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Review

Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression

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ABSTRACT

The AGC family of serine/threonine kinases (PKA, PKG, PKC) includes more than 60 members that are critical regulators of numerous cellular functions, including cell cycle and differentiation, morphogenesis, and cell survival and death. Mutation and/or dysregulation of AGC kinases can lead to malignant cell transformation and contribute to the pathogenesis of many human diseases. Members of one subgroup of AGC kinases, the protein kinase C (PKC), have been singled out as critical players in carcinogenesis, following their identification as the intracellular receptors of phorbol esters, which exhibit tumor-promoting activities. This observation attracted the attention of researchers worldwide and led to intense investigations on the role of PKC in cell transformation and the potential use of PKC as therapeutic drug targets in cancer diseases. Studies demonstrated that many cancers had altered expression and/or mutation of specific PKC genes. However, the causal relationships between the changes in PKC gene expression and/or mutation and the direct cause of cancer remain elusive. Independent studies in normal cells demonstrated that activation of PKC is essential for the induction of cell activation and proliferation, differentiation, motility, and survival. Based on these observations and the general assumption that PKC isoforms play a positive role in cell transformation and/or cancer progression, many PKC inhibitors have entered clinical trials but the numerous attempts to target PKC in cancer has so far yielded only very limited success. More recent studies demonstrated that PKC function as tumor suppressors, and suggested that future clinical efforts should focus on restoring, rather than inhibiting, PKC activity. The present manuscript provides some historical perspectives on the tumor promoting function of PKC, reviewing some of the observations linking PKC to cancer progression, and discusses the role of PKC in the pathogenesis of cancer diseases and its potential usage as a therapeutic target.

1. Introduction

The protein kinase C (PKC) family is comprised of a group of serine/threonine kinases that are evolutionary conserved and expressed in a wide variety of species across phyla. The biological role of PKC has been intensely investigated in a wide range of normal physiological systems and in a variety of pathological conditions. These studies revealed the involvement of PKC enzymes in an array of metabolic processes in almost all cell types. In addition, PKC enzymes are implicated in multiple signal transduction networks that convert environmental cues into cellular actions [1,2]. The PKC-regulated signals are translated within cells into critical physiological processes regulating gene expression, cell-cycle progression, cell migration, proliferation and differentiation, cell survival and apoptosis. The distinct functions of individual PKC isoforms in cellular processes are regulated by the receptor-triggered translocation of individual isoforms to specific intracellular compartments. Absence of PKC and/or its dysregulation may therefore lead to different pathologies, including diabetes [3,4], heart failure [5,6], Alzheimer and Parkinson diseases [7,8], allergy [9,10], inflammatory diseases [11,12], and a range of autoimmune diseases [13–16]. Furthermore, PKC has been implicated in a range of cancer diseases in different organs [17–19]. Based on these observations, it has been assumed that PKC isoforms might serve as potential drug targets for the treatment and management of different human diseases. A number of examples where distinct PKC isoforms were found to contribute to cell transformation, tumor progression and metastasis formation of distinct types of cancer diseases are described in the following chapters.

Studies in animal models substantiated the assumptions on the role of individual PKC isoforms in specific diseases, and provided initial evidence for the therapeutic effects of selected PKC-inhibitory drugs [20–24]. However, despite the promising results obtained using PKC modulators in in vitro studies and in animal disease models, clinical trials using a range of PKC inhibitors and modulators in patients with different cancer diseases showed very limited success [18].

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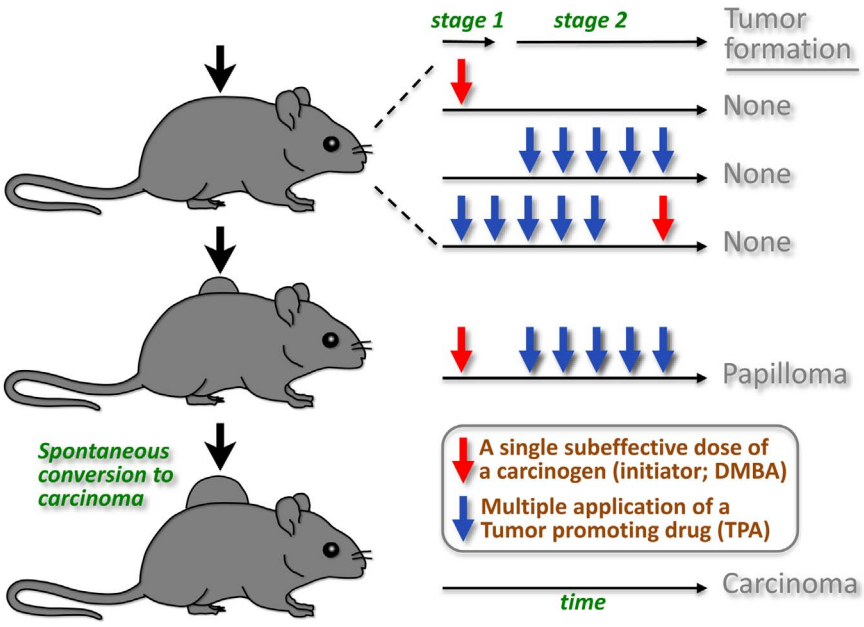

Fig. 1. A model of the two-stage chemical-induced carcinogenesis.
Topical application of a single subthreshold dose of a carcinogen (initiator), such as 7,12-dimethylbenz[*a*]anthracene (DMBA), which can mutagenize proto-oncogenes, such as Ras, followed by repetitive epicutaneous applications of a tumor promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), results in the formation of benign papillomas that may eventually progress into malignant SCC. The effect of the tumor promoter is reversible, since repeated epicutaneous applications of the tumor promoter before the treatment with the carcinogen do not result in tumors. In contrast, the effect of the carcinogen is irreversible; it causes skin tumor formation even if the promotion step is delayed by up to one year.

2. PKC is the cellular receptor for tumor-promoting phorbol esters

Phorbol esters are natural products extracted from the seed oil of *Croton tiglium*, a plant genus of the family *Euphorbiaceae* [25]. The "co-carcinogenic" activity of the croton oil was initially observed in the early 1940s, following application of the croton oil on mouse skin [26]. These studies demonstrated that application of a single sub-carcinogenic dose of hydrocarbon followed by repeated application of croton oil led to the formation of skin tumors. These observations led to the "two-stage hypothesis" of chemical-induced skin carcinogenesis [27], and initiated an era of intense investigation focusing on the potent tumor promoting activity of phorbol esters.

In the classical "two-stage model of carcinogenesis", a single topical application of a subeffective dose of a carcinogen (an 'initiator' drug, such as the 7,12-dimethylbenz[*a*]anthracene (DMBA)) on the mouse skin, followed by repeated applications of a tumor promoter (such as phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA)), resulted in the formation of benign skin papillomas, which could progress into a carcinoma (see Fig. 1). The effect of the carcinogen was irreversible, and it could cause tumor formation even when the promotion step was delayed by up to one year. In contrast, the tumor promoters were not carcinogenic by themselves. They are considered to be pro-inflammatory drugs and their biological effect on the skin is reversible since repeated epicutaneous applications of tumor promoters prior to the treatment with the initiator drug, did not result in tumors.

The relevance of phorbol esters to the potential regulation of PKC became clear only in the early 1980s, following the discovery of PKC by Nishizuka and coworkers [28,29]. The PKC was discovered in 1977 and originally considered a cyclic nucleotide-independent protein kinase that phosphorylates serine and threonine residues on histones, and undergoes activation by proteolysis. Additional studies by Nishizuka's group led to the understanding that an intact PKC enzyme can be activated by diacylglycerol (DAG) in a Ca^{2+}- and phospholipid-dependent manner [30]. Shortly after, it was discovered that the generation of DAG occurs in activated cells by receptor-stimulated hydrolysis of phosphoinositides [31], suggesting that DAG functions as a physiological second messenger that links activating agonist-bound receptors to PKC activity.

Structural similarities between DAG and the phorbol esters led Nishizuka to compare the effects of the two compounds on PKC activity and to discover that DAG and phorbol ester could interchangeably activate PKC with similar kinetics [2,32].

The observation that phorbol esters could operate as tumor promoters in the two-stage model of chemical carcinogenesis was made independently by several investigators [25,33–36], and ignited a race for the identification of the cellular target for phorbol esters. Before long, scientists found that the enzymatic activity of PKC and the phorbol ester binding ability reside in the same cellular fractions, and additional purification studies demonstrated that PKC and the phorbol ester receptors represent a single entity [37].

3. PKC structure and function

The fact that PKC represents a family of enzymes became clear in 1986, when the first PKC genes were cloned, including the PKCα, β, and γ isoforms [38–40]. Alignment of their amino acid sequences revealed an overall homology in four regions, referred to as conserved (C) domains, which are interspersed by five variable (V) segments (see Fig. 2). Further studies led to the cloning of six additional PKC genes and the identification of additional PKC proteins that are produced by alternative splicing of the PKCβ [41] and the PKCδ genes [42–44].

