The Pros and Cons of Targeting Protein Kinase c (PKC) in the Management of Cancer Patients

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Abstract: In the last years, PKC has become an attractive target for the treatment of cancer patients given its widely described role in carcinogenesis and tumor promotion. Despite the extensive research conducted on these phorbol ester receptors there is only limited knowledge about the contribution of each individual PKC isozyme in malignant transformation, mainly due to the different roles of each isozyme and their tissue-specificity. This diversity provides the unique opportunity to develop specific pharmacological agents, but the complex nature of the signaling pathways activated by different PKCs challenges selective drug therapies. Currently, several classes of PKC inhibitors including small molecule kinase inhibitors, biologic modulators, and anti-sense oligonucleotides are being evaluated for the treatment of different cancers where PKC isozymes were found to be deregulated as lung, colon, skin, prostate, and breast malignancies. In this article we will review which PKC isoforms are deregulated in different human cancers and summarize the mechanism of action of some of the major PKC modulators, analyzing the strengths and weaknesses of each one in the clinical setting.

Keywords: Cancer, clinical trials, PKC.

INTRODUCTION

Phorbol esters are natural products that have attracted the scientist's attention due to their high potency as tumor promoters in the multi-stage model of mouse skin carcinogenesis. In the early 1980s, protein kinase C (PKC) was identified as the high affinity receptor responsible for the carcinogenic effect of these products [1], becoming a major player in the oncology research field. Later, it was shown that the three-dimensional conformation of phorbol esters was analogous to that of diacylglycerol (DAG), a phospholipidic second messenger that endogenously activates PKC [2].

The PKC family comprises 11 structurally-related isozymes of phospholipid-dependent serine/threonine kinases that influence diverse cell functions through phosphorylation of target proteins. Since every isoform has different substrate specificity, each one modulates an overlapping but different array of fundamental physiological processes including proliferation, differentiation, apoptosis, and malignant transformation [3-5].

Over the last two decades, abundant evidence has emerged demonstrating the deregulation of PKC activity and /or expression in several malignancies uncovering a central role for PKC in the signaling pathways leading to tumor progression [4, 6-8]. Consequently, PKC has become a therapeutic target for the treatment of cancer. Nevertheless, it is still necessary a better understanding of the specific role of each isozyme in the modulation of the different steps leading to malignant transformation.

In recent years, substantial advances in this subject where achieved allowing the development of isoform-specific therapeutic tools that, in the form of small molecules [9-13] and *antisense oligodeoxynucleotides* [14, 15], specifically modulate the kinase activity or the expression of different PKC isozymes.

PKC CLASSIFICATION AND STRUCTURE

Based on their primary sequence and cofactor dependency, this kinase family can be classified into three distinct categories [16, 17]. *Classical* PKC isozymes (α, β, and γ), which are responsive to both, elevated intracellular Ca^{2+} levels and the second messenger DAG (or phorbol esters); *novel* PKC isozymes (δ, ε, η, and θ), that are activated by DAG but are Ca^{2+} insensitive; and *atypical* PKC isozymes (ζ and λ/ι), which are unresponsive to Ca^{2+} and DAG [3, 5, 18] and are mainly regulated by trans-phosphorylation mechanisms. These differences in co-factor dependency are considered to be a direct consequence of their distinct three-dimensional conformation.

All PKC isozymes are composed of two functional domains: an N-terminal regulatory region, and a C-terminal catalytic region. The function of the regulatory region is to keep the enzyme in an inactive conformation. This inhibition involves binding of the catalytic region to a motif named pseudo-substrate that resembles the three-dimensional conformation of the specific substrate. The inactive conformation is achieved due to a hinge region (V3 domain), that links the regulatory to the catalytic domain. This inhibition of the kinase activity exerted by the regulatory region is released upon the binding of an activator.

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The structure of classical PKCs includes four conserved domains (referred as C1–C4) interrupted by five variable regions (V1-V5). The C1 region contains cysteine-rich zinc-finger-like motifs responsible for phosphatidylserine, DAG and phorbol esters binding. Immediately after the C1 domain there is the autoinhibitory pseudosubstrate sequence. The C2 region is rich in acidic residues and binds Ca^{2+}. The C3 and C4 regions form the ATP- and substrate-binding lobes. Novel PKCs lack the C2 region and therefore are Ca^{2+} insensitive. On the other hand, atypical PKCs having only one cysteine-rich zinc-finger-like motif, are dependent on phosphatidylserine but not affected by DAG, phorbol esters or Ca^{2+}. The differences among isozymes structure are summarized in Fig. (**1**).

PKC ACTIVITY REGULATION

As mentioned above, the increment of intracellular Ca^{2+} and DAG production are involved in classical PKCs activation. The increased level of intracellular Ca^{2+} favors the binding of classical PKCs to cell membranes in a process called pre-targeting. This cation acts synergistically with DAG; a well known lipidic second messenger, generated in the plasma membrane in response to different extracellular stimuli. Binding of DAG to the C1 domain of PKC increases the affinity for phosphatidylserine, stabilizing the PKC-membrane interaction. This sequence of events leads to a massive conformational change that exposes the kinase domain and allows the phosphorylation and activation of downstream effectors [19]. In this sense, due to the lack of calcium pre-targeting, novel PKC isoforms translocate to membranes in a slower manner than classical PKCs. A schematic representation of classical PKC activation is depicted in Fig. (**2**).

