a natural diterpenoid isolated from *P. grandidentatus*, with potent anticancer activity, led us to search for new anticancer agents in *Plectranthus* species. Particularly, it was evaluated the *in vitro* anti-proliferative activity of the abietane diterpenoid 6,7-dehydroroyleanone (DeRoy; Fig. 3.3.1), obtained from the *P. madagascariensis,* and of their synthetic derivatives, 6,7-dehydro-12-*O*-pro-2-yn-1-yloxy-royleanone (FRP19) and 6,7-dehydro-12-*O*-(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyl)oxy)-royleanone (FRP20), in a panel of human cancer cell lines. Additionally, it was evaluated the anti-proliferative activity of other extracts obtained from *Plectranthus* spp. plants.
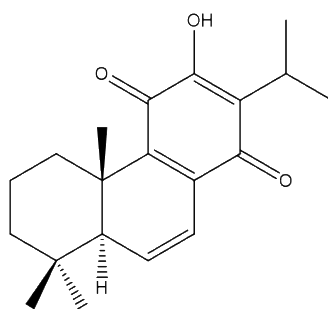
Figure 3.3.1: Chemical structure of abietane diterpenoid 6,7-dehydroroyleanone.

3.3.1. Anti-proliferative activity of DeRoy and synthetic derivatives in human cancer cells

Human colon adenocarcinoma HCT116, breast adenocarcinoma MCF-7, and NSCLC NCI-H460 cells were treated with 1.56 - 50 μM DeRoy, FRP19 or FRP20 for 48 h, and the tumor growth inhibitory activity was evaluated by SRB assay. The IC_{50} values obtained showed that DeRoy was an inhibitor of the growth of the three cancer cell lines (Table 3.3.1). These results led us to test two derivatives of DeRoy (FRP19 and FRP20), with different alkyl substituents based on the C-12 hydroxyl group of the starting material, as described (292). However, contrary to what was expected, the obtained derivatives did not show

improved *in vitro* anticancer activity, being less potent than the starting natural material, in all tested cancer cell lines.

Table 3.3.1. IC$_{50}$ values of DeRoy and derivative compounds in human cancer cells.

Compound	IC$_{50}$ [μM]		
	HCT116	MCF-7	NCI-H460
DeRoy	6.68 ± 1,37	6.12 ± 1,62	3.23 ± 0.11
FRP19	9.72 ± 0,39	10.51 ± 0,41	10.91 ± 0.05
FRP20	≥ 50	≥ 50	≥ 50

Results were obtained by SRB assay after 48 h treatment. Data are mean ± SEM of four independent experiments.

3.3.2. Anti-proliferative activity of *Plectranthus* spp. extracts in human cancer cells

The anti-proliferative activity of 16 extracts, obtained from different *Plectranthus* spp. plants, was studied in human HCT116, MCF-7 and NCI-H460 cells. These human cancer cells were treated with 1.56 – 50 μg/mL plant extracts for 48 h, and the tumor growth inhibitory activity was evaluated by SRB assay. The IC$_{50}$ values obtained for each extract (Table 3.3.2), showed that the majority of the extracts tested were able to inhibit the growth of the three human cancer cells (Table 3.3.2). In fact, only the extract P.R-A. was ineffective in all tested cancer cells. P.M.L-A. and P.ST-A. were the extracts with the highest anti-proliferative activity (IC$_{50}$ values lower than 10 μg/mL), followed by extracts P.S-A., P.HE-A., P.HA-A., P.A-A., and P.AB-A. (IC$_{50}$ values lower than 20 μg/mL), in all tested cancer cells. Interestingly, while HCT116 cells showed to be quite sensible, MCF-7 cells proved to be rather resistant to *Plectranthus* spp. extracts. Particularly, P.Z-A. was quite effective against HCT116 and NCI-H460 cells, while presenting low anti-proliferative activity against MCF-7 cells. An interesting exception occurred with the P.E.A. extract, which was ineffective against NCI-H460 cells and presented a high anti-proliferative activity against HCT116 and MCF-7 cells.

Table 3.3.2. IC$_{50}$ values of *Plectranthus* spp. extracts in human cancer cells.