All PKC isoforms share a highly conserved carboxy-terminal region that includes an ATP-binding domain and a catalytic domain (Fig. 2). A short variable (V3) hinge region links the PKC catalytic domain to a membrane-targeting amino-terminal regulatory region, which possesses a pseudosubstrate sequence plus two discrete membrane targeting regulatory elements. The pseudosubstrate is similar in sequence to the optimal PKC substrate sequence, but lacks the serine/threonine phosphoacceptor residue [45,46]. In resting state, the pseudosubstrate motif interacts with the substrate-binding cleft within the catalytic domain, thereby blocking its access to potential substrates. Most of the variations between the PKC isoforms are found in the regulatory region, which includes the two major regulatory domains, the DAG/phorbol ester-binding domain, termed C1, and the phospholipids and Ca2+-binding domain, termed C2. The ability of the C1 domain to bind DAG/phorbol esters was established through a series of deletional studies and site-directed mutagenesis [47–49]. Based on the binding characteristics of the C1 domain, PKC has been considered as the major cellular receptor for phorbol esters. However, later studies identified additional phorbol ester-binding cellular proteins, including the chimaerins [50,51], RAS guanyl nucleotide-releasing protein (RASGRP) [52,53] and Munc13 [54,55], which possess a C1 domain, and respond to DAG/phorbol ester-binding by membrane translocation [56]. These findings indicated that pharmacological studies which utilize phorbol esters as

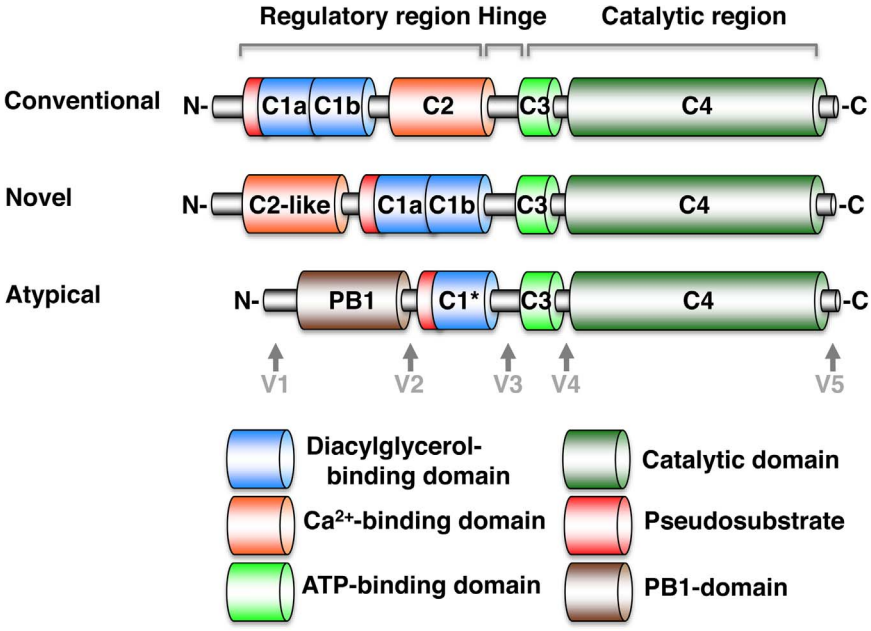

Fig. 2. A schematic representation of the structure of mammalian protein kinase C (PKC) family members.
Mammalian PKC isoforms are grouped into three structurally and functionally distinct subfamilies according to their regulatory domains, and include the conventional (classical) isoforms (cPKC), novel isoforms (nPKC), and atypical isoforms (aPKC). The carboxy-terminus in all isoforms includes an ATP-binding domain and a catalytic domain, which are linked via a hinge region (V3) to a membrane-targeting amino-terminal regulatory region. A pseudosubstrate motif in the amino-terminus resembles the optimal PKC substrate sequence, but lacks the serine/threonine phosphoacceptor residue. In resting state, it interacts with the substrate-binding cleft within the catalytic domain, thereby blocking its access to potential substrates and inhibiting its catalytic activity. Inhibition of the catalytic domain is relieved upon recruitment of the enzyme to the plasma membrane, where its two discrete regulatory elements interact with membrane constituents. These include diacylglycerol (DAG)-binding tandem C1 domains (in cPKC/nPKC), a Ca^{2+}-dependent phospholipid-binding C2 domain (in cPKC/nPKC), and a partitioning defective 6 (Par6)-CDC42 binding Phox/Bem 1 (PB1) domain (in aPKC). The single C1 (C1*) domain in aPKC does not bind DAG, and the C2-like (C2*) domain in nPKC does not bind Ca^{2+} or phospholipids. The aPKC PB1 is a protein-protein binding domain that mediates heterodimerization or homo-oligomerization. The human PKCι and mouse PKCλ are orthologs (having 98% amino acid sequence identity) and are referred to as PKCι/λ.

PKC Subfamily	PKC Isoforms	Activators
Conventional	PKCα, βI, βII, γ	Diacylglycerol, lipid, Ca^{2+}
Novel	PKCδ, ε, η, θ	Diacylglycerol, lipid
Atypical	PKCγζ, ι/λ,	PIP_3, Par6-CDC42

activators of PKC must be complemented by alternative approaches that distinguish between cellular effects induced by phorbol esters via the activation of PKC versus those induced by phorbol ester via the activation of other C1 domain-containing proteins [57].

Although tumor-promoting phorbol esters are potent activators of a majority of the PKC isoforms and mimic some of the effects induced by DAG, the two compounds differ significantly with respect to the duration of their induced activation signals. The transient nature of DAG, which is produced following receptor triggering, dictates the short-term activation of PKC. Within a short time, DAG undergoes phosphorylation by diacylglycerol kinase (DGK), which catalyzes the conversion of DAG to phosphatidic acid (PA). In contrast, phorbol esters and some other pharmacological activators of PKC induce a constitutive, long-term activation of the enzyme. The phorbol esters are non-physiological compounds and therefore resist all intracellular degradation processes. The mechanism by which cells attenuate the phorbol-ester-induced chronic PKC signal is by promoting the proteolytic degradation of PKC [58,59].

The extent of homology between the regulatory elements in individual PKC isoforms and their respective cofactor requirements allowed categorizing PKC members into three major subfamilies [60,61].

The first subfamily includes the conventional PKC (cPKC) isoforms, PKCα, βI, βII, and γ, which possess two functional cysteine-rich zinc finger structures (C1a and C1b) arranged in tandem near the cPKC amino terminus [62–64], plus a single adjacent C2 domain which binds phospholipids in a Ca^{2+}-dependent manner [63,65,66]. The majority of cPKC in resting cells resides in the cytosol in an inactive state. Signals leading to the transient formation of DAG promote cytosol-to-membrane translocation and upregulation of cPKC activity. Although DAG binding to the C1 domain occurs at a relatively low affinity, translocation of cPKC to the membrane is made possible by the coordinated binding of the C1 domain and the Ca^{2+}-regulated phospholipid-binding C2 domain. All cPKC isoforms can mediate high affinity binding of phorbol esters, such as TPA, and respond by prolonged activation.

The second subfamily includes the novel PKC (nPKC) isoforms, PKCδ, ε, η, θ, which also possess C1 and C2 domains, but in opposite order; the C2 domain (instead of the C1 domain in cPKC) is located at the amino-terminus. The C1 domain possesses two functional cysteine-rich zinc finger structures that mediate high affinity binding of DAG/phorbol ester. In contrast, the C2-like domain lacks the essential Ca^{2+}-binding residues. The membrane translocation capability of nPKC is therefore independent of Ca^{2+} and phospholipids [67,68]. Nevertheless, despite the functional differences between the nPKC and cPKC C2 domains, members of the two subfamilies bind membranes with comparable affinities [69,70]. Apparently, the two orders of magnitude higher affinity of the nPKC C1 domain to DAG compensates for the lack of involvement of its C2 domain in the translocation process [69,70].

The third subfamily includes atypical PKC (aPKC), PKCζ and λ/ι (the human aPKCι is referred to as aPKCλ in mice) that require neither Ca^{2+} nor DAG for their activity. The aPKC isoforms C1 domain includes a single cysteine-rich membrane-targeting structure but lacks essential features for DAG/phorbol ester recognition. However, it can interact with phosphatidylinositol 3,4,5 trisphosphate (PIP3) [71,72] or ceramide [73,74]. In addition, the Phox/Bem domain 1 (PB1) N-terminal domain of aPKC can mediate homodimeric interactions or heterodimerization with other PB1 domain-containing proteins [75,76]. aPKC activity is regulated predominantly by PB1-mediated protein-protein interaction [75,76] and by 3-phosphoinositide-dependent protein kinase 1 (PDPK1 should not to be confused with pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1))-mediated phosphorylation [77].

4. Regulation of PKC activity

Activation of PKC is strictly dependent on a posttranslational 'maturation' process involving a series of phosphorylation steps of PKC [78,79]. The first step is mediated by PDPK1, which phosphorylates a critical threonine at the activation loop of conventional [80], novel [81], and atypical [82] PKC. The resulting change in conformation

leads to exposure of the 'turn' and the 'hydrophobic' motifs at the C-terminus, and autophosphorylation leads to stabilization of the enzyme. This 'mature' form of enzyme is then 'primed' for activation and resides predominantly in the cytosol in an inactive conformation due to the interaction of its C-terminal catalytic domain with the N-terminal pseudosubstrate motif. Activation of PKC under physiological conditions is made possible by extracellular agonists, such as growth factors, hormones, cytokines or antigens, following interaction with their cognate G protein-coupled receptors or receptor tyrosine kinases. Receptor engagement then initiates the activation of effector molecules, such as the membrane-associated phospholipase C (PLC), which hydrolyses the membrane phospholipid phosphatidylinositol 4,5-bisphosphatet (PIP_2) to form two hydrolytic products: DAG and inositol 1,4,5-trisphosphate (IP_3).

