Perspectives of Protein Kinase C (PKC) Inhibitors as Anti-Cancer Agents

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Abstract: Although the role of serine/threonine protein kinase C (PKC) in malignant transformation is known from decades, an anti-PKC based approach in cancer therapy was hampered for the difficulties in developing pharmacological compounds able to selectively inhibit specific PKC isoforms. In this review, the role of PKC-ε and PKC-δ in promoting and counteracting tumor progression in different types of cancer, respectively, will be discussed in relationship with promising therapeutic perspectives based either on small molecule inhibitors or on natural compounds. Among a miriad of molecules able to modulate PKC activity, we will focus on the role of the enzastaurin and briostatin-1, which already entered clinical trials for several human cancers.

Key Words: PKC, apoptosis, differentiation, tumorigenesis.

INTRODUCTION

Pioneer experiments performed in the early 1940s with phorbol esters (phorbol-12-myristate-13-acetate or 12-O-tetradecanoylphorbol 13-acetate) in the mouse skin carcinogenesis model first demonstrated that these natural products display tumour-promoting activity [1]. However, only in the early 1980s protein kinase C (PKC) was identified as the high-affinity intracellular receptor for the phorbol esters [2-3], which mimics the activity of the lipid diacylglycerol (DAG) [4,5]. The characterization of many structurally related PKC isozymes in the last 25 years added a high level of complexity in understanding their individual role in carcinogenesis [reviewed in 6]. PKC isozymes have been classified into three groups: classical PKCs (PKCs: PKC-α, PKC-βI, PKC-βII and PKC-γ), novel PKCs (PKCs: PKC-δ, PKC-ε, PKC-η and PKC-θ) and atypical PKCs (PKCs: PKC-ζ and PKC-ι) [7]. Moreover, PKC-μ and PKC-ν were recently added to the PKC super-family based on homology within the catalytic domain. PKC isoforms consist of an N-terminal regulatory domain and a C-terminal catalytic domain. The membrane targeting regulatory domains C1 and C2 of classical PKCs confer binding to the lipid second messenger DAG, phorbol esters and phosphatidylserine (C1) as well as to Ca^{2+}. Similarly, novel PKCs contain the C1 domain and a novel C2 domain; they are regulated by DAG but not by Ca^{2+}. In contrast, atypical PKCs are not regulated by either DAG or by Ca^{2+}. Despite the fact that DAG binding to the C1 domain at the plasma membrane is generally regarded as a key event for classical and novel PKC activation, this does not fully explain the diverse intracellular localization of PKC isozymes [7]. Indeed, it has demonstrated that in response to either differentiation [8] or apoptotic [9-10] stimuli, distinct PKC isozymes can redistribute from the cytosol to the nuclear membrane or within the nuclear compartment. Although this nuclear redistribution is likely important for conferring functional selectivity to the different PKC isoforms, the significance of nuclear localization and the nuclear targets of PKC are incompletely understood.

INVOLVEMENT OF PKC-ε AND -δ IN TUMORIGENESIS

After the initial data between PKC and mouse skin carcinogenesis [1], studies in various normal and cancer cells confirmed the involvement of distinct PKC isozymes in promoting cell survival, proliferation and malignant transformation [reviewed in 11]. Although the relative contribution of individual isozymes to malignant transformation is incompletely understood, the widely expressed PKC-ε and PKC-δ isozymes seem to play opposite roles on malignant transformation and this review will mainly focus on their role in carcinogenesis. In particular, it has been shown that over-expression of PKC-ε promotes the *in vitro* survival and proliferation of different tumour cell models, and confers a tumorigenic phenotype in nude mice [12,13]. Perhaps more importantly, PKC-ε is able to promote an invasive metastatic tumour-cell phenotype [14,15]. On the other hand, PKC-δ mediates apoptosis in different cell types, and, conversely, its knock-down promotes a transformed phenotype [16-18]. Of note, a potential mechanism by which phorbol esters can promote malignant transformation is by inducing the depletion of PKC-δ [19]. Animal models have provided important informations on the role of PKC-ε and PKC-δ in promoting and inhibiting carcinogenesis, respectively. As an example, the strikingly different pattern of responses triggered by the PKC isoforms cited above has been shown in models of skin carcinogenesis [20-22]. Transgenic mice over-expressing PKC-δ are resistant to phorbol-ester-induced tumour promotion, whereas transgenic mice over-expressing PKC-ε develop squamous cell carcinoma with significant metastatic potential and show increased sensitivity to UV-radiation-induced carcinogenesis [20-22]. Finally, it should be men-

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tioned that although mutations in PKC isozymes are rare occurrences in tumour genetics, PKC-ε is up-regulated in various types of cancers, whereas PKC-δ is often down-regulated [reviewed in 11].