After activation of different cell membrane receptors, DAG levels increase rapid and transiently. This peak may be followed by a second and more sustained increase in DAG production. This biphasic effect may be related to the production of DAG by two different sources; the first wave is dependent on phospholipase C activity and the second, responsible for the prolonged phase of PKC activation, would depend on phospholipase D action.

The mechanism of PKC activation by phorbol esters is similar to the above mentioned for DAG. In fact, phorbol esters and DAG interact with PKC in the same domain since both activators bind to cysteine-rich regions. However, phorbol esters are more potent and metabolically more stable than DAG, resulting in a longer-lasting PKC activation.

A prerequisite for PKC activation is that it must be phosphorylated in order to be able to become active [20]. This process, termed PKC maturation, involves three phosphorylation steps. The first one is performed by a PKC kinase named phosphoinositide-dependent kinase 1 (PDK1). After that, PKC is autophosphorylated on two sites at the C-terminus end, one at the turn motif (Thr-638 in human PKC) and the other at the hydrophobic site (Ser-657 in human PKC). This first autophosphorylation step is involved in the stabilization of the enzyme while the second is important for the release of the newly synthesized PKC to the cytosol. Mature PKC may now be activated by DAG and Ca^{2+}.

A sustained activation of PKC results in the downregulation of the protein levels through a poorly understood mechanism. Ubiquitin/proteasome-dependent and independent pathways have been described for PKC degradation [21].

PKC DOWNSTREAM SIGNALING PATHWAYS

Upon PKC activation, several signaling cascades are triggered. These different signaling pathways modulate various physiological processes primary leading to cell proliferation or apoptosis modulation. Consequently, PKC deregulation could induce malignant transformation. However, it is important to recognize that PKC isoforms present redundant, complex and even opposing effects in tumor biology.

The main pathway activated by PKC is the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) / extracellular receptor kinase (ERK) pathway, widely implicated in the transcription of genes involved in cell proliferation. Upon activation, PKCα phosphorylates

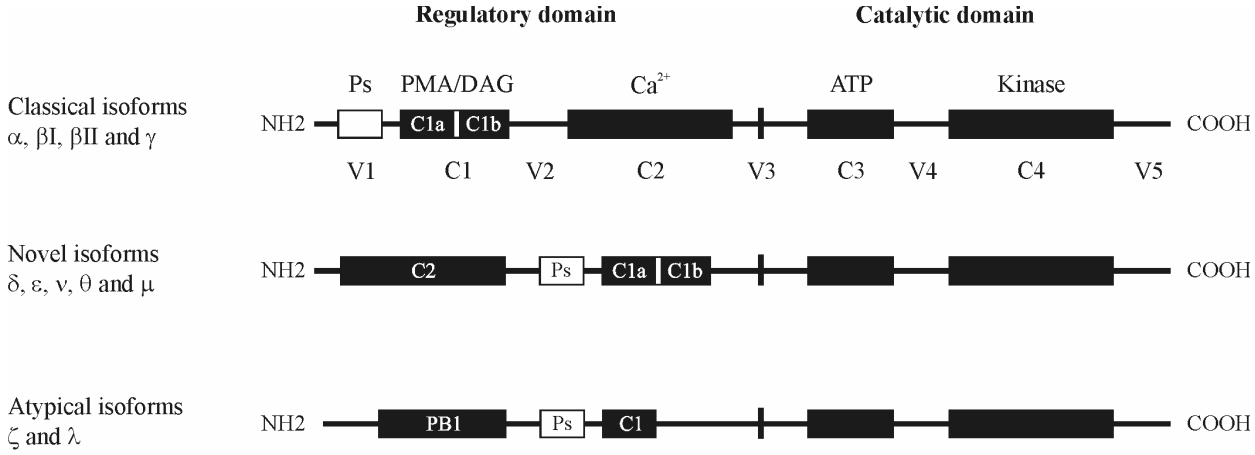

Fig. (1). Structure of PKC isoforms. PKCs are classified according their structure and co-factor dependency. Novel PKCs presents a C2 like domain unable to bind Ca^{2+}.The C1 domain of atypical PKCs does not have the conserved residues required for DAG binding. This isoforms also present a PB1 domain involved in regulatory functions. PS: Pseudosubstrate; V: variable region; C: conserved region.