The increase in the cytoplasmic free Ca^{2+} concentration occurs by IP_3 interaction with the inositol trisphosphate receptors (InsP3R), which operate as IP_3-gated Ca^{2+} channels and promote the rapid release of Ca^{2+} from intracellular stores at the endoplasmic reticulum (ER).

An increase in the membrane-associated DAG promotes low affinity DAG binding to the C1 domain in cPKC, followed by coordinated binding of the cPKC C2 domain to membrane phospholipids in a Ca^{2+}-dependent manner. This step causes cytosol-to-membrane translocation of cPKC, and involves changes in its conformation that release the catalytic domain from the pseudosubstrate, allowing for substrate binding and phosphorylation and activation of downstream signaling cascades.

A similar mechanism regulates the activation of nPKC, in which the affinity of the C1 domain to DAG is two orders of magnitude higher than that of the C1 domain of cPKC [69,83]. The higher affinity to DAG enables nPKC to respond to agonists by membrane translocation and activation in the absence of elevated intracellular Ca^{2+} concentration.

The transient nature of DAG appears to control both the initiation and the termination of the PKC-regulated signaling cascades. Following its formation by PIP_2 hydrolysis, DAG undergoes phosphorylation by DGK, which catalyzes its conversion to PA and terminates the PKC-induced signals. The reversal of activity of selected PKC isoforms may also involve their dephosphorylation, ubiquitination, and degradation [84–86].

Conformational changes in PKC, which occur during its transition between inactive and active states, determine the ability of the distinct isoforms to interact with their specific anchoring/scaffolding proteins. These interactions define the subcellular location of individual isoforms, and determine their accessibility to specific substrates and confer functional selectivity [87]. Recent studies suggested that the C1a domain plays a critical role in controlling the targeting of individual PKC isoforms to specific intracellular compartments [88,89].

5. PKC in cancer diseases

The understanding that PKC is the long sought-after receptor for tumor-promoting phorbol esters established the potential role of PKC in carcinogenesis and prompted a series of studies aimed at understanding the mechanisms by which PKC contributes to tumorigenesis. Numerous observations from basic studies and clinical trials have implicated PKC in the pathophysiology of diverse types of cancer diseases Table 1. In addition, increased expression of selected PKC isoforms was observed in different types of cancer, including cancer of the breast [90], lung [91], brain [92], ovaries [93], bladder [94], kidney [95], and the gastrointestinal system [96]. Despite the fact that abnormal expression of PKC is a common characteristic of neoplasia, the causal relationships between the altered expression of PKC and the transformation process or the disease progression are not clear.

The PKC genes appear to be distinct from classical protooncogenes, since mutations in PKC genes do not lead to their activation and/or induction of cell transformation. However, because of the central role that PKC plays in the maintenance of cellular homeostatic mechanisms, it seems possible that an altered regulation of PKC contributes to tumor promotion by inducing the erroneous expression of protooncogenes or by synergizing with oncogenes and altering signaling pathways that control cell behavior. For example, PKC-mediated regulation of the protooncogene product rapidly accelerated fibrosarcoma (c-Raf; also termed Raf-1) [97], may contribute to the c-Raf-induced cell transformation, since the c-Raf serine/threonine kinase is a gatekeeper of the mitogen-activated protein kinase (MAPK) pathway [97]. This pathway regulates the expression of genes that control the cell cycle, promote cell proliferation and migration, and inhibit apoptosis. The tight regulation of this pathway by PKC maintains normal cell behavior under strict control.

Table 1
Potential effects of individual PKC isoforms in cancer.

PKC	Proposed role of PKC	References
PKCα	Promotes cell proliferation, migration, survival (C)	[143]
	Promotes cell proliferation, antiapoptotic (Gl)	[124]
	Inhibits cell spreading and motility (B)	[136]
	Promotes cell migration, intravasation and metastases (B)	[269]
	Protects from drug resistance (B, C)	[270,271]
PKCβ	Promotes angiogenesis (B, Gl)	[125,128]
	Promotes angiogenesis and tumor cell growth (Pr)	[127]
	Promotes cell growth, survival, antiapoptosis (Le)	[272–274]
PKCγ	Promotes indirectly tumor cell motility and metastasis formation (B)	[275]
PKCδ	Inhibits cell proliferation, promotes apoptosis (Gl)	[124]
	Promotes apoptosis (K)	[245–247,251]
	Promotes cell invasion and migration (Li)	[276]
PKCε	Promotes cell proliferation (L)	[91]
	Promotes cell survival, antiapoptosis (Gl, L, Ly)	[115]
	Promotes cell invasion, motility (H)	[122]
PKCη	Increase resistance to apoptosis (L, Le)	[277,278]
	Promotes senescence (B)	[279]
PKCθ	Promotes cell proliferation, antiapoptosis (G)	[280]
PKCζ	Promotes cell proliferation, migration, invasion (Gl)	[281]
	Promotes cell survival, metastasis (P)	[282]
PKCι/λ	Promotes cell proliferation, invasion and survival (Gl, L)	[129]
	Represses cell senescence (B, Gl)	[283]

*The abbreviations indicate the origin of the tissue or cell line in which the studies were performed.
B, breast; C, colon; G, gastrointestinal stromal tumor (GIST); Gl, glioma/glioblastoma; H, head and neck squamous cell carcinoma (HNSCC); K, keratinocytes; L, lung; Le, leukemia; Li, Liver; Ly, lymphoma; P, pancreas; Pr, prostate.

5.1. Involvement of PKCε in cancer diseases

One of the PKC isoforms most intensively studied in the context of oncogenesis, which showed the greatest oncogenic potential among members of the PKC family, is PKCε [98,99]. This PKC isoform was found to be abnormally upregulated in the majority of primary human non-small cell lung cancer (NSCLC) [91]. In addition, elevated PKCε expression was found to correlate with increased disease recurrence and decreased overall survival of head and neck squamous cell carcinoma (HNSCC) patients [100].

The first hint that PKCε might be involved in tumorigenesis evolved from sudies of a small cell lung cancer (SCLC) cell line, NCI-N417, which was found to abnormally express high levels of PKCε, including a truncated form of constitutively active PKCε [101]. Further studies by Mischak et al. [102] demonstrated that overexpression of PKCε in NIH3T3 cells led to significant changes in the growth rate and the adhesion properties of the cells. The overexpressed PKCε led to increased anchorage-independent cell growth, and augmented the rate of cell multiplication in vitro. In addition, in contrast to the nontransfected cells, the PKCε-overexpressing NIH3T3 cells were able to grow and produce solid tumors following their inoculation into athymic nude

mice. Similar observations were made in PKCε overexpressing rat embryonic fibroblasts, which multiplied in vitro at a much faster rate and at a higher saturation density, and exhibited lower dependency on serum. Inoculation of these cells into athymic nude mice also resulted in a high rate of tumor formation [99].

5.2. The role of PKCε in chemical carcinogenesis

Using the two-stage chemical carcinogenesis model, PKCε transgenic mice that were initiated with DMBA and promoted with TPA developed a significantly higher number of squamous cell carcinoma (SCC) tumors, compared to wild type treated mice [103]. DMBA alone induced SCC tumors in approximately one quarter of the PKCε transgenic mice, but in none of the wild-type littermates. The skin carcinomas in the PKCε transgenic mice, which were pathologically graded as moderately differentiated SCC, metastasized to the regional lymph nodes within 3 weeks of appearance. In contrast, the skin tumors in the wild type mice, which were graded as well-differentiated SCC, were restricted to the dermis and did not metastasize. These studies demonstrated that overexpression of up to 18-fold higher levels of PKCε were insufficient for the spontaneous production of tumors. However, the high PKCε expressors were significantly more sensitive to the effects of the tumor initiating and promoting agents, suggesting that PKCε acts as a positive regulator of the transformation process.

5.3. PKCε as a regulator of apoptosis

PKCε is a critical regulator of cellular responses mediated by TNF-related apoptosis-inducing ligand (TRAIL; Apo2 ligand), a member of the tumor necrosis factor-α (TNFα) superfamily and promoter of apoptosis in a variety of tumor cells [104]. Activation of PKCε was found to protect cells from TRAIL-induced apoptosis in various types of cancer, including cancer of the breast [105], glioma [106,107], melanoma [108], erythroleukemia [109], and acute myeloid leukemia (AML) [110].

PKCε has also been implicated in the negative regulation of apoptosis, and was found to inhibit both the mitochondrial (intrinsic) and receptor-mediated (extrinsic) pathways of programmed cell death [111,112]. The intrinsic pathway is usually triggered by genotoxic stress, which permeabilizes the mitochondia and induces the release of apoptotic factors, such as cytochrome c and apoptosis inducing factor (AIF) into the cytosol, leading to activation of caspase proteases which mediate cell apoptosis [113].