Extract	IC$_{50}$ [µg/mL]		
	HCT116	MCF-7	NCI-H460
P.M.L-A.	5.72 ± 0.59	3.47 ± 0.15	5.39 ± 0.48
P.ST-A.	3.78 ± 0.49	8.35 ± 0.57	8.75 ± 0.70
P.S-A.	6.64 ± 0.12	15.13 ± 0.85	17.43 ± 0.89
P.HE-A.	10.72 ± 0.86	14.14 ± 0.07	15.53 ± 1.74
P.HA-A.	13.41 ± 0.46	16.57 ± 1.20	16.09 ± 2.10
P.A-A.	16.67 ± 0.10	14.59 ± 1.70	15.45 ± 0.41
P.AB-A.	11.74 ± 1.78	13.38 ± 2.56	18.27 ± 0.84
P.J-A.	4.99 ± 0.20	12.85 ± 1.76	22.56 ± 0.46
P.O-A.	18.25 ± 2.65	16.55 ± 0.19	27.52 ± 2.11
P.MV-A.	17.23 ± 1.87	13.37 ± 1.49	41.22 ± 0.18
P.M-A.	24.29 ± 1.00	27.23 ± 1.27	25.90 ± 2.26
P.E-A.	4.35 ± 0.08	12.23 ± 0.90	≥ 50
P.Z-A.	8.08 ± 0.19	≥ 50	10.76 ± 2.03
P.BA-A.	10.58 ± 1.01	≥ 50	31.79 ± 0.31
P.F-A.	29.05 ± 0.63	≥ 50	34.88 ± 1.06
P.R-A.	≥ 50	≥ 50	≥ 50

IC$_{50}$ values were determined after 48 h treatment using the SRB assay. Data are mean ± SEM of four independent experiments.

3.3.3. Discussion

For many years, *Plectranthus* species have been recognized by their medicinal relevance in the treatment of diverse diseases (282). In a previous work, the phytochemical analysis of *P. madagascariensis* identified the abietane diterpenoid DeRoy as the main component of the essential oil of this species (291). Previous studies with this natural occurring compound demonstrated its potent cytotoxic and antioxidant activity (325).

In the present work, it is evidenced its anti-proliferative activity against human cancer cells. Further studies are still required to elucidate the molecular mechanism of action underlying its tumor growth inhibitory activity. Despite this, DeRoy revealed to be a

promising starting material for the development of a library of new compounds with improved *in vitro* antitumor activity. However, the strategy used to modify the DeRoy scaffold was not well-succeeded, since all the synthesized derivatives exhibited lower antiproliferative activity against human cancer cells than the starting material.

Regarding to *Plactranthus* spp. extracts, the majority of the extracts tested in this work revealed growth inhibitory effect against human cancer cells. Particularly, P.M.L-A. and P.ST-A. showed to be promising sources of new potential anticancer drug candidates. Consequently, further studies will be performed with these extracts in order to identify the bioactive compounds.

As a whole, the results herein obtained reinforced the therapeutic value of *Plectranthus* genus as an attractive source of new anticancer agents, and identified new compounds and raw materials that may conduct to the discovery of new effective anticancer agents.

CHAPTER 4

General Discussion

Final conclusions and Future perspectives

Despite an evident progress in diagnosis and treatment, the worldwide cancer mortality is increasing every year (1). Great efforts have been made to improve the efficacy of anticancer drugs in clinical use. However, this has shown to be a challenge, as demonstrated by the disappointing results achieved in the clinical translation of an impressive number of new drugs.

As a critical player in carcinogenesis, PKC early became an attractive therapeutic target in cancer (68, 212). For long, PKC-targeting drugs were developed based on the assumption that PKC positively contributes to cancer development and progression. Therefore, many PKC inhibitors were tested in clinical trials with no success (212). More recently, cumulating data have supported that PKC primarily function as tumor suppressor, indicating that PKC-targeting strategies should be focused on restoring, rather than inhibiting, PKC activity (77). On the basis of all controversy, it is the inability of addressing the isozyme-specific function in the different cancer subsets due to the reduced specificity of available pharmacological PKC modulators.