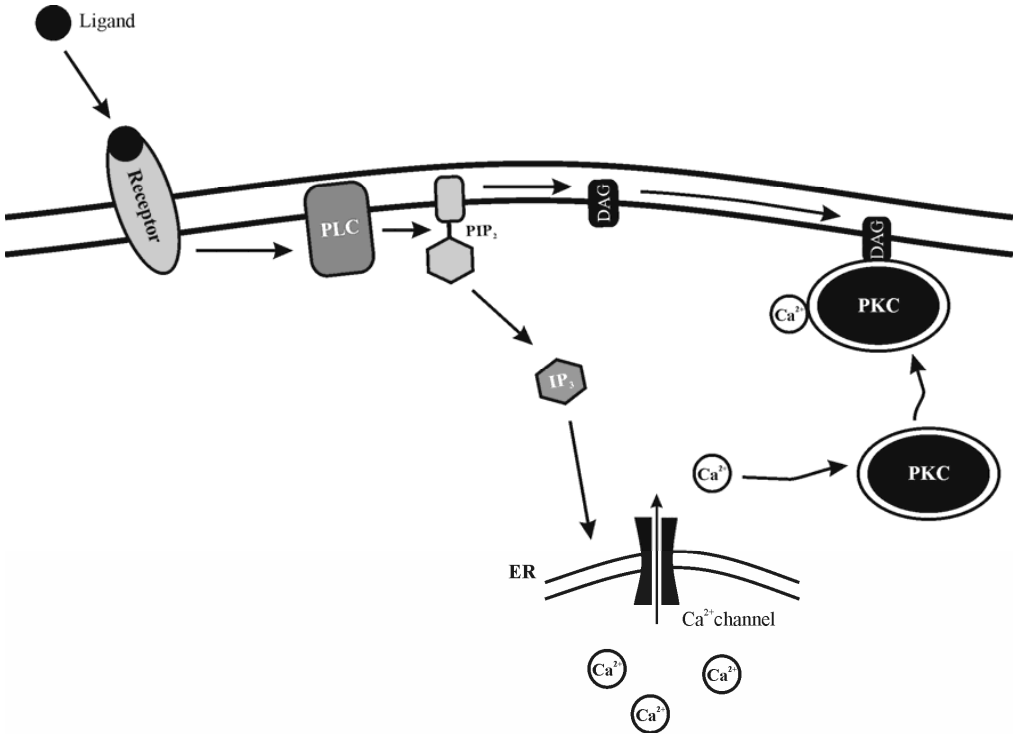

Fig. (2). Regulation of classical PKC isoforms. Stimulation of membrane receptors activates phospholipace C (PLC), which cleaves phosphoinositol-4,5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 induces the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER) to the cytoplasm. Ca^{2+} favors the interaction of classical PKCs with cell membranes, where PKC interacts with DAG. Binding to DAG increases de affinity for phosphatidylserine, stabilizing the PKC-membrane interaction. Both Ca^{2+} and DAG act synergistically in the process of PKC activation.

and activates the serine/threonine kinase Raf1 [22]. On the other side, there are evidences indicating that the same PKC isoform can activate other MAP kinases such as JNK and p38, inducing cell cycle arrest [23]. Although PKCδ has generally been proposed as a growth inhibitory or pro-apoptotic PKC isoform we, and others, have demonstrated that this isoform can also activate the MEK/ERK pathway through a direct interaction with Raf1 in a Ras independent fashion [24, 25]. PKCε is considered to be a transforming oncogene in many cellular models mediating ERK signaling activation in most cell systems. However, in some instances, PKCε-mediated ERK activation could lead to cell death [26]. An atypical PKC isoform (PKCζ) has also been reported as an activator of the MEK/ERK pathway but in this case the mechanism appears to be independent of Raf1 and would probably involve a direct interaction with MEK [27, 28].

Activation and upregulation of PKCα was demonstrated in many prostate cancer cells. It is also known that PKCα activation is likely to suppress the apoptosis induced by different physical or chemical agents. In this sense, signaling through phosphatidylinositol-3-kinase (PI3K) and its downstream kinase Akt has been widely implicated in cell survival [29], therefore the relationship between PKC activation and Akt function has been subject of intense investigation. Some studies have shown that PKCs activate Akt in endothelial and myeloid cells, an effect that has been attributed in most cases to the classical PKC isoforms [30, 31]. Nevertheless, it was also shown that in normal mammary cells PKCδ overexpression conferred resistance against cytotoxic drugs throughout a substantial elevation in the levels of activated Akt [25]. On the other hand, other studies have shown that treatment of PC12 prostate cancer cells with PMA attenuated IGF-1-induced Akt activation. This attenuation was completely blocked by the PKC inhibitor Gö6983 [32]. Accordingly, the overexpression of PKCζ in MDA-MB-468 breast cancer cells inhibited growth-factor-induced increases in Akt phosphorylation and activity [33]. A similar effect has been recently reported in keratinocytes, where Akt inactivation in response to PMA did not involve any classic isoforms, but the novel PKCδ and PKCε [34]. In sum, the mechanisms underlying the role of PKC on regulating Akt activity are not yet fully understood, but it is clear that there is a strict isoform- and cell type-dependency.

Recent studies have shown that PKC is involved in the regulation of nuclear factor-κB (NF-κB)-dependent gene expression. NF-κB is a transcription factor that regulates the expression of a wide variety of genes, especially those involved in survival, such as Bcl-2 and caspase inhibitors. The function of this transcription factor depends on the release from its inhibitor (IκB), which sequesters it into the cytoplasm. Upon phosphorylation, IκB is degraded and NF-κB can translocate into the nucleus to induce the transcription of its target genes [35].

Knockout mice studies have revealed that PKCβ controls NF-κB activation in B cells in response to B-cell stimulation [36]. However, it has been proposed that in T cells NF-κB activation is mediated by PKCθ [37]. A growing body of evidence indicates that PKCδ, in particular its kinase activity, is involved in NF-κB activation [38, 39]. Furthermore, it

has been described that DAG can mediate NF-κB activation by promoting both PKCε and PKCδ translocation, favoring IκB degradation [40].