Studies by Lau et al. demonstrated that a genotoxic stress promotes the translocation of the activating transcription factor 2 (ATF2) to the mitochondria, where it increases membrane permeability and promotes apoptosis [114]. Nuclear localization of ATF2 is directed by PKCε-mediated phosphorylation of ATF2 at Thr52, an effect which is attenuated during a genotoxic stress. The negative effect of PKCε on genotoxic stress-inducing apoptosis was demonstrated in melanoma cells, where high levels of PKCε negatively regulated the ATF2 nuclear export and function at the mitochondria, thereby attenuating apoptosis. Overexpression of PKCε was found to inhibit TRAIL-induced apoptosis in MCF-7 breast cancer cells by inhibiting the activation of caspase 8 and 9 and the release of the mitochondrial cytochrome c [105].

The extrinsic pathway of apoptosis, also termed death receptor-mediated pathway, is triggered by extracellular ligands of the TNF family that engage their cognate cell surface death receptors. Triggering of the receptor-linked signaling pathway leads to the activation of caspase 8 and the formation of death-inducing signaling complex (DISC) [115,116].

The extrinsic pathway of apoptosis was found to be sensitive to inhibition by TPA-mediated activation of PKC. Melanoma cells that express low levels of PKC were found to be sensitive to TRAIL-induced apoptosis, while PKCε overexpression enabled the TPA-treated cells to gain resistance to TRAIL-induced apoptosis [108]. This effect was dependent on the enzymatic activity of PKCε, since expression of a dominant-negative PKCε had an opposite effect. Similarly, introduction of a PKCε inhibitor into U937 histiocytic lymphoma cells increased the sensitivity of the TPA-treated cells to TNFα-induced apoptosis [117]. TRAIL-induce apoptosis in glioma cells was found to be associated with a rapid caspase-dependent cleavage and down-regulation of PKCε [107]. Indeed, the cells' sensitivity to TRAIL could be reversed by PKCε overexpression, while expression of a caspase-resistant PKCε mutant (D383A) provided better cell protection from the TRAIL-induced apoptosis. Overall these studies indicate that both the cleavage and the down-regulation of PKCε contributed to the TRAIL-induced apoptosis in the glioma cells.

An additional PKCε-downstream protein that plays a role in TRAIL-induced apoptosis is protein kinase B (PKB; also termed Akt), an important positive regulator of cell growth and survival [118]. PKB was found to undergo selective downregulation in glioma cells following cell exposure to TRAIL or after silencing of PKCε [107]. Overexpression of PKCε inhibited the apoptosis induced by TRAIL and prevented the downregulation of PKB. The assumption that the anti-apoptotic activity of PKCε is mediated by PKB was further substantiated in MCF-7 human breast cancer cells where TNF promoted the transient phosphorylation and activation of PKB, an effect that was more prominent in PKCε overexpressing cells [119]. siRNA-mediated silencing of PKCε decreased TNF-induced PKB phosphorylation and activation, and induced a concomitant increase in cell death. In addition, depletion of PKB in MCF-7 cells abolished the anti-apoptotic effect of PKCε in TNF-treated cells. These findings substantiate the role of PKB as a downstream target of PKCε, which is essential for the transduction of the survival signal following tumor cell exposure to TRAIL.

5.4. Regulation of tumor cell growth and motility by PKCε

In addition to its positive role in the regulation of cell growth and survival, PKCε also promote cellular functions that increase cell motility and, following cell transformation, the ability to metastasize to remote tissues and organs. This was demonstrated in NIH3T3 fibroblasts where PKCε overexpression promoted polarized cell morphology and increased the cells' ability to invade and migrate through Matrigel-coated polycarbonate matrices [120]. Furthermore, the PKCε-overexpressing cells produced metastases in various organs following their inoculation into nude mice.

A different approach for testing the role of PKCε in cancer cells was employed by Pan et al. who performed targeted disruption of PKCε in the human breast cancer cell line, MDA-MB231. These cells express high levels of PKCε and exhibit an aggressive phenotype. However, silencing of PKCε significantly decreased their in vitro proliferative, invasive and motile activity [121]. Furthermore, inoculation of the PKCε knocked-down MDA-MB231 cells into nude mice resulted in a retarded growth of the local tumors and lower incidents of lung metastases. These results suggest that PKCε plays an important role in promoting an aggressive and invasive phenotype in breast cancer.

The above results were further substantiated using an aggressive HNSCC cell line, where targeted disruption of PKCε decreased the invasive and motile phenotype of the cells [121,122]. Knockdown of PKCε was found to inhibit RhoA and RhoC activity, suggesting that a PKCε-Rho GTPase signaling axis plays a role in the promotion of the invasive and motile phenotype of HNSCC [122].

5.5. Involvement of PKCα and PKCβ isoforms in cancer diseases

A host of studies implicate additional PKC isoforms in a wide range of cancers. The ubiquitously expressed PKCα isoform, which is activated in response to many different physiological stimuli, is implicated in cancer progression due to its ability to upregulate cell growth and migration and promote anti-apoptotic activity [123,124]. PKCβ isoforms have been implicated in the progression of breast cancer [125],

colon cancer [126], prostate cancer [127], and glioblastoma [128], and PKCι promoted lung cancer cell growth and tumorigenicity [129], and exhibited oncogenic properties in NSCLC [130,131] and ovarian cancer [132].

More comprehensive reviews describing many of the studies showing the potential role of individual PKC isoforms in tumor formation and progression have been published elsewhere [96,133–135].

The effects of PKC enzymes on cancer appear to be cell type-specific, since a single PKC isoform may exert opposite effects in different cancers. For example, PKCα was found to increase tumor cell motility in colon carcinoma [123], but inhibited cell motility in breast cancer [136].

The expression levels of PKC isoforms in cancer cells can also vary drastically. For example, the expression of PKCβII in colon cancer, was found to increase [137,138], remain unchanged [139,140] or decrease [139,141,142], with no direct correlation to the disease stage or severity. In addition, PKCα was found to promote colon cancer cell proliferation in some studies [143], and exhibit opposite effects in others [144].

The sometimes-contradictory effects of PKC on tumor cell behavior might reflect PKC-mediated regulation of signaling pathways that are affected in an opposite way by different oncogenes within the different cancer cells tested. In other cases, they might reflect the utilization of two or more different experimental systems. Furthermore, some conclusions might be based on information which is only partially accurate.

For example, PKCα was shown to increase cell motility of the human carcinoma cell line SW480 in which the PKCα-specific inhibitor Gö6976, slowed down the spontaneous and norepinephrine-induced cell locomotion in a three-dimensional collagen lattice [123]. In contrast, PKCα was found to inhibit the motility of the breast cancer cell line MDA-MB-231, whereby TPA selectively activated the PKCα isoform and inhibited cell spreading and motility [136]. The opposite assumptions regarding the role of PKCα in the regulation of cell locomotion were based on the utilization of different experimental systems and pharmacological modulators of PKCα. Furthermore, independent studies questioned the specificity of the reagents used, and provided evidence for their broad-spectrum activity. They demonstrated that Gö6976 is an ATP-competitive inhibitor of PKCα and PKCβ [145], which inhibits additional kinases, including the Janus kinase 2 (Jak2) [146], fibromyalgia syndrome (fms)-like tyrosine kinase 3 (FLT3) [146], and protein kinase D (PKD) [147], while TPA acts as a DAG homologous that can activate all cPKC and nPKC isoforms [148,149], as well as other C1 domain-containing proteins, such as chimaerins, RasGRP and Munc13 [50–55]. It is therefore clear that findings obtained using pharmacological modulators of PKC must be substantiated by alternative approaches that utilize genetic tools.

5.6. Involvement of atypical PKC isoforms in cancer diseases

Members of the aPKCs family have also been implicated in oncogenic transformation and altered expression or mutation in aPKC genes were found to modulate tumor cell migration, growth, survival and ability to invade remote tissues [150].

Increased expression and/or activity of PKCζ was found to promote tumorigenesis in several different tissues, including prostate [151,152] lung [152,153] and pancreas [154]. Other studies demonstrated that PKCζ may operate as a tumor suppressor in prostate cancer, where PKCζ knockdown, or expression of a kinase-inactive PKCζ enhanced the growth rate and invasive capabilities of the tumor cells in vitro [155]. Tumor suppressing activity of PKCζ was also observed in other tissues, including the intestine, where loss of PKCζ enhanced intestinal tumorigenesis in mice, and low levels of PKCζ correlated with a poor prognosis in human colorectal cancer patients [156]. Furthermore, an inactivation mutation in PKCζ (S514F) has been identified in human colon cancers [153,157]. The mechanisms by which PKCζ affects tumorigenesis are unclear, and are likely to reflect the specific PKCζ-upstream regulators and/or PKCζ-interacting molecules that are mutated or altered in each of the distinct cells that underwent transformation.