ROLE OF PKC-ε AND -δ IN CELL CYCLE AND APOPTOSIS

The role of PKC in modulating cellular proliferation is well documented, although again the role of specific PKC isozymes in controlling cell-cycle events is still quite controversial [23]. The early dogma that PKCs were mitogenic kinases has been proved to be only partially correct, as there is a strict isozyme and cell-type dependency for this effect. The realization that PKC isozymes could mediate growth-inhibitory responses caused a reassessment of the role of PKCs in mediating tumour promotion and as potential therapeutic targets. Activation of PKC-δ causes proliferation defects both in G1 and G2 phases of the cell cycle through the modulation of cyclin expression or the activity of cyclin-dependent kinases with a great degree of cell-type variability [23]. PKC-δ down-regulates the expression of cyclins A, D1 and E, but also up-regulates p27 expression. Although there are no reports of increased cancer susceptibility in PKC-δ-null mice, these animals show enhanced B-cell proliferation [24].

Perhaps one of the clearest examples of the differential responses conferred by PKC isozymes is the contrasting role of PKC-ε and PKC-δ in survival and apoptosis. Early studies in hematopoietic cells showed that over-expression of PKC-ε, but not PKC-δ, protects cells from apoptosis induced by cytokine depletion through the induction of the anti-apoptotic protein BCL-2 [25]. Although PKC-ε has been shown to be involved also in commitment of hematopoietic progenitors along the megakaryocytic lineage [26,27], its major role in protecting normal haematopoietic cells from deprivation of serum and/or growth factors was independently confirmed by our group in a factor-dependent hematopoietic cell line [28] and, more recently, by a different group of investigators on primary hematopoietic cells [29]. The possibility that the anti-apoptotic activity of PKC-ε might be confined to normal hematopoietic cells was excluded by many studies, performed on a variety of different tumour cell types, which have established a strong link between PKC-ε and suppression of apoptosis [30-40], a key event in the context of cancer (Table **1**). Of particular interest, PKC-ε was highly expressed in glioma cell lines and high-grade gliomas, and its silencing induces apoptosis in glioma cells and primary glioma cultures [32]. At variance to factor-dependent hematopoietic cells [25], no significant correlation between PKC-ε levels and BCL-2 or BAX (BCL2-associated X protein) expression was found in glioma cells. Rather, it was shown that PKC-ε depletion reduces total Akt expression [32] and that PKC-ε over-expression prevents apoptosis induced by tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a death inducing ligand belonging to the TNF superfamily of cytokines [41]. Over-expression of PKC-ε in lung cancer cells was sufficient to up-regulate pro-survival factors BCL-

Table 1. Opposite Effects of PKC-ε and PKC-δ in Different Cancer Cell Types

Biological effect	Cell type	References
PKC-ε		
anti-apoptotic/pro-metastatic	head and neck squamous cell carcinoma	[14]
anti-apoptotic	skin cancer	[20-21]
anti-apoptotic	brain tumors	[30, 32]
anti-apoptotic	thyroid cancer	[31]
anti-apoptotic	melanomas	[33]
anti-apoptotic	lung cancer	[36-39]
anti-apoptotic	ovarian carcinomas	[40]
anti-apoptotic/pro-metastatic	breast cancer	[45, 119]
pro-angiogenic	normal endothelial cells	[51-52]
PKC-δ		
pro-apoptotic	colon cancer	[18]
pro-apoptotic	skin cancer	[22, 128]
pro-apoptotic	prostate cancer	[44]
pro-apoptotic/anti-metastatic	breast cancer	[46-47]
pro-apoptotic	glioma cells	[108]
pro-apoptotic	osteosarcomas	[128]