Besides being involved in the above described signaling pathways, PKCδ seems to be the main isoform involved in apoptosis induction and growth inhibition in a large number of cell types [41]. Overexpressing PKCδ in NIH3T3 cells leads to an important reduction in their proliferation rate [42, 43]. This effect has also been reported in other cell types, including glial [44], endothelial [45] and breast cancer [46] cells. Several reports have also shown that activation of PKCδ promotes apoptosis of malignant colon [47] and prostate [48] cell lines. The proposed underlying mechanism suggests that activated PKCδ translocates to mitochondrial membrane and induces cytochrome c release and subsequent caspases activation. This mechanism, described for the cervical cancer HeLa cell line, involves PKCδ phosphorylation and inhibition of phospholipid scramblase 3, an enzyme that mediates cardiolipin transport from the mitochondrial inner membrane to the outer membrane. The disruption of this process ultimately leads to the lost of transmembrane potential inducing an apoptotic response [49]. It has been reported that PKCδ itself is a substrate of caspase 3. When caspase 3 is activated, PKCδ is cleaved and the 40 kDa catalytic fragment is released. The existence of such a feedback loop between PKCδ and caspase 3 suggest that PKCδ may regulate its own cleavage in response to apoptotic stimuli by activating caspase 3 [50]. However, the involvement of PKCδ on apoptosis induction is not a universal phenomenon. There are several studies showing that PKCδ could also act as a positive regulator of growth in mammary cells [4, 25], and moreover a pro-survival role for PKCδ in breast and lung cancer has also been reported [51, 52]. Different authors have assigned this contradictory behavior to differences in the functionality between PKCδ 40 kDa catalytic fragment and PKCδ holoenzyme, being the first pro-apoptotic whereas the holoenzyme induces the opposite effect [50, 53]. Moreover, cellular and/or tissue context dependency are not to be discarded.

Altogether, we conclude that a careful understanding of the specific targets of each PKC isoform in the different cell contexts is essential for the development of PKC modulators for cancer treatment in order to avoid undesired effects.

PKC AND CANCER

Changes in the expression of PKC isozymes have been reported in numerous human cancers including lung, colon, skin, prostate, and breast [4, 7, 8, 51, 54-56] malignancies among others.

In many instances a correlation between elevated PKC protein levels and aggressiveness has been reported [57-59]. For example, there is strong evidence for the involvement of PKC isozymes in breast cancer progression [60]. Indeed, PKCα overexpression correlates with the lack of estrogen receptor expression in mammary tumor cells, an indicator of poor prognosis [58, 59]. In addition, several classical and novel PKC isoforms have been associated with an enhanced expression of matrix metalloproteinases (MMP) and urokinase-type plasminogen activator (uPA) [61-63]; enzymes that play crucial roles in tissue remodeling as well as in cancer initiation and progression [64]. Moreover, PKCβ has become an important player in breast cancer cell proliferation since specific inhibitors could significantly reduce both, the *in vitro* and *in vivo* growth potential of breast cancer cell lines [10, 65]. In addition, there has been considerable interest in understanding the role of PKCδ in breast cancer cells as some studies suggested that this PKC mediates anti-proliferative responses while others concluded that PKCδ enhances proliferation and survival. For example, PKCδ inhibitors or dominant-negative mutants, impaired phorbol ester-induced arrest in G1 in SKRB-3 breast cancer cells [66]. Conversely, a few studies suggested that PKCδ could act as a positive regulator of growth in tumor-derived mammary cells [4, 67], probably through the activation of mitogenic pathways [4, 68]. Additionally, a pro-survival role for PKCδ in breast cancer cells has also been reported [51, 52].

Atypical ι and ζ PKC isoforms are involved in several malignancies. It has been described that PKCζ overexpression in mammary cells affects several critical pathways implicated in the modulation of invasive and metastatic phenotype. This includes an enhanced expression of proteases and alterations in the adhesive, spreading and migratory potential [28]. On the other side, PKCι was found to be genomically amplified and/or overexpressed in ovarian cancers and in several ovarian malignancies where elevated levels are associated with a decreased survival [69].

The role of PKC isoforms in human colon cancer remains controversial. Several studies have shown an increased PKC activity in human colonic carcinomas in comparison to the adjacent normal counterparts, whereas others have found opposite results [60]. In this sense, while PKCα expression is decreased in different colonic tumors under the effect of specific carcinogens [70], other classical isoform (PKCβII) is found elevated during the early stages of colon carcinogenesis. Additionally, transgenic mice overexpressing PKCβII presented colonic epithelium hyperproliferation; being this PKC isozyme sufficient to increase the susceptibility to colon carcinogenesis [71]. Contradictory data can also be found regarding the effect of PKCδ in colon carcinogenesis. While it has been proposed that the inhibition of this isoform in Caco-2 cells enhances proliferation [72], the PKCδ inhibitor Rottlerin decreased colon carcinoma SW116 cell proliferation [60]. Atypical PKC isoforms also participate in colon cancer, PKCζ expression could inhibit colon cancer cell growth and its downregulation contributed to tumorigenesis [73].

Drugs that target PKC are being evaluated in patients with non-small cell lung cancer (NSCLC). The main PKC target of these clinical trials is PKCα, but its role in these malignancies remains unclear since there are only a few reports in the literature. One of them points out a cooperative interaction between PKCα and PKCε in the modulation of MMPs production by the stroma in response to different stimuli [74]. It has also been shown that in NSCLC, novel PKC isoforms can promote malignant progression, being PKCδ and PKCε involved in the apoptosis prevention [52, 75]. The atypical isoform PKCι could be considered an oncogene and its expression is highly required for maintenance of NSCLC transformed phenotype since its inhibition alters

its growth potential [76]. It is important to note that this PKC isoform is overexpressed in all NSCLCs and its expression could be used as a survival marker of lung cancer patients.