Accumulating data indicates that PKCι is an oncogene and a prognostic marker in human tumors, and in many cases, a predictive of poor clinical outcome. Elevated expression of PKCι has been demonstrated in many different human cancers originated in tissues, such as the lung [131], pancreas [158], colon [159], breast [160], and ovaries [161,162]. The PKCι gene, PKCι, is located on chromosome 3q26, a frequently amplified genomic region in human cancers, where PKCι overexpression is frequently correlated with increased copy number of PKCι. For example, PRKCι copy number gain was observed in > 70% of the lung squamous cell carcinoma (LSCC) [131] and epithelial ovarian cancers [162], and in smaller proportions in other major forms of human cancer, although alternative mechanisms that promote PKCι expression and/or activation have been suggested (reviewed [163,164]).

Studies of PKCι in cell lines and animal models demonstrated that PKCι is required for the transformed phenotype of colon [159], ovarian [93], lung [165], and other types of cancer, and provided evidence for the involvement of PKCι in multiple stage tumorigenesis, including the initiation, progression and metastasis formation (reviewed in [164]).

The role of PKCι in tumorigenesis depends on the cell type and mechanism of transformation, and involves multiple PKCι-regulated signaling pathways. For example, the PKCι-dependent signaling pathway that drives oncogenic growth of human NSCLC cells is mediated by the PKCι-Par6- Ect2 complex, which activates the MEK/ERK signaling axis [166,167]. Studies in rat intestinal epithelial cells demonstrated that PKCι is involved in the *Ras*-induced cell transformation processes, which are likely to contribute to colon carcinogenesis [159], while in LSCC and ovarian serous carcinoma (OSC), PKCι drives the transformed cell growth via a PKCι-dependent Hedgehog signaling [130], and PKCι-dependent YAP1 signaling pathways [168], respectively.

More recent studies demonstrated the involvement of PKCι in the maintenance of normal pluripotent stem cells through a NOTCH-dependent pathway [169], and again demonstrated that loss of PKCι results in a marked increase in the number of pluripotent cells [169,170].

These findings suggest that additional studies and extra precautions should be taken before applying PKCι inhibitors in cancer therapy, since such drugs might suppress the growth of the majority of tumor cells, on the one hand, but promote cancer stem cell propagation on the other hand.

6. Drug targeting and modulation of PKC

Ever since its discovery as the cellular receptor for tumor promoting agents, PKC has been the subject of intensive research in cancer diseases. The frequently observed disregulation of PKC enzyme activity in cancer cells implicated PKC in cell transformation and tumorigenesis, and generated interest in the development of PKC-based drugs for anticancer therapy. These efforts turned out to be very challenging, since most cells express multiple types of PKC isoforms, which share substantial sequence homology and overall structural similarities. In addition, distinct PKC isoforms within a single cell might exhibit opposite or antagonistic biological effects. For example, overexpression of PKCα and PKCβ in the rat embryo fibroblast cell line R6 exhibits redundant effects on the expression of early response genes, but opposite effects on cell growth stimulation, suggesting that the two PKC isoforms may have opposing effects on tumorigenesis [171,172].

The most common drugs that target PKC include compounds that interact with the PKC ATP binding site or the DAG-binding C1 domain. Additional modulators include competitive or allosteric inhibitors of PKC interaction with binding partners and PKC isoform-specific antisense oligonucleotides (Table 2).

Table 2
Selected approaches for inhibition/regulation of PKC in cancer cells[a].

Mechanism of inhibition	Compound/Drug	Target	Type of cancer	References
Upstream regulators of PKC	UCN-01	PDPK1	ovarian cancer	[177]
	AZD2014	mTOR	clear cell renal cancer	[284]
	temsirolimus (CCI-779)		breast cancer	[285]
	everolimus (RAD001)		renal cell carcinoma	[286]
ATP competitive Inhibitors	midostaurin (PKC142)	pan-PKC	acute myeloid leukemia	[191,287]
	sotrastaurin (AEB071)	PKCβ	chronic lymphocytic leukemia	[24]
	enzastaurin (LY317615)	PKCβ	B-cell lymphoma	[192,193]
			pancreatic cancer	[288]
			lung cancer	[289]
DAG mimetic Compounds	bryostatin 1 (NSC339555)	cPKC/nPKC	ovarian cancer	[290,291]
			pancreatic cancer	[292]
Inhibitor of PKC interaction with binding partners	aurothiomalate (ATM)	PKCι	non-small-cell lung cancer	[293]
			ovarian cancer	[293]
			pancreatic cancer	[293]
Antisense oligonucleotide inhibitors	aprinovarsen (ISIS-3521; LY900003)	PKCα	colorectal cancer	[294,295]
			prostate cancer	[296]
			non-Hodgkin's lymphoma	[297]
			non-small-cell lung cancer	[298]
			ovarian carcinoma	[299]

[a] The table includes representative compounds/drugs in each subgroup and selected cancer diseases in which they were tested. Only compounds/drugs that have been used in clinical studies (phase I, II, or III) are included in the table.

6.1. Inhibition of upstream regulators of PKC

Regulation of PKC activity is mediated by phosphorylation of conserved motifs within its activation loop, which is required for the catalytic competence of PKC, as well as for the regulation of its stability and ability to interact with other molecules that determine its subcellular location [78,173,174]. A critical upstream pan-PKC regulator is PDPK1, a master regulator of kinases that might serve as a generic target for inhibition of all PKC isoforms [78,173]. A single PDPK1 isoform has been reported in humans and targeted deletion of PDPK1 in mice resulted in embryonic lethality [175]. However, PDPK1 hypomorphic mice that express ~10% of the normal level of PDPK1 displayed no obvious deleterious phenotype [175] suggesting that PDPK1 inhibitors might be useful in cancer therapy. A potent but nonselective PDPK1 kinase inhibitor [176], UCN-01, was tested in phase I/II clinical trials in advanced cancer patients, but did not yield significant therapeutic effects [177]. The mammalian target of rapamycin (mTOR) enzymes are also involved in the phosphorylation of residues essential for the activation of all cPKC and nPKC isoforms [178,179], as well as other effector proteins, such as AKT, p4EBP1 and pS6RP [180]. An inhibitor of mTOR, AZD2014 [181], increased the radiosensitivity of human glioblastoma stem-like cells (GSC) and prolonged the survival of irradiated athymic nude mice bearing a GSC-derived brain tumor xenograft [182]. In addition, AZD2014 exhibited dose-dependent antiproliferative effects on ER+ breast cancer cell lines in vitro, and inhibited tumor growth in vivo, in severe combined immunodeficiency (SCID) mice bearing a human breast cancer xenograft [183]. AZD2014 was found to inhibit the growth of additional types of tumor cells and is currently being evaluated in more than 20 clinical trials for the treatment of different types of cancer (https://clinicaltrials.gov/ct2/results?term=AZD2014&Search=Search).

Two additional randomized phase III studies using mTOR inhibitors, temsirolimus and everolimus, demonstrated short-term survival benefits, but the responses were not durable and the patients ultimately progressed because of the emergence of drug resistance (reviewed in [184]).

Competitive inhibitory peptides possessing sequences of PKC, which are phosphorylated by PDPK1 or mTOR, are likely to be non-selective, since PDPK1 and mTOR phosphorylate conserved sequences that are shared by all PKC isoforms. Other PKC upstream kinases, such as the Lck and Src protein tyrosine kinases (PTKs), which phosphorylate PKCθ [185] and PKCι [186], respectively, appear to be highly selective regulators of individual PKC isoforms. Therefore, competitive inhibitory peptides possessing the relevant PKC isoform-specific sequences might operate as selective inhibitors of PKCθ-dependent functions in T cells [185], and inhibitors of activity and transport of PKCι to the nucleus [186].

6.2. ATP-competitive small-molecule inhibitors

The most efficient kinase inhibitors are the ATP-competitive small-molecules that mediate high affinity interaction with the highly conserved ATP binding motif within the N-terminal extremity of the catalytic domain. This ATP is then catalyzed by the kinase domain which transfers the ATP gamma phosphate to an amino acid residue in a substrate protein. However, the ATP binding site and its immediate vicinity share high sequence homology and structural similarity among kinases, and therefore the design of selective inhibitors, even for a subset of the ~500 human protein kinases [187], is an extremely difficult challenge.

The most studied ATP competitive kinase inhibitor is staurosporine, a natural product of *Streptomyces staurosporeus* bacterium, which was discovered 40 years ago [188]. Staurosporine is a water-soluble compound and a potent inhibitor of PKC that is effective at nanomolar concentrations. However, it binds to all PKC isoforms, as well as to other serine/threonine kinases [189], and its lack of selectivity precludes its clinical application.

Functional analysis of a semi-synthetic derivative of staurosporine, termed midostaurin (PKC142), demonstrated potent inhibition of all PKC isoforms.

The effect of midostaurin was tested in 25 clinical trials on patients with different types of cancer, either as a single agent or in combination therapy (https://clinicaltrials.gov/ct2/results?term=Midostaurin), and the results suggested some improvement in survival of patients with selected types of disease [190,191].

A synthetic compound, termed enzastaurin (also known as LY317615) is structurally similar to staurosporine and initially reported as a selective PKCβ inhibitor [192]. Later studies demonstrated that enzastaurin inhibits additional PKC isoforms as well as other serine/threonine kinases. Phase I and II clinical trials using enzastaurin showed some promising effects (https://clinicaltrials.gov/ct2/results?term=LY317615), but a recent phase III clinical trial, which assessed enzastaurin hydrochloride as a monotherapy in the prevention of relapse in patients with diffuse large B-cell lymphoma (DLBCL), was

disappointing, and showed no increase, compared to placebo, in disease-free survival of patients [193].