XL and X-linked inhibitor of apoptosis (XIAP) [38]. The pro-survival effect of fibroblast growth factor 2 (FGF2) in these cells involves the formation of a PKC-ε–BRAF–S6K2 complex that presumably regulates the translation of mRNA species involved in cell-death regulation. Interestingly, increased PKC-ε expression has been specifically linked to chemotherapy resistance, as exemplified in etoposide-treated and doxorubicin-treated non-small-cell lung carcinoma cells [39]. Moreover, in these cells the expression of PKC-ε blocks mitochondrial-dependent caspase activation and inhibits cytochrome *c* release. Cisplatin-resistant ovarian carcinoma cells also show increased PKC-ε expression [40]. Similarly to glioma cells, the expression levels of PKC-ε in melanoma, lung cancer and hematopoietic cells were related to their resistance to TRAIL-induced apoptosis [33-35]. Although obtained in *in vitro* models, these findings are particularly relevant since phase I and phase II clinical trials indicate that both recombinant TRAIL and antibodies to TRAIL receptors are usually well tolerated and are promising new anticancer biotherapeutic [41,42]. The inherited resistance of cells expressing high levels of PKC-ε to TRAIL-based therapy suggests that compounds able to inhibit PKC-ε activity should sensitize to TRAIL cytotoxicity.

The interplay between PKC and TRAIL has been further confirmed in a recent study in which it has been demonstrated that PKC-δ can trigger an autocrine apoptotic loop through the secretion of TRAIL in prostate cancer cells [43]. In keeping with previous observations that several cell types undergo apoptotic cell death in response to phorbol-ester treatment [6], the apoptotic effect of phorbol esters in prostate cancer cells was entirely dependent on PKC-δ and was greatly impaired upon inhibition or RNAi depletion of TRAIL death receptors [44] (Table (**1**)).

PKC INVOLVEMENT IN METASTASIS AND ANGIOGENESIS

Although data concerning the role of individual PKC in the metastatic process and in angiogenesis should be interpreted cautiously, PKC-ε activation seems to positively affect motility, invasion and metastasis. For example, inhibition of PKC-ε in MDA-MB-231 breast carcinoma cells drastically decreases tumour growth and metastasis [45], while PKC-δ negatively modulates breast cancer cell migration [46]. PKC isozymes have also been implicated in regulating the secretion or expression of matrix metalloproteinase protein 9 (MMP9), with down-regulation of PKC-δ in MCF-7 breast-cancer cells increased cell motility with a corresponding increase in MMP9 secretion [46,47].

An increasing amount of data indicates that PKC is involved in mediating vascular endothelial growth factor (VEGF)-induced angiogenesis. Several PKC isoforms are activated in response to VEGF receptor activation, and PKC has been implicated as an important mediator of VEGF-induced proliferation of endothelial cells [48,49]. The most solid evidence points to PKC-βII isozyme as being the most relevant PKC involved in angiogenesis, not only in cell culture models but also in an *in vivo* setting [50]. Additional studies, however, have demonstrated that down-regulation of PKC-ε abrogated VEGF-stimulated phosphorylation of Akt at Ser473, eNOS at Ser1179 and VEGF-stimulated Erk1/2 phosphorylation [51]. PKC-ε knockdown also decreased and abolished VEGF-stimulated DNA synthesis [51]. In addition, VEGF receptor-2 (VEGFR2) tyrosine phosphorylation and expression of VEGFR2 protein and mRNA were markedly decreased during knockdown of PKC-ε [51]. A key role of PKC-ε in promoting endothelial cell survival has been further supported by its ability to promote cytoprotection of human vascular endothelium against apoptosis [52] (Table (**1**)). These studies are particularly interesting since they were performed using myristoylated inhibitory peptides [52]. In endothelial cells infected with an adenovirus expressing a constitutively active form of PKC-ε (Adv-PKC-ε-CA) a 3-fold, PKC-ε-dependent, increase in Bcl-2 expression was evidenced, with no significant change in Bcl-XL, Bad, Bak, or Bax. The induction of Bcl-2 inhibited apoptosis induced not only by serum starvation but also by etoposide. A number of studies have shown that inhibition of the phosphoinositide 3-kinase/Akt pathway attenuated serum- and VEGF-induced protection against apoptosis and sensitized endothelial cells to TRAIL-mediated apoptosis [53-55].