LNCaP cells are a widely used model of androgen-dependent human prostate cancer. The high responsiveness to phorbol esters and the fact that these cells express elevated levels of PKCα, δ, and ε make them attractive to study the role of PKCs in prostate carcinogenesis. Upon treatment of LNCaP cells with PMA, a marked inhibition on cell viability is observed [56], and the activation of both PKCα and PKCδ was described as responsible for this effect [77]. It has been proposed that the activation of PKCα and PKCδ promotes the dephosphorylation and inactivation of the survival kinase Akt [78], whereas PKCδ also promotes apoptosis through release of death receptor ligands [77]. On the other hand, PKCε isozyme appeared to be increased in prostatic adenocarcinoma specimens, as compared with control benign tissues [79]. In fact, PKCε expression in LNCaP cells was sufficient to increase cell proliferation and is thought to have oncogenic potential in that model [80]. Moreover, PKCε isozyme was described in prostate malignancies while its overexpression sensitized cells to the induction of apoptosis by bryostatin-1 [81].

It is commonly accepted that PKCs play a central role in hepatocellular cancer (HCC) development. PKCα seems to play a key role in these malignancies since it is overexpressed in several HCC, and its levels constitute a disease prognosis marker that could be related to tumor size and TNM staging [82]. The reduction of PKCα expression levels in this class of tumors could be associated with a less proliferative, migratory and invasive phenotype. For this reason targeting PKCα promises to be a useful tool for the treatment of HCC pathologies [60]. Other PKCs, such as PKCδ and PKCι are also overexpressed in hepatocellular malignancies but their roles remain to be elucidated [82].

Six PKC isoforms are mainly expressed both in mouse and human skin: α, δ, ε, η, μ, and ζ. Using a PKCε transgenic mouse model, Aziz and co-workers [83] have shown that the epidermal levels of this isoform are closely related to the susceptibility of squamous cell carcinoma (SCC) development by ultraviolet radiation (UVR) exposure. Besides, it has been described that PKCδ may inhibit transformed keratinocyte growth by inducing apoptosis. This PKC isoform may function as a tumor suppressor in human SCC because its loss in transformed cells could provide a growth advantage by preventing apoptosis [84]. Furthermore, Li and co-workers have demonstrated that the activation of both PKCδ and PKCε negatively regulates Akt phosphorylation and kinase activity in mouse keratinocytes, acting as modulators of cell survival pathways in response to external stimuli [34].

Renal cell carcinoma (RCC) is typically resistant to chemotherapy and radiation therapy [85]. The finding that several PKC isoforms (α, δ, ε and ζ) are expressed in renal cancer [86] renews the expectations for a treatment for this malignancy. PKCα is decreased in RCC compared to normal renal tissue, probably indicating that this PKC isoform displays growth inhibitory functions in the renal epithelium [86] as already described in prostate cancer. PKCε levels are increased in RCC and a straight correlation with the progression of the disease was described by Brenner et al [86]. Recently, the same research group has proposed that PKCδ is involved in malignant RCC adhesion, migration and proliferation [87]. These parameters are also modulated by PKCδ in mammary and pancreatic cell lines, exerting important alterations in tumor progression [88, 89]. PKCζ has also been involved in RCC and its expression was associated, among other factors, with VEGF expression, a key player in the angiogenesis induction [85].

In pancreatic cancer, PKCα, PKCβ, and PKCδ levels have been found increased as compared to normal tissues [90]. It has been described that PKCδ overexpression in pancreatic tumor-derived cell lines may induce a more aggressive phenotype *in vivo*, through the modulation of cell proliferation and survival, involving PI3K and ERK signaling pathways [89]. Moreover, PKC inhibition sensitizes cells to the pro-apoptotic effects of chemotherapeutic agents such as gemcitabine [89].

Finally, regarding hematopoietic neoplasms, PKCδ, PKCι, PKCμ, and PKCζ expression was detected in multiple myeloma cell lines [91] and PKCβ, PKCγ, PKCδ, and PKCζ expression was detected in chronic lymphocytic leukemia cells patients [60].

In sum, unambiguous alterations in different PKC isozymes expression and/or activity could be detected both, in cancer patients and in tumor-derived cell lines, indicating that a clinical improvement would be achieved by targeting PKC. Although, since different PKCs may exert differential responses and even the same isoform can act differently depending on the cellular context, it is necessary to carefully understand the signaling events controlled by every PKC in each cell type in order to avoid non-desired effects in a clinical setting. The constant increase in the knowledge of PKC-associated processes would provide new opportunities for the rational design of more specific PKC modulators as therapeutic agents.

PKC AND THE MULTI-DRUG RESISTANCE (MDR) PHENOTYPE

The MDR phenotype is one of the main factors responsible for the failure of many chemotherapeutic approaches in patients suffering from a variety of blood and solid cancers. Earlier studies have shown that the exposure of different human breast cancer cell lines to phorbol esters led to the expected increased in PKC activity together with an enhanced resistance to cytotoxic drugs. Moreover, agents known to revert the MDR phenotype were also shown to inhibit PKC activity [92].

Recent studies have suggested that the activity of multidrug transporters, which are responsible for the MDR phenotype, could be modulated by different PKC isozymes. Most of PKC isoforms are able to phosphorylate the 170 kDa P-glycoprotein (Pgp) transporter *in vitro* probably altering this transporter's activity [93]. In fact, there is evidence that PKC activity correlates with Pgp phosphorylation and with an increased MDR phenotype in tumors [94]. The association of PKC isoforms with MDR seems to be cell type-dependent. While in most cell models MDR is mainly attributed to PKCα [95-97]; in P388 leukemia cells PKCα does not significantly affect the MDR phenotype although another clas-

sical isoform, PKCβ, does it so [98]. Moreover, in human ovarian carcinoma cells the inhibition of both PKCα and PKCβ is required to reverse MDR-mediated resistance to paclitaxel [99]. As it is commonly observed in the PKC family, once more the effects of a particular isoform appear to be cell type- and context-dependent.