A structurally distinct ATP-competitive small-molecule inhibitor of PKC, termed rottlerin (mallotoxin), is a natural product isolated from the Asian tree *Mallotus philippensis*. Rottlerin was originally reported to selectively inhibit PKCδ [194], but further studies revealed that it modulates several additional enzymes, including a potassium channel opener, which regulates the mitochondrial membrane potential [195].

High throughput screening methods are currently being employed in order to identify newer ATP-competitive small-molecule inhibitors, and structure-based drug design strategies are utilized to improve and optimize lead compounds. It is hoped that modern state-of-the-art knowledge-based approaches will lead to future discoveries of more selective and potent ATP-competitive inhibitors.

6.3. C1 domain-binding DAG mimetic compounds

Phorbol ester and related diterpenes are the first group of compounds that were found to exhibit pharmacological properties similar to those of the lipid second messenger DAG, due to their ability to interact with the PKC C1 domain. Because of their greater affinity and stability compared to DAG, these compounds activate PKC in a constitutive manner, and eventually lead to its degradation [2].

Bryostatin 1 is a representative of a second group of highly potent C1 domain-binding compounds, which can mediate short-term activation of cPKC and nPKC without the induction of tumor-promoting activity. The bryostatin 1 is a macrocyclic lactone, which was originally isolated from the marine bryozoan, *Bugula neritina* [196]. It antagonizes the tumor-promoting effects of phorbol esters [197], apparently by promoting the rapid degradation of the cellular PKC [59]. Bryostatin 1 was found to promote differentiation and inhibit the growth of several types of freshly isolated human leukemia cells [198–200]. The potential anti-cancer effect of bryostatin 1 was tested in multiple clinical trials (https://clinicaltrials.gov/ct2/results?term=bryostatin), but the results showed very limited success. The low concentration of bryostatin 1 in the bryozoans makes its large-scale production unrealistic, and therefore, current studies utilize chemically synthesized bryostatin 1, as well as structurally related analogs.

An additional group of DAG analogues was designed through a pharmacophore-guided approach, based on the 3D structure of the PKCδ C1b domain in complex with TPA. These DAG lactones show 3–4 orders of magnitude higher affinity for PKC isoforms, and a greater specificity to PKCα and PKCδ, compared with the natural DAG [201]. Two of these compounds, HK434 and HK654, were found to induce apoptosis in the human prostate cancer cell line, LNCaP, with a higher potency than DAG and PDBu [201].

The structure of the C1 domain in all cPKC and nPKC isoforms is highly conserved, predominantly at the DAG binding motif, making it difficult to develop isoform-selective compounds that target the C1 domain. However, the lower levels of sequence identity on the exterior of the C1 domain [201] might be advantageous in the future design of isoform-selective C1 domain-binding compounds.

6.4. C2 and PB1 domain-directed inhibitors

The C2 domain in cPKC/nPKC and the PB1 domain in aPKC are involved in various protein-protein interactions and are essential for the recruitment of activated PKC isoforms to distinct subcellular compartments. The fact that the subcellular localization of PKC isoforms changes following their activation suggests that localization of activated PKC is controlled by the ability of the enzyme to interact with unique isoform-specific anchoring proteins [202]. The first binding partner of activated PKC was identified in 1991 and termed receptor for activated C-kinase (RACK) [203]. Soon after, additional RACK proteins were discovered, including PKC isoform-specific RACKs, such as RACK1, which interacts with PKCβII, but not with PKCβI [204], despite the fact that the two enzymes originate by alternatively splicing of a single gene.

Another PKC binding partner is heat shock protein 90 (HSP90), which can interact through different sites and for different purposes with either PKCδ or PKCε isoform. The HSP90 chaperone interacts with the PKCε C2 domain and allows the shuttling of PKCε into the mitochondria [205]. Binding of HSP90 to PKCδ is mediated by a distinct mechanism and promotes the shuttling of PKCδ to the nucleus [206]. A cell permeable 7-mer peptide, derived from the PKCε C2 domain, affects HSP90 interaction with PKCε, but has no effect on HSP90 interaction with PKCδ [205].

Additional binding proteins were found to interact with unique sequences in the PKC C2 domain, and peptides that correspond to these binding sequences were shown to act as competitive inhibitors [18,207].

The aPKC isoform-specific PB1 domain is found in PKCι and PKCλ, and is involved in PB1-PB1 protein-protein interactions. PB1 domain-mediated interaction of PKCι with the adaptor protein partitioning defective 6 (Par6) [75] is essential for activation of the ERK1/2 signaling pathway, which is required for the transformed growth of NSCLC cells in vitro, and for tumor formation in vivo [129]. In search of small molecule chemical inhibitors of the PKCι-Par6 interaction, Stallings-Mann et al. [208] utilized a drug screening assay based on fluorescence resonance energy transfer (FRET) to identify compounds that disrupt the PB1-PB1 domain-mediated binding of PKCι to Par6. Aurothiomalate (ATM), which was identified in this screen, was found to be a selective inhibitor of the PB1-PB1 domain interaction between PKCι and Par6 [167,209]. In addition, ATM inhibited the transformed growth of NSCLC cells by selectively targeting the PKCι PB1 domain.

6.5. Additional inhibitors of PKC interaction with binding protein

In addition to the PKC binding partners that interact with the C1, C2 and PB1 domains, other molecules were found to interact with intervening regions within the PKC protein [210]. For example, PKCθ in resting T lymphocytes resides in the cytosol, but is translocated to the center of the immunological synapse following T cell antigen receptor (TCR) engagement by peptide-bound MHC molecules expressed in the surface of antigen-presenting cells (APCs) [211]. The redistribution of PKCθ is made possible by interaction of a specific motif within the PKCθ V3 domain with the immunological synapse-residing CD28 coreceptor via interaction with the Lck PTK, which functions as an intermediate protein. A peptide possessing the unique V3 domain motif, which is required for PKCθ-CD28 interaction, inhibited PKCθ sequestration to the immunological synapse [211], suggesting that this inhibitory peptide might be useful as a specific inhibitor of PKCθ-dependent signaling events in normal T cells during their activation, and in hyperproliferative or leukemic T cells in which cell growth is positively regulated by PKCθ.

A different peptide that corresponds to the PKCβII V5 domain inhibits PKCβII binding to RACK1 [212] and reduces human prostate cancer growth by targeting both angiogenesis and tumor cell growth [127]. In addition, a peptide that corresponds to the PKCε C1a-C1b domain-intervening sequence competes with PKCε binding to actin [213], while a peptide that corresponds to the PKCε C2-C1 domain-intervening sequence competes with PKCε binding to HSP90 [205].

Additional proteins were found to interact with selected PKC isoforms, including the A-kinase anchor proteins (AKAP) [214], annexins [210], 14-3-3 [215], and PKC-interacting cousin of thioredoxin (PICOT) [216]. However, binding motifs within the binding partners and the effect of competitive binding inhibitors on these interactions have not always been identified.

Finally, another group of PKC binding partners includes the substrate interacting with C-kinase (STICK) proteins [217], the myristoylated alanine rich c-Kinase (MARCKS) [218] and AKAP79 [214], which interact with the PKC regulatory domain and are substrates for