With this thesis, great advances were reached concerning the PKC pharmacology and cancer therapy with the discovery of a new:

i) PKC isozyme-selective agent

The identification of isozyme-selective PKC modulators has been the main determining factor for the success of PKC-targeting therapy. In this thesis, a previously developed yeast-based PKC screening assay (275) was used to analyze the effect of a small chemical library of royleanone derivatives on PKC activity (Chapter 3.1). Using this approach, the compound Roy-Bz was identified as a potential PKCδ-selective activator. The selective activation of PKCδ by Roy-Bz was thereafter confirmed in an *in vitro* kinase assay using recombinant PKC isozymes and in human colon cancer cell lines (Chapter 3.1). Additionally, molecular docking studies supported the binding of Roy-Bz to the PKCδ-C1-domain (Chapter 3.1).

ii) Anticancer drug candidate by targeting PKC

The anticancer potential of the PKCδ-selective activator Roy-Bz was also validated in the present thesis (Chapter 3.1 and 3.2). In colon cancer cells, Roy-Bz potently inhibited the proliferation by inducing an apoptotic cell death (Fig. 5.1) and presented an anti-migratory effect, in a PKCδ-dependent manner. It also impaired the colonosphere growth and formation with depletion of stemness markers. Notably, *in vivo*, Roy-Bz showed a PKCδ-dependent antitumor activity, with no apparent toxic side effects. Beyond these results, it was demonstrated that Roy-Bz potentially targets tumor glucose metabolism by

dual inhibition of glycolysis and OXPHOS (Chapter 3.2). Altogether, these data demonstrated that Roy-Bz was a promising anticancer drug candidate.
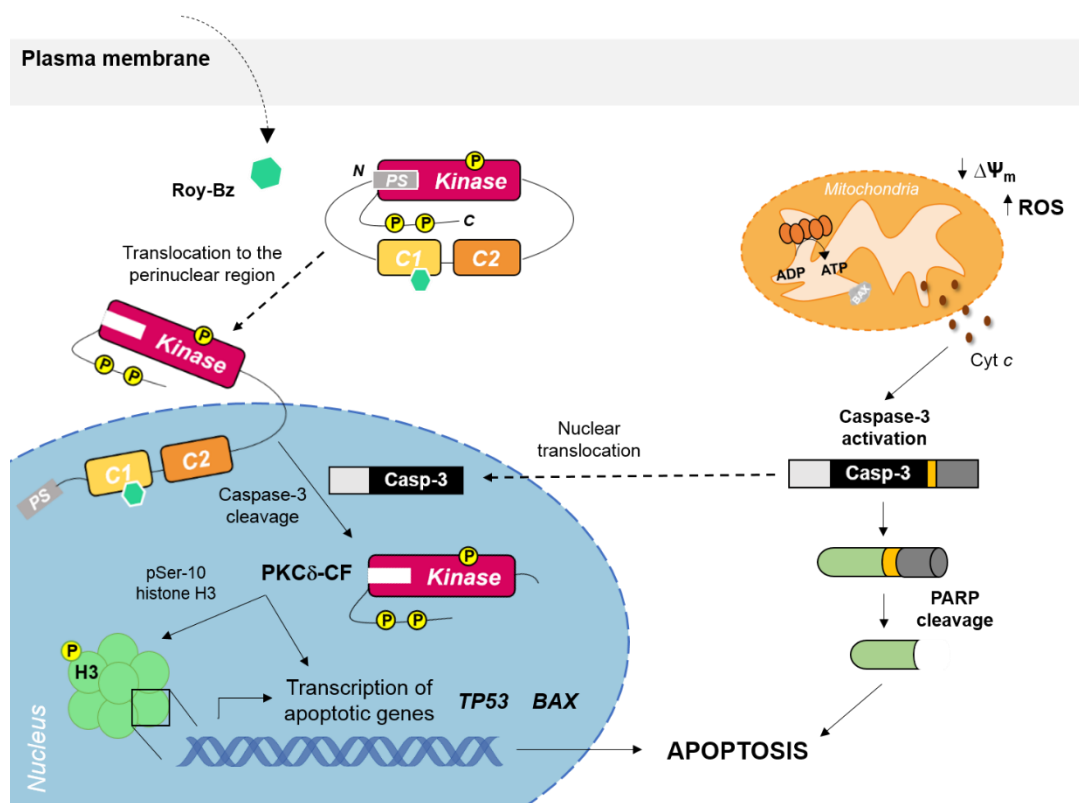
Figure 5.1: Putative molecular mechanism of action of Roy-Bz-induced apoptosis. Roy-Bz binds to the PKCδ-C1 domain, inducing its translocation to the perinuclear region. In the nucleus, PKCδ is cleaved by caspase-3, resulting in the generation of the PKCδ-CF, which in turn phosphorylates nuclear substrates, such as of histone H3 on Ser-10, that promotes apoptosis. Additionally, the transcription of apoptotic genes, namely *TP53* and *BAX*, activates a mitochondrial apoptotic pathway, with subsequent cyt *c* release, activation of caspase-3, PARP cleavage, and subsequent apoptosis induction.