PKC INHIBITORS

Staurosporine is a microbial alkaloid with a potent growth inhibitory activity originally isolated in 1977 from bacterium Streptomyces staurosporeus. This drug was the first PKC inhibitor that attracted scientist attention and although it has poor isoform specificity it was used as a leading compound for the development of more specific and powerful drugs.

The increase in knowledge about PKC isoform three-dimensional structures has allowed the development of inhibitors that specifically block the ATP or the DAG binding sites as well as the development of antisense strategies. All this efforts were performed aiming to use isoform-specific PKC inhibitors in the clinical management of cancer patients. In this section we will sum up the information about the different PKC inhibitors being tested or used in the clinic, and the main agents under investigation. This information is summarized in Tables **1** and **2**.

UCN-01

UCN-01 or 7-hidroxy-staurosporine is a staurosporine analogue that competes for the ATP binding site, in the kinase domain of PKCs. UCN-01 was selected for clinical development because of its potent antiproliferative activity *in vitro* in several cell lines and its antineoplastic activity in xenograft systems [100]. The precise mechanism of UCN-01 antitumor activity is still not fully understood. It was described that besides inhibiting PKC activity this drug also inhibits other kinases including cyclin dependent kinases and phosphoinositide-dependent kinases [101, 102]. UCN-01 induces cell cycle arrest and apoptosis, and sensitizes tumor cells to DNA damaging agents [103, 104]. At clinically relevant concentrations, UCN-01 was shown to act synergistically with thioTEPA, mitomycin C, cisplatin, melphalan, topotecan, gemcitabine, Xudarabine, and 5-Xuorouracil. In particular, preclinical studies with UCN-01 and topoisomerase inhibitors demonstrated a synergistic effect [100]. This drug presented antitumor activity against non-Hodgkin's lymphomas, myosarcomas, melanomas and lung cancer. Phase II clinical trials using UCN-01 as monotherapy or in combination with other chemotherapeutic agents are ongoing nowadays [105].

Midostaurin

Midostaurin, also called PKC412, is a staurosporine derivative (N-benzoylated staurosporine) that has undergone several phase I clinical trials as a PKC and tyrosine kinase inhibitor. Its specificity as a PKC inhibitor involves the inhibition of classical (α, β and γ) and novel (δ, ε and η) isoforms [60]. One of the main advantages of this drug is that it is almost devoid of cytotoxic side effects, it could be administered orally and, just like the majority of the staurosporine analogs, it showed a longer half-life than it was predicted from preclinical studies [106]. PKC412 displayed clinical activity as a single agent in leukemias and NSCLC [107]. Additionally, *in vitro* studies indicated that the simultaneous presence of noncytotoxic concentrations of PKC412 with doxorubicin causes a 3-fold increase in cell death in colonic- and fibrosarcoma-derived murine cell lines [108]. Likewise, the same treatment significantly inhibited the subcutaneous growth of a drug-resistant malignant colon derived cell line *in vivo* [108, 109].

In patients, it has been demonstrated that high doses of midostaurin can induce the downregulation of ERK2 levels probably through the inhibition of classical PKCs [110, 111]. Preclinical studies have shown that PKC412 may act as a radio-sensitizer for human tumor xenografts throughout the blockade of the PI3K/Akt pathway [11]. In addition, it was described that midostaurin was able to reverse the Pgp-mediated MDR phenotype of tumor cells *in vitro* [109, 112]. Moreover, it was recently described as an inhibitor of other kinases including c-KIT, FLT-3, VEGF-R1 and 2, and PDGFR thus, also eliciting anti-angiogenic effects [105, 111].

Enzastaurin

Enzastaurin, also known as LY317615, is an acyclic bis-indolylmaleimide that displays a potent and selective ATP competitive inhibition of PKCβ. This drug also inhibits other PKC isoforms but in a lesser degree, being approximately 20-fold more powerful for PKCβ I and II, than to other PKCs [113]. Moreover, enzastaurin prevents angiogenesis by altering the vascular endothelial growth factor receptor (VEGFR) signaling cascade [114]. This phenomenon was observed both *in vitro*, since enzastaurin exposure inhibited the proliferation of VEGF-stimulated umbilical vascular endothelial cells, and *in vivo* by reducing intratumoral blood vessels of nude mice bearing human tumor xenograft [115]. In addition, Enzastaurin also induced tumor cell apoptosis and reduced proliferation by inhibiting the PI3K/Akt pathway [114, 116]. In sum, enzastaurin displays antitumor activity by the modulation of different pathways involved in tumor progression.

Preclinical studies have shown that enzastaurin treatment caused growth inhibition and apoptosis induction in a large number of tumor cells including myeloma, lymphoma, small cell lung cancer, colon and renal cancers [117, 118]. In several tumor models, enzastaurin as a single agent was not sufficient to ensure a better clinical outcome. This data, together with the knowledge that the toxicity profiles of enzastaurin and other cytotoxic agents are non-overlapping favored the development of different phase I studies combining enzastaurin with gemcitabine and cisplatin in patients with advanced or metastatic cancer. The enzastaurin addition did not increase the toxicity of gemcitabine/cisplatin chemotherapy, and a phase II study of the combination regimen was recommended [114]. Another several phase II and III clinical trials have been started for the use of enzastaurin in the treatment of gliomas, lymphomas and NSCLC and the efficacy of the treatment with this drug should emerge briefly.