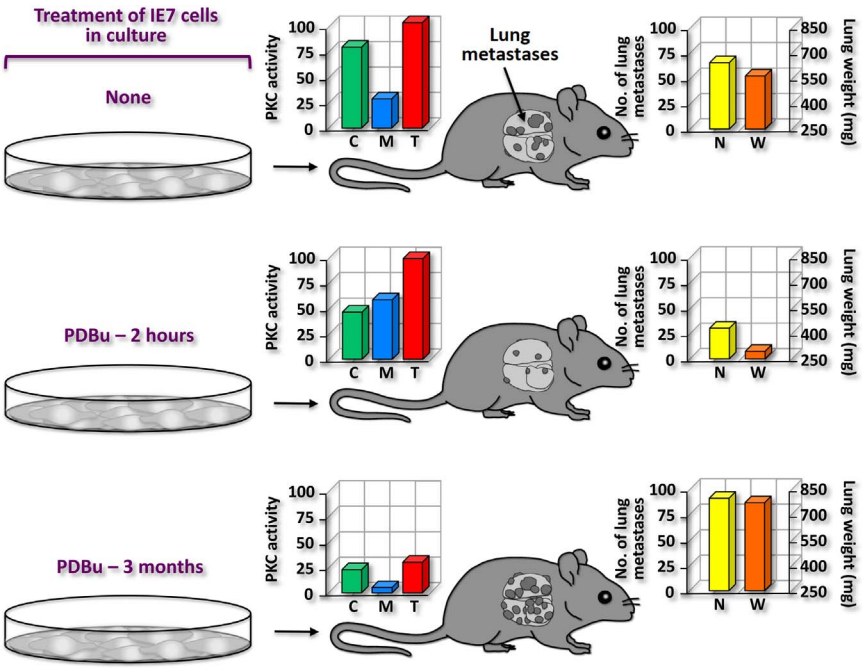

Fig. 3. Treatment of cancer cells with phorbol esters shows an inverse correlation between the effect on PKC activity and the cells' ability to form lung metastases.
The IE7 is a metastatic subline of the murine methylcholanthrene-induced T10 fibrosarcoma. Intravenous injection of IE7 cells into syngeneic C57BL/6J mice results, within three weeks, in the formation of a large number of lung metastases and an increase in the lung weight (upper panels). Short term (2 h) in vitro treatment of the cells with the PKC-activating tumor promoter phorbol 12,13-dibutyrate (PDBu) did not alter the total amount of cellular PKC protein, but promoted its redistribution by allowing the translocation of a fraction of the cytosolic PKC into the membrane, and increased the overall PKC activity within the cells (middle panels). Intravenous injection of these cells into syngeneic mice resulted in a decrease in the number of lung metastases, demonstrating an inverse correlation between PKC activity in the cells and their ability to form lung metastases (middle panels). Long-term (3 months) exposure of IE7 cells to PDBu resulted in PKC degradation, a decrease in total amount of cellular PKC, and a marked decrease in the membrane-bound active PKC (lower panels). Intravenous injection of these cells into syngeneic mice resulted in a significant increase in the number and size of lung metastases. These results demonstrate once more an inverse correlation between the overall PKC activity in the cells and the cells' ability to form lung metastases, and suggest a negative role for PKC in tumorigenesis. Abbreviations: C, cellular fraction; M, membrane fraction; T, total cell lysate; N, number of metastases; W, weight of lungs.

PKC. The STICKs mediate additional interactions with phosphatidylserine and F-actin, suggesting their involvement in multisite interactions that help PKC targeting.

6.6. Antisense oligonucleotide inhibitors

Antisense therapy is based on the utilization of oligonucleotides possessing complementarity sequences to mRNA transcripts (sense sequence) of genes of interest. The mRNA is inactivated by the binding of the oligonucleotides which turns off the relevant gene. The PKCα-specific expression inhibitor, ISIS-3521, is a 20-mer antisense phosphorothioate oligonucleotide, which was shown to down-regulate PKCα expression in cancer cell lines and human xenograft models [219,220]. ISIS-3521 was tested in phase I and phase II clinical studies in metastatic melanoma, breast cancer, and NSCLC (http://clinicaltrials.gov/ct2/results?term=ISI+3521). It demonstrated activity as a single agent, but was more effective in combination with chemotherapy [220].

7. Tumor suppressing activity of PKC

The recognition that immortalized cancer cells are capable of continuous proliferation and self-renewal led to the suggestion that the induction of cancer cell differentiation might serve as an efficient strategy of cancer therapy. The differentiation therapy assumes that restraining the continuous growth and proliferation of cancer cells by reactivation of their differentiation and maturation programs will initially reduce the tumor mass, and eventually lead to apoptosis of the differentiated tumor cells [221–223]. This strategy was shown to be effective in several different types of leukemia, such as acute promyelocytic leukemia (APL), where all-trans-retinoic acid (ATRA) treatment led to a complete remission [224,225]. However, implication of similar strategies in other cancers appears to be extremely challenging. This is due to the enormously large number of cancer-inducing mutations in different oncogenes, which arrest tumor cell differentiation using many different tactics. Therefore, the induction of differentiation and maturation of different types of tumors will require the development of numerous tailor-made unique strategies.

Studies performed more than three decades ago demonstrated that blockage of leukemia cell differentiation can be reversed by certain chemicals that modulate PKC functions. The first demonstration was made in studies of the human myeloid cell line HL-60, which was derived from the peripheral blood leukocytes of an acute promyelocytic leukemia (AML) patient [226,227]. In vitro treatment of HL-60 cells with the tumor promoting agent TPA induced the cells to differentiate into adherent, monocyte/macrophage-like cells, that ceased proliferation [228]. TPA-induced cell differentiation was also demonstrated using the KG-1 myeloid leukemia cells, which were blocked at the myeloblast-promyelocyte stage of maturation [229]. These experiments were performed in the late 1970s when PKC was considered as the only TPA receptor in cells, and they therefore suggested that PKC activation by TPA might have a negative role on cancer progression.

This assumption was further substantiated using the PKCβ-specific inhibitor LY379196, which prevented the TPA-induced differentiation of the HL-60 cells [230]. Furthermore, characterization of an HL-60 mutated subline, termed phorbol ester tolerant (PET) [231], which is defective in the ability to differentiate in response to TPA, revealed that the cells express low levels of PKCβ mRNA. Restoration of PKCβ in the PET cells restored their ability to respond to TPA by differentiation and growth arrest, indicating that PKCβ is both necessary and sufficient for phorbol ester-induced differentiation of HL-60 promyelocytes [232].

Additional support for the assumption that PKC has a negative effect on tumor growth and/or aggressiveness came from studies on the IE7 metastatic subline of the murine methylcholanthrene-induced T10 fibrosarcoma. Intravenous injection of IE7 cells into syngeneic C57BL/6J mice resulted within three weeks in a large number of lung metastases and a significant increase in the lung weight [233]. Short term in vitro treatment of the cells with the PKC-activating tumor promoter phorbol 12,13-dibutyrate (PDBu) led to a partial cytosol-to-membrane translocation of PKC that correlated with an overall increase in PKC activity [234,235], with no change in the total amount of the cellular PKC. Intravenous injection of these cells to syngeneic mice resulted in a significant decrease in the number of lung metastases (see Fig. 3). An even more significant decrease was observed following the treatment of IE7 cells with TPA or phorbol-12,13-didecanoate (PDD) tumor promoters, while cell treatment with non-tumor promoting phorbol esters, such as 4α-phorbol 12,13-dibutyrate (4α-PDB), had no effect on the ability of IE7 cells to metastasize [233]. These results indicated that an increase in the fraction of activated PKC in IE7 cells correlates with a decreased ability of the tumor cells to form lung metastases.

An additional experiment using the IE7 cells was based on previous findings that long-term cell exposure to certain PKC-activating drugs resulted in degradation of PKC and decrease in PKC-dependent cellular responses [58,59,86]. In this experiment, IE7 cells were cultured in vitro in the presence of PDBu for three consecutive months. The constitutive long-term activation of PKC resulted in its degradation, leading to a ~4-fold decrease in the total amount of cellular PKC and a very marked decrease in the membrane-bound PKC (~3-fold lower levels compared to those in resting cells). Intravenous injection of these cells to syngeneic mice resulted in a significant increase in the number and size of lung metastases (see Fig. 3). These results indicate that a reduction in PKC expression and activity correlates with an increased metastatic ability of the tumor cells and an increase in their growth rate and/or resistance to host-mediated anti-tumor immunity.

A study by Boyle and co-workers [236] tested whether intra-lesional injection of a PKC-activating diterpene ester would affect the tumor growth in intact mice. They have found that a single injection of the novel diterpene ester EBC-46 led to a rapid ablation of the B16-10 melanoma in the syngeneic C57BL/6J mice, and ablation of the SK-MEL-28 and MM649 human melanoma in athymic BALB/c mice. This study demonstrated once again that activation of PKC could lead to inhibition of tumor cell growth.

Somewhat similar studies were performed in human patients with advanced myelocytic leukemia, which were refractory to several different anti-leukemia drugs, where intravenous injection of TPA led to a marked decrease in bone marrow myeloblasts and a temporary remission of disease symptoms [237,238].

Recent studies by Antal and coworkers provided more direct evidence for the role of PKC as a tumor suppressor [239]. They tested whether PKC imposes a positive or a negative effect on cancer progression by analyzing the effect of mutations in PKC genes on the loss or gain of PKC functions in human cancers. By examining a total of 48 mutations in PKC that were identified in human cancers, the authors discovered that the majority of mutations led to reduced or silenced PKC activity. None of the mutations turned on PKC activity. The mutations were not restricted to a specific PKC isoform or functional domain, and decreased PKC-mediated signals by altering the conformation, cofactor binding-ability or catalytic activity of the enzyme. Therefore, it was concluded that PKC isoforms function as tumor suppressors, and suggested that PKC-based therapies should focus on restoring, instead of inhibiting, PKC activity in the cancer cells.

7.1. Tumor suppressor activity of PKCδ

PKCδ is the first PKC isoform shown to have tumor suppressing properties. Activation of PKCδ was found to inhibit cell cycle progression, while down-regulation of PKCδ facilitated tumor promotion. In addition, PKCδ responded to DNA damage-induced stress by activation of programmed cell death mechanisms.

PKCδ and PKCε exhibited different and sometimes opposite effects in earlier studies where the biological effects of these two PKC isoforms were compared. While PKCε inhibited apoptosis and promoted cell growth and survival [106], PKCδ inhibited cell growth and exhibited pro-apoptotic activity [240]. These two PKC isoforms were also active during myocardial ischemia, where PKCδ negatively regulated mitochondrial function and induced apoptosis of cardiomyocytes, while PKCε exhibited opposite effects by protecting the mitochondrial function and preventing cell death [241].