Final conclusions and future perspectives

The establishment of Roy-Bz as the first small-molecule selective activator of PKCδ may contribute to unravel fundamental aspects of the pharmacology of PKCδ. In fact, besides the elucidation of the structural requirements underlying the selectivity of compounds to PKCδ, Roy-Bz will improve the structure-based design of new PKC isozyme-selective agents. Roy-Bz may also provide relevant insights concerning the PKCδ biology

in human diseases, including cancer. Most importantly, encouraging applications can be envisaged for Roy-Bz in cancer therapy, particularly in the treatment of CRC that is a leading cause of cancer incidence and death worldwide (3). Actually, cumulated data have shown reduced PKCδ levels in colon cancer tissues, compared to normal ones (146), and have demonstrated a tumor suppressive role of PKCδ in colon cancer (146-150). The therapeutic value of Roy-Bz in CRC is further emphasized by the limited therapeutic options available for the treatment of patients with advanced or metastatic CRC. Currently, the first-line treatment for metastatic CRC is still based on conventional chemotherapeutic agents, such as irinotecan or oxaliplatin combined with a fluoropyrimidine and leucovorin (FOLFIRI or FOLFOX regimens), with modest therapeutic efficacy (326-328). The PKCδ-targeting therapy with Roy-Bz, alone or in combination with other therapeutic agents, may represent a new hope in CRC treatment. However, besides CRC, other types of cancer in which PKCδ has been implicated in growth inhibition and apoptosis induction, such as prostate and glioma, may also benefit from Roy-Bz therapy (133, 144).

Finally, in this thesis it is reinforced the potential of *Plectranthus* genus as a promising source of new compounds with anticancer activity (Chapter 3.3). In particular, it was identified one abietane diterpenoid (DeRoy) and two extracts (P.M.L-A. and P.ST-A.) with anti-proliferative activity in distinct cancer cell lines. These data may give rise to additional works with the subsequent identification of more effective anticancer drugs.

In conclusion, with the discovery of Roy-Bz, it is possible to open the way to a new era on PKC biology and pharmacology. Notably, Roy-Bz will contribute to the redefinition of PKC isozymes as feasible therapeutic targets in precision medicine. Besides this, the work carried out under this thesis gives rise to new therapeutic opportunities in cancer treatment by providing a new effective anticancer drug candidate.

CHAPTER 5

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Appendix

Fig. 1. PKCδ expression levels in yeast cells
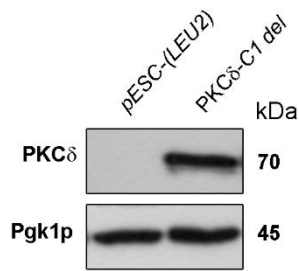
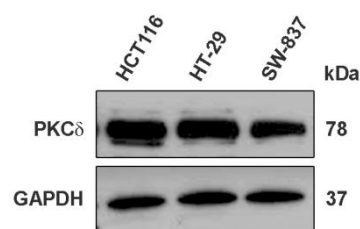

Western blot analysis of PKCδ expression levels in yeast cells transformed with pESC-(*LEU2*) (empty vector) or pESC-PKCδΔC1cells after 48 h incubation in selective induction medium. Immunoblot represents one of three independent experiments; Pgk1p was used as loading control.