Bryostatin

Bryostatin are a family of 20 macrolic lactones that act as classical and novel PKC agonists by interacting with their

Table 1. PKC Inhibitors

Drug Name	Class	Molecular Targets	Inhibition Mechanism
UCN-01	7-hudroxy-staurosporine (staurosporine analogue)	PKCs, CDKs, PKDs	Competes for the ATP-binding site
Midostaurin (PKC412)	N-benzoylated staurosporin (staurosporine analogue)	PKCs, PI3K/Akt, VEGF, PDGFR, c-KIT, FLT-3	Competes for the ATP-binding site
Enzastaurin (LY317615)	Enzastaurin hydrochloride (Bisindolemaleimide analogue)	PKCβ	Competes for the ATP-binding site
Bryostatin 1	Macrolic lactone	Activator of PKC, topo II, TNFα, IL6. Also acts as PKC antagonist	Interacts with the regulatory domain
Phorbol 12-Myristate 13-Acetate (PMA)	Phorbol ester	Activator of PKC. Also binds to chimaerins, Ras-GRP, Unc-13/Munc-13, PKD. Also acts as PKC antagonist	Interacts with the regulatory domain
ISIS 3521 (aprinocarsen or LY900003)	antisense phosphorothioate oligonucleotide	PKCα	Induces PKCα mRNA degradation by endogenous RNAses
Safingol	Dihydrosphingosine, L-threo enantiomer	PKCs	Interacts with the regulatory domain
Tamoxifen	Estrogen receptor antagonist	PKCs	Unknown (non-selective PKC inhibitor at micromolar concentrations)

Table 2. Clinical Trials Involving PKC Inhibitors.

Drug Name	Combination	Phase	Conditions
Midostaurin (PKC412)	RAD001 (mTOR inhibitor)	I	Relapsed, refractory or poor prognosis AML or MDS
	Daunorubicin, and Cytarabine	I	Newly Diagnosed AML
	Monotherapy	I / II	AML and MDS with either wild-type or mutated FLT3
	Itraconazole	I / II	AML and MDS
	Monotherapy	I / II	Relapsed or Refractory Pediatric Leukemia
	Monotherapy	II	Aggressive Systemic Mastocytosis and Mast Cell Leukemia
	Monotherapy	II	Acute Myeloid Leukemia and Patients With Myelodysplastic Syndrome With Either Wild Type or Mutated FLT3
	Daunorubicin, and Cytarabine	III	Newly Diagnosed AML
ISIS 3521 (LY900003)	Gemcitabine and Carboplatin	II	Advanced, Previously Untreated Non-Small Cell Lung Cancer
	Carboplatin and Paclitaxel	III	Patients With Non-Small Cell Lung Cancer
UCN-01	Fluorouracil	I	Advanced or Refractory Solid Tumors
Bryostatin 1	Paclitaxel and Cisplatin	I	Advanced Solid Tumors
	All-Trans Retinoic Acid (ATRA)	II	AML and MDS
Phorbol 12-Myristate 13-Acetate (PMA)	Monotherapy	I	Chronic Myeloproliferative Disorders, Leukemia, Lymphoma, Multiple Myeloma and Plasma Cell Neoplasm, Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Diseases
	Dexamethasone & Choline Magnesium Trisalicylate	II	Relapsed or Refractory Acute Myeloid Leukemia
Enzaustaurin (LY317615)	Monotherapy	I	B-Cell Chronic Lymphocytic Leukemia
	Monotherapy	II	Mantle-Cell Lymphoma
	Monotherapy	II	Relapsed or Refractory Diffuse Large B-Cell Lymphoma

regulatory domain (N-terminal) [119]. These natural products are potent PKC activators but, as already described, a sustained PKC activation induced by a chronic treatment in the presence of other activating ligands results in the PKC downregulation via proteosomal degradation. Under these circumstances, bryostatin could be considered as an antagonist. Besides modulating PKCs, some additional anti-tumor mechanisms where described. This mechanism includes the stimulation of cytokine production (TNFα and IL6), the activation of cytotoxic T lymphocytes [106] and the inhibition of topoisomerase II phosphorylation [120]. Preclinical studies have shown an *in vitro* and *in vivo* activity of bryostatin against a variety of tumors including melanoma, leukemia, lymphoma and lung cancer [120]. Phase I clinical trials have demonstrated anti-tumor activities against melanoma and ovarian cancer [106]. Conversely, phase II clinical trials with colorectal cancer and melanoma patients showed no efficacy [121]. Moreover, the results obtained by combining bryostatin 1 with other cytotoxic drugs have also been disappointing [105].

Phorbol 12-Myristate 13-Acetate (PMA)

As mentioned before, the chronic treatment with different PKC activators may induce a time-dependent decrease in PKC levels. This concept led to the clinical use of PMA, the best known PKC activator [5], for cancer treatment.

PMA was first found in the croton plant, a shrub found in Southeast Asia. It has been widely studied as a differentiation agent, a tumor promoter, and a modulator of multiple cellular signaling pathways. In human melanoma-, prostate-, breast-, colon-, and lung-tumor-derived cell lines PMA can induce cell growth inhibition, apoptosis stimulation, and cell differentiation [122, 123]. Moreover, the establishment of PMA as a hematopoietic cell-differentiating agent [124] has promoted the development of phase I clinical trials for blood malignancies [97].

At this point it is important to note the existence of critical differences in the biological responses to bryostatin 1 and PMA. While PMA is a potent tumor promoter in the mouse skin model, bryostatin 1 is not. This indicates that even though these two drugs achieve the same molecular effect (PKC downmodulation); they differ in their ultimate biological response [125, 126].