NEW PROMISING PKC MODULATORS ENROLLED IN CLINICAL TRIALS OF HUMAN CANCERS: ENZASTAURIN AND BRIOSTATIN

A large variety of structurally and mechanistically distinct anti-PKC agents have been identified or developed in recent years, but we will focus our attention on enzastaurin and briostatin, taking into consideration that these compounds have entered clinical trials (Table (**2**)). Staurosporine, an alkaloid produced by *Streptomyces* bacteria, is one of the best-known pan-PKC inhibitor in *in vitro* models [56]. Although the poor kinase selectivity of staurosporine impeded the development of clinical applications, this molecule served as lead compound for the synthesis of more PKC-selective analogues, i.e. 7-hydroxystaurosporine or UCN-01 [57] and N-benzoyl-staurosporine or CGP 41251 [58] with improved selectivity for specific PKC isoforms [59]. Recently, a novel more specific analogue of staurosporine, enzastaurin (Fig. (**1**)), was introduced in this scenario. This molecule was originally described as a selective PKC-βII inhibitor able to impair VEGF-driven tumour growth with a corresponding decrease in neovascularization in a mouse xenograft model [60]. Nevertheless, although the first and best-characterized activity of enzastaurin is the inhibition of the PKC-βII isoform, increasing experimental evidence clearly suggests that enzastaurin also potently inhibits PKC-ε [61]. Moreover, while enzastaurin was initially developed for its potential antiangiogenic properties, preclinical studies demonstrate also direct anti-tumour activity against both solid tumours and hematological malignancies [61-70]. Clinical trials studies have demonstrated that enzastaurin was well tolerated when used alone or in combination with conventional chemotherapeutic drugs [71]. Several clinical trials have studied the possible use of enzastaurin in treatment of patients with different type of cancers, including leukemias and lymphomas together with R-CHOP chemotherapy (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone). Most prominently, enzastaurin is under evaluation for the treatment and prevention of relapse in diffuse large B cell lymphomas (DLBCL). Importantly, a multicen-

Table 2. Clinical Studies on the Anti-Cancer Activity of Enzastaurin and Bryostatin-1

Drugs	Concentrations	Outcome	References
Enzastaurin	525-700 mg/die	advanced solid tumors (n=47) 45% stable disease	[62]
	500 mg/die	diffuse large cell lymphomas (n=55) 56% freedom from progression or complete remission	[65]
	500 mg/die + capecitabine (1000 mg/m^2)	advanced solid tumors (n=) 18% stable disease	[71]
Bryostatin-1	25-40 μg m^2/die/72h (weekly for 4 cycles)	metastatic renal cell carcinoma (n=32) 25% stable disease or partial remission	[83]
	25-40 μg m^2/die/72h (weekly for 4 cycles)	advanced sarcoma (n=12)/head and neck cancer (n=12+n=14) no response	[84-85]
	25-40 μg m^2/die/72h (weekly for 4 cycles)	metastatic melanoma (n=37) no response	[86]
	25-40 μg m^2/die/72h (weekly for 4 cycles)	metastatic colorectal cancer (n=28) no response	[87]
	40 μg m^2/die/72h (weekly for 4 cycles)	multiple myeloma (=9)/ non-Hodgkin's lymphoma (n=17) no response	[88-89]
	40 μg m^2/1h i.v. + paclitaxel 80 mg/ m^2/ 2h i.v.	gastric cancer (n=35)/esophageal cancer (n=22) partial response 27-29%	[91-92]

ter phase II study has demonstrated that treatment with enzastaurin was well tolerated and associated with prolonged free from progression in a small subset of patients with relapsed or refractory DLBCL [www.clinicaltrials.gov, accessed December 29 2008]. These promising preliminary data granted enzastaurin orphan-drug status by the European Medicines Agency for the treatment of patients with DLBCL (Table (**2**)). Enrolment into a phase III clinical trial is ongoing. Since it is unlikely that enzastaurin will enter into the clinical practice as single pharmacological agent, it is particularly noteworthy that enzastaurin displayed a strong synergistic *in vitro* cytotoxicity when combined with bortezomib and a moderate synergistic or additive cytotoxicity when combined with melphalan or lenalidomide in multiple myeloma cell models and retained cytotoxicity when multiple myeloma cell lines were co-cultured with multipotent mesenchymal stromal cells [66-67]. Besides being active in multiple myeloma, some preliminary evidences indicate that enzastaurin also shows significant *in vitro* and *in vivo* antitumoral activity in Waldenstrom's macroglobulinemia, a low-grade lymphoplasmocytic lymphoma [68]. Moreover, enzastaurin enhances the *in vitro* antitumor activity of rituximab, bortezomib, fludarabine and dexamethasone, strongly supporting the potential therapeutic value of using enzastaurin in combination with these agents [69-70].