Comparison of the effects of PKCδ and PKCε isoforms in NIH3T3 cells demonstrated that overexpression of PKCε induced anchorage-independent growth and neoplastic transformation. Inoculation of the PKCε-overexpressing cells into athymic nude mice led to tumor formation in 100% of the cases. In contrast, overexpression of PKCδ induced significant changes in cell morphology, slowed down their growth, reduced the cell density at confluency and did not promote the anchorage-independent growth [102].

Somewhat similar effects of PKCδ were observed in Chinese hamster ovary (CHO) cells, in which TPA treatment led to growth arrest of PKCδ-overexpressing cells, which accumulated in the G2/M phase of the cell cycle. In contrast, overexpression of PKCα, PKCβII or PKCζ in the CHO cells had almost no effect on the in vitro growth rate of the cells [242].

The effect of PKCδ was also tested in a line of mouse myeloid progenitor cells 32D, which do not differentiate in vitro, even in the presence of TPA. Upon PKCδ overexpression, these cells acquired the ability to differentiate into mature macrophages within 2–6 h post TPA stimulation [243].

Studies by Perletti et al. [244] compared the effects of PKCδ overexpression on two related epithelial cell lines derived from rat colon: the D/WT cell line which exhibits anchorage-dependent growth and does not form tumors in vivo, and a D/WT-derived v-*src* transformed cell line, termed D/*src*, that exhibits neoplastic phenotype. Overexpression of PKCδ inhibited the growth of the D/*src* cells and induced morphological changes toward the D/WT phenotype. In contrast, stable transfection of a kinase-defective PKCδ mutant into D/WT cells induced morphological and growth changes characteristic of partial transformation. It was concluded therefore that PKCδ could reverse the transformed phenotype and act as a suppressor of D/*src* cell growth.

PKCδ was shown to have tumor suppressor activity in UV-induced apoptosis of human keratinocytes [245–247], where PKCδ undergoes activation by caspase 3-mediated proteolysis [245]. Similar proteolytic mechanisms for activation of PKCδ were observed in additional types of cells that underwent apoptosis [248–250]. Furthermore, PKCδ overexpressing transgenic mice were found to be resistant to skin chemical carcinogenesis, while the in vitro response of their keratinocytes to TPA promoted an apoptotic response [251,252].

Since the induction of programmed cell death could serve as a general anti-cancer protective mechanism, the latter studies suggested that activated PKCδ might function as a tumor suppressor protein that leads to the elimination of the transformed cells by the induction of their apoptotic death.

This assumption was further substantiated by analysis of the contribution of PKCδ to cell responses in different experimental systems. For example, the PKCδ inhibitor rottlerin induced a transformed phenotype in c-Src-overexpressing rat fibroblasts [253] and HaCaT human keratinocytes [254], while downregulation or inactivation of PKCδ was often associated with neoplastic cell transformation [255].

Ras mutations are frequently observed in SCCs [256], and studies in Ha-*ras*-transformed cells demonstrated an inverse correlation between the activities of activated Ha-*ras* and PKCδ [255]. Expression of activated Ha-*ras* in HaCaT cells selectively reduced the expression of PKCδ with no apparent effect on the expression levels of other PKC isoforms, including PKCα, ε, and ζ [257]. Interestingly, downregulation of PKCδ expression in the Ha-*ras*-transformed HaCaT cells was induced indirectly via the effect of transforming growth factor alpha (TGFα). This was demonstrated by the addition of recombinant TGFα to HaCaT cells, which selectively inhibited PKCδ expression, whereas anti-TGFα antibodies reversed this effect.

In a somewhat different experimental system, the expression of oncogenic Ha-*ras* in epidermal keratinocytes was found to stimulate the tyrosine phosphorylation and inhibition of the enzymatic activity of PKCδ [258]. This effect appeared to be mediated indirectly by Ha-*ras*-induced expression of TGFα, since addition of TGFα to normal cells could recapitulate the effect of Ha-*ras* on PKCδ [259].

The human gene for PKCδ is located on chromosome 3p21.31 at a region that is frequently lost in poorly differentiated SCCs and in squamous cell carcinoma in situ (SCCIS), an early form of skin cancer also known as Bowen's disease (BD) [260,261]. Re-expression of PKCδ in activated Ha-*ras*-expressing HaCaT cells induced cell apoptosis in vitro and suppressed tumor cell growth in vivo in athymic nude mice [262]. Comparative immunohistochemical studies of PKCδ expression in normal human skin and SCCs/SCCIS revealed a loss of PKCδ

expression in approximately one third of the human skin cancers evaluated, indicating that loss of PKCδ expression is an important cellular mechanism that enables Ha-*ras* to induce cell transformation. These studies further promote the assumption that PKCδ functions as a tumor suppressor in certain types of human skin cancer.

While the pro-apoptotic functions of PKCδ support a tumor suppressor role for this PKC isoform, it should be emphasized that PKCδ participates in the regulation of additional cellular functions, and that under certain physiological conditions, it can also exhibit anti-apoptotic effects and even promote tumor cell survival. For example, PKCδ promotes the growth of human pancreatic cancer [263], and inhibition of PKCδ with rottlerin, which blocks DNA damage-induce apoptosis, was found to potentiate TNF receptor-induced apoptosis in HeLa cells [264]. The latter observation was corroborated by genetic studies performed in the human glioma cell line A172, where depletion of wild type PKCδ by transfection of either a kinase-deficient PKCδ mutant or PKCδ-specific small interfering RNA (siRNA) resulted in increased TRAIL-induced cell apoptosis, whereas overexpression of PKCδ provided cell protection from apoptosis [265]. Further studies demonstrated that in certain oncogenic context PKCδ could function as a growth promoter of tumor cells, as was demonstrated in K-ras dependent non-small cell lung cancer cells [266,267].

It appears therefore that in different cell types PKCδ can operate as a bi-functional regulator of tumor cell growth and death, and that future PKCδ-targeting strategies in cancer therapy will require predetermined knowledge on the specific function of PKCδ in the particular cancer cell type.

7.2. Tumor suppressor activity of PKCβII

In a recent study by Antal et al. [239], the authors tested whether a PKC loss-of-function mutation might confer a survival advantage for tumor cells. They utilized a patient-derived colon cancer cell line (HCT116) with a heterozygous loss-of-function mutation in the PKCβ gene, and demonstrated that restoration of the PKCβ gene function, using CRISPR-mediated genome editing, led to a significant increase in the basal level of PKCβ activity, concomitantly with decreased anchorage-independent cell growth in vitro. More important, the correction of PKCβ loss-of-function led to a slower growth rate of the tumor cells in vivo, following injection into nude mice, further demonstrating that loss of PKCβ function enhances tumor growth.

In a follow-up study performed on human colon cancer, Dowling et al. [139] provided a mechanistic explanation for the role of PKCβII in tumor suppression. They demonstrated that activation of PKCβII promoted the downregulation of signals induced by IGF-1, a critical regulator of cell survival and a major player in colorectal carcinogenesis [268].

Analysis of PKC gene expression in normal and cancer tissue samples from colon cancer patients revealed a significant and selective reduction of PKCβII in colon cancer cells. Tissue microarray analysis substantiated these findings and demonstrated a reduction in PKCβII protein expression in both the epithelia and the stroma of the diseased tissue. Furthermore, a direct correlation was observed between the expression level of PKCβII in the normal tissue of colon cancer patients and the patients' ten-year survival rate.

The tumor suppressive activity of PKCβII was also demonstrated using the HCT116 colorectal carcinoma cell line, in which overexpression of PKCβII led to down regulation of colony-forming ability and inhibition of migration and invasion of the tumor cells in in vitro assays.

The above studies suggested that targeting of PKC in cancer patients using a variety of PKC inhibitory drugs might be counteractive, and that future therapies should consider focusing on restoring, rather than inhibiting, PKC activity. Further studies are required in order to clarify whether the suppressor activity of PKC is a general phenomenon in all types of cancers, and whether the ability to suppress tumor growth is a common trait that is shared by all or only a selected group of PKC isoforms.

8. Conclusions and future perspectives

PKC enzymes play a major role in numerous metabolic and signaling pathways, and are involved in the regulation of cell growth, migration, differentiation and apoptosis. Considering the central role of PKC in a wide range of human diseases in general, and in cancer diseases in particular, it is obvious that the PKC enzymes represent important targets for drug development. The ability to target PKC enzymes in cancer therapy requires a better understanding of the regulatory networks relevant to individual PKC isoforms, which might vary between different types of normal cells and even between tumors that originate from the same tissue but carry distinct oncogenes and/or mutations. Future efforts to target PKC, using inhibitors and modulators directed against specific motifs in individual PKC isoforms, blockers of PKC-mediated phosphorylation of single types of substrates, or inhibitors of membrane-associated PKC-binding proteins (such as RACK or RICKs) represent promising avenues for the treatment of cancer diseases. Additional efforts are required for the development of tumor cell-specific drug delivery means, to avoid non-specific damage to vital tissues and organs.

Since inhibition or modulation of individual PKC isoforms in cancer cells may lead to the activation of alternative signaling routes, it is expected that PKC-based therapy will be effective when combined with additional drugs that target such elusive 'alternative signaling pathways', and conceivably with the assistance of additional and conventional treatment modalities.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the author.

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