ISIS 3521

ISIS 3521 also known as aprinocarsen or LY900003 is a 20-mer antisense phosphorothioate oligodeoxynucleotide complementary to the 3'-untranslated region of the mRNA for human PKCα. The phosphorothioate backbone (a sulfur substitution of nonbridging oxygen on the backbone) provides relative resistance to exonuclease- and endonuclease-mediated degradation and increases the stability of the oligodeoxynucleotide in serum and tissue. This single-stranded antisense DNA forms a non-covalent bond with the specific PKCα mRNA sequence inhibiting translation and rendering the complex susceptible to degradation by the nuclease RNaseH [127]. Selective sequence- and concentration-dependent inhibition of PKCα expression by ISIS 3521 has been demonstrated *in vitro* and *in vivo*. The activity of ISIS 3521 depends both on the expression level and the role (proliferative *vs*. pro-apoptotic) of PKCα in the specific tissue, since this isoform has shown different and even opposing functions in different cell contexts. In nude mice, ISIS 3521 inhibited the progression of different mammary, pancreatic, lung, bladder, and colon cell lines [128].

ISIS 3521 has been investigated in phase I, II and III clinical trials. Administered as a single agent, ISIS 3521 did not show clinical activity in non-Hodgkin's lymphoma or in advanced ovarian carcinoma. Even though, it has been described that this drug enhances the *in vitro* sensitivity to cisplatin and carboplatin of human cell lines. Although the mechanism of this sensitization is not yet fully understood, these finding argues for the development of further clinical studies using a combination with platinum-based regimens [127, 129].

Conversely, encouraging results in phase II clinical trials were described for lymphomas, NSCLC, and ovarian cancer [105]. Despite these promising results, no differences against the control group were observed in a small phase II trial in metastatic breast cancer patients [106]. Ultimately, a phase III clinical trial using ISIS 3521 alone or in combination with other cytotoxic drugs for NSCLC has also been unsatisfactory [130].

Safingol

Safingol is an L-threo enantiomer of dihydrosphingosine considered to be the first PKC-specific inhibitor to enter in clinical trials. Safingol inhibits PKC activity by interfering with the function of its regulatory domain. *In vitro* studies have demonstrated that safingol increases the activity of different chemotherapeutic agents enhancing chemotherapy-induced apoptosis. *In vivo*, safingol significantly enhanced the antitumor effect of doxorubicin and cisplatin, although as a single agent it only had modest antitumor activity. In pilot clinical studies in animals, nontoxic levels of safingol in serum could be achieved and were associated with chemopotentiation of doxorubicin in animals [131].

Tamoxifen

Anti-estrogen drugs such as tamoxifen and its analogs have been described as PKC inhibitor drugs in a non-selective fashion at micromolar concentrations, doses several-fold higher than those typically attained during the treatment of breast cancer. The mechanism of PKC inhibition at high concentrations remains unknown. Anti-estrogen drugs are in phase I and II clinical trials for several malignancies including gliomas and prostate cancer [106, 132].

CONCLUSIONS AND PERSPECTIVES

PKC isozymes are critical players in many signaling pathways involved in the control of cell fate. Based on this affirmation, alterations in PKC signaling would lead to malignant transformation and tumor progression. Indeed, altered PKC expression and/or activation can be detected in many human cancers, being PKC expression used as an indicator of poor prognosis in several malignancies. For these reasons, targeting PKC and/or its downstream effectors in

the management of different human cancer has become an appealing option already tested in many models to-date.

Preclinical *in vitro* studies using several PKC inhibitors have shown high efficacy in the control of cell proliferation or apoptosis induction in numerous cell lines. But to move on to the clinical setting, drugs must first be tested and validated in patients, where the efficacy is still limited and not much success has been yet achieved.

Nevertheless, PKC could still be considered a legitimate target for cancer therapy. The complexity and tissue specificity of the different PKC signaling pathways, together with the lack of appropriate patient selection remain as major issues in the development of PKC inhibitors for use in the clinic.

It may also be possible that agents that target a single PKC isoform might not be potent enough in order to inhibit tumor growth; in consequence, combining PKC inhibitors with conventional cytotoxic drugs may be the path to follow to attain a positive clinical response.

Another complication is that some PKC isoforms present pleiotropic responses (e.g.: PKCδ may be growth stimulatory *vs*. growth inhibitory). This paradoxical behavior greatly impacts in the rational design of isozyme specific PKC modulators as therapeutic agents. Therefore, it is imperative to elucidate the molecular events controlling such disparate effects to avoid non-desired effects in clinical settings. A better understanding of the mechanisms associated with the control of these processes should provide new opportunities for the rational design of PKC modulators as therapeutic agents.

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ABBREVIATIONS

PKC = Protein kinase C

DAG = Diacylglycerol

ERK = Extracellular signal-regulated kinase

PI3K = Phosphatidylinositol-3-kinase

MAPK = Mitogen activated protein kinase

PMA = Phorbol 12-myristate 13-acetate

MEK = Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase

MMPs = Matrix metalloproteinases

uPA = Urokinase-type plasminogen activator

PMA = Phorbol 12-myristate 13-acetate

SCC = Squamous cell carcinoma

NSCLC = Non-small cell lung cancer

UVR = Ultraviolet radiation

HCC = Hepatocellular cancer

RCC = Renal cell carcinoma

VEGF = Vascular endothelial growth factor

BCNU = 1,3-bis (2-chloroethyl)-1-nitrosourea

MDR = Multi-drug resistance

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