Bryostatin-1 (Fig. (**1**)) is a macrocyclic lactone derived from the symbiontic proteobacterium *Candidatus Endobugula sertula*, which potently binds the regulatory domain of PKC [72]. The mechanism of action of bryostatin-1 involves the initial activation of PKC, followed by its rapid down-regulation. The unique activity of bryostatin-1 is due, at least in part, to its selectivity for PKC-δ and PKC-ε, and in particular the protection of PKC-δ from down-regulation [73-77]. Since it has been previously demonstrated that recombinant TRAIL displays potentially important pro-apoptotic and maturative effects in myeloid leukemic cells [78-81] and neuroblastoma cells [82], and that the intracellular levels of PKC-ε are critical determinants in regulating the survival/ apoptotic response of hematopoietic cells [29], it will be of interest to evaluate whether combination of bryostatin-1 with recombinant TRAIL or anti-TRAIL receptor agonistic antibodies will potentiate the anti-leukemic activity of TRAIL. Although generally well tolerated, with commonly reported side effects myalgia, fatigue, nausea, headache, vomiting, anorexia, anaemia and lymphopenia, bryostatin-1 trials indicate minimal single-agent activity (Table (**2**)) with the best result obtained in renal cell carcinoma where weekly treatment produced partial remission in 25% of patients [83]. By contrast, bryostatin-1 is not recommended for use as a single agent for the treatment of variety of cancer, including sarcoma [84] head and neck cancers [85], melanoma [86], colorectal cancer [87], multiple myeloma [88] and progressive non-Hodgkin's lymphoma [89] (Table (**2**)). However, the combinations with standard chemotherapy are providing encouraging results and indicate a new direction in cancer therapy [90-92]. As the role of PKC in gastric cancer development has been well established [90], it is noteworthy that some of the most encouraging results with bryostatin-1 were obtained in treatment of gastric cancers [91]. Sequential paclitaxel plus bryostatin-1 resulted in a superior response rate than would be expected of paclitaxel alone in patients with

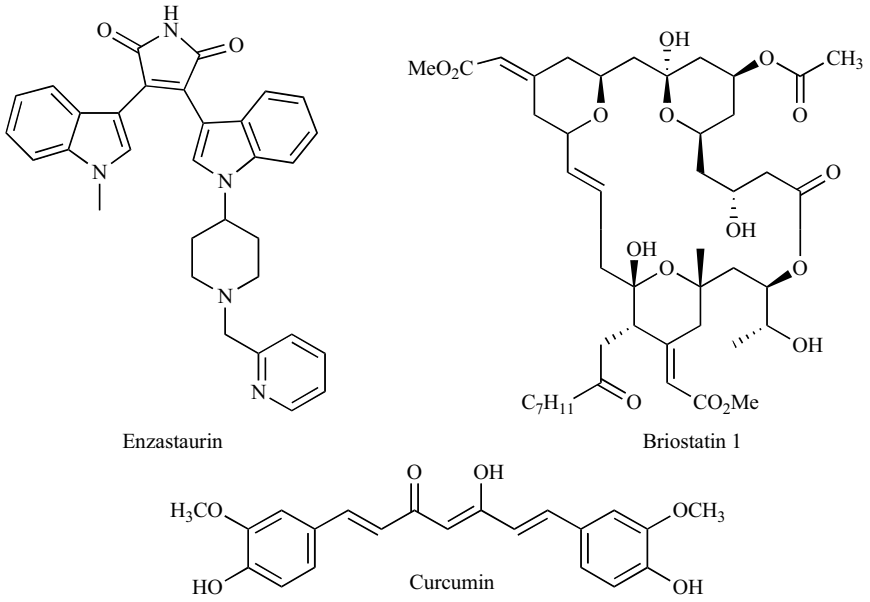

Fig. (1). Structures of the novel PKC inhibitors enzastaurin, bryostatin-1 and curcumin.

untreated, advanced gastric or gastroesophageal junction adenocarcinoma. However a following phase II clinical trial to determine the response rate and toxicity profile of sequential paclitaxel and bryostatin-1 in patients with advanced esophageal cancer, highlighted the severe toxicity, especially myalgias, observed in treated patients [92] (Table (**2**)). Bryostatin-1 has been administered with full dose of fludarabine for the treatment of chronic lymphocytic leukemia (CLL) and indolent lymphomas. The combination was moderately active in patients with persistent disease following prior treatment. It has been suggested that, in view of the activity of monoclonal antibodies such as the anti-CD20 monoclonal antibody rituximab in the treatment of CLL and indolent lymphomas, the combination of bryostatin 1 and fludarabine with rituximab could warrants future consideration [93]. The development of a serum assay for bryostatin-1 and additional mechanistic studies would be useful for future bryostatin-1 clinical trials [94].