**Fig. 2. PKCδ-knockdown in HCT116 colon cancer cells**
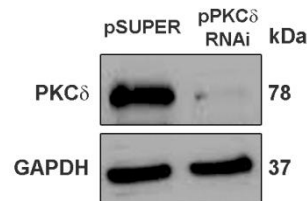
Efficiency of PKCδ-knockdown was assessed by Western blot analysis after 24 h post-transfection with control plasmid (pSUPER) or pSuperPKCδ.RNAi. Immunoblots represent one of three independent experiments; GAPDH was used as loading control.

Fig. 3. PKCδ expression levels in colorectal cancer cells

Western blot analysis of PKCδ expression levels assessed in HCT116, HT-29 and SW-837 cells after 48 h incubation. Immunoblots represent one of three independent experiments; GAPDH was used as loading control.

Fig. 4. PKCδ expression levels in colorectal cancer cells
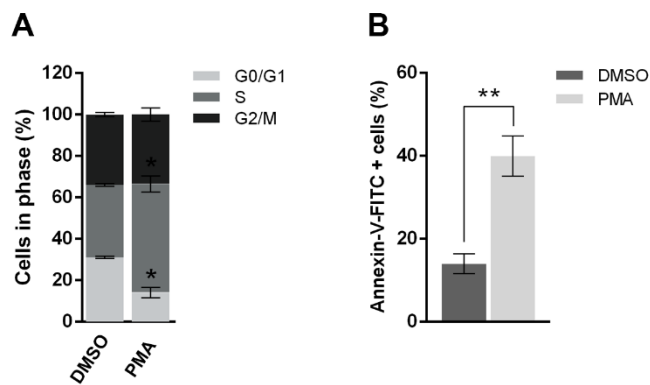
(**A**) Cell cycle (**B**) and apoptosis were analyzed after 24 h treatment with 8 μM PMA or vehicle in HCT116 cells. Data are mean ± SEM of four independent experiments; values significantly different from vehicle: (*p<0.05, **p<0.01), unpaired Student's t-test.

Table 1: Antibodies used in Western blot.

Antigen	Final Dilution	Supplier
Primary antibodies		
Caspase-3 (#9662) (rabbit polyclonal)	1:300	Cell Signaling Technology (Werfen/IZASA, Carnaxide, Portugal)
Phospho-Histone H3 (Ser10) (#9701) (rabbit polyclonal)	1:100	
Histone H3 (#9715) (rabbit polyclonal)	1:1000	
PKCδ (G-9) (mouse monoclonal)	1:300	Santa Cruz Biotechnology (Frilabo)
PARP (C2-10) (mouse monoclonal)	1:1000	
p53 (DO-1) (mouse monoclonal)	1:5000	
BAX (2D2) (mouse monoclonal)	1:100	
Bcl-2 (C-2) (mouse monoclonal)	1:200	
Cytochrome *c* (A-8) (mouse monoclonal)	1:50	
HCAM (CD44) (F-4) (mouse monoclonal)	1:200	
COX IV (F-8) (mouse monoclonal)	1:500	
GAPDH (6C5) (mouse monoclonal)	1:10000	
Survivin (ab76424) (rabbit monoclonal)	1:10000	Abcam (Cambridge, United Kingdom)
ALDH2 (ab108306) (rabbit monoclonal)	1:1000	
MMP-9 (ab38898) (rabbit polyclonal)	1:200	
Histone γH2AX (phospho-Ser139) (rabbit monoclonal)	1:50	
Secondary antibodies		
Anti-mouse horseradish-peroxidase (HRP)-conjugated	1:5000	Santa Cruz Biotechnology
Anti-rabbit horseradish-peroxidase (HRP)-conjugated	1:5000	

Table 2: Antibodies used in immunohistochemistry.

Antigen	Final Dilution	Supplier
Primary antibodies		
Caspase-3 (AB3623) (rabbit polyclonal)	1:100	
HK2 (MABN702) (mouse monoclonal)	1:50	Merck Millipore
TIGAR (AB10545) (rabbit polyclonal)	1:200	
Ki-67 (SP6) (rabbit monoclonal)	1:200	
BAX (6A7) (mouse monoclonal)	1:200	Pierce Thermo Scientific
VEGF (VG1) (mouse monoclonal)	1:200	
GLUT1 (ab652) (rabbit polyclonal)	1:200	Abcam
MCT4 (H-90) (rabbit polyclonal)	1:50	Santa Cruz Biotechnology