Bryostatin-1 Analogues

Two major problems that have impeded clinical advancement of bryostatin-1 are the low yields from the natural source and the difficulties in selectively modifying bryostatin-1 to obtain analogues with ameliorate features. Thus, relatively few bryostatin-1 derivatives have been prepared [95]. Wender *et al*. have described a simplified approach for the function-oriented synthesis of simplified analogues of bryostatin (bryologs) that are superior to bryostatin in numerous assays including growth inhibition in a variety of human cancer cell lines and in animal models [96]. In this approach, the structural features of the complex bryostatin-1 influencing its activity are recapitulated on a simplified scaffold to produce a functional analogue that is designed in a rapid, step-economical and practical synthesis that can be scaled to meet clinical needs. These studies showed that, in contrast to the natural bryostatins, the C7 region of the bryologs can play a significant role in binding affinity and could be potentially exploited for improved pharmacological function such as PKC selectivity [97]. In addition, this group described the synthesis of a new class of bryostatin analogues that contain the complete oxycarbocyclic core ring system of bryostatin-1, assembled *via* a highly efficient, functional-group-tolerant, and stereoselective prins-driven macrocyclization. These authors stated that these tetrahydropyranyl B-ring analogues are among the most potent and efficacious analogues to date, exhibiting nanomolar and picomolar activities in PKC affinity assays as well as in cellular antiproliferation assays [98].

EMERGING NATURAL MOLECULES THAT AFFECT PKC-DEPENDENT PATHWAYS AND SHOW POTENTIAL APPLICATIONS IN CANCER THERAPY

Naturally derived products, in particular herbal extracts, have been widely used in the past to treat a variety of human diseases including cancer and are attracting considerable attention in modern medicine. Increasing literature reports the effects of natural products, or derivatives, as activator or inhibitor of PKC-dependent pathways (Table (**3**)). The structure of curcumin, which already entered clinical trials, similarly to enzastaurin and bryostatin-1, is reported in Fig. (**1**), while the structure of some of the emerging natural molecules with PKC modulatory effects, for which only *in vitro* and preclinical studies are available, is reported in Fig. (**2**).

The most extensively studied natural product in the recent years is curcumin (diferuloylmethane), which derives from the plant *Curcuma longa*. Curcumin is a constituent of turmeric, a gold-colored spice commonly used in the Indian subcontinent, not only for health care but also for the preservation of food and as a yellow dye for textiles. Extensive research within the last half century has proven that the tumeric activities are due to curcumin [99], which exhibits low toxicity and growth-suppressive activity against a variety of cancer cells and possesses certain chemo-preventive proper-

Table 3. Natural Molecules with Anti-PKC Activity and Potential Use in Cancer Therapy

Compound	Biological Activity	Concentrations	References
Curcumin	inhibitor of Ca^{2+}-dependent PKC isoforms	nanomolar to micromolar	[99-103]
Peplin/ ingenol 3-angelate	activator of PKC-δ	nanomolar	[104]
Decursin	inhibitor of PKC-α and -βII	micromolar	[105-107]
Penta-acetyl geniposide	activator of PKC-δ	micromolar	[108]
Bufalin	activator of PKC-δ	nanomolar	[109-111]
Yuanhuacine	antagonist of phorbol ester receptor	micromolar	[112-113]

ties. As described in a recent excellent review [100], curcumin is a potent inhibitor of several intracellular pathways involved in cancer transformation. It is considered that PKC, mTOR, and EGFR tyrosine kinase are among the major upstream molecular targets for curcumin intervention, whereas the nuclear oncogenes, such as c-Jun, c-Fos, c-Myc, CDKs, FAS, and iNOS might act as downstream molecular targets for curcumin actions. Thus, although Ca^{2+}-dependent PKC isoforms represent a major target of curcumin [101], curcumin differs from more specific PKC inhibitors since its anticancer activity is mediated through the impairment of multiple intracellular pathways, that play a pivotal role in the regulation of several basic cellular processes, including differentiation, proliferation, cell cycling, apoptosis and inflammation [102]. Curcumin was studied in several clinical trials for the treatment of human cancers. Phase I clinical trials have shown that curcumin is safe even at high doses (12 g/day) in humans, but exhibit poor bioavailability. In fact, oral dosing of 4-8 g of curcumin in humans showed peak plasma levels of 0.41-1.75 mM after 1 hour of dosing. Similarly, in a different human clinical trial, 3.6 g of curcumin *via* oral route was found to produce a plasma curcumin level of 11.1 nmol/L after an hour of dosing [102]. Major reasons contributing to the low plasma and tissue levels of curcumin appear to be due to poor absorption, rapid metabolism, and rapid systemic elimination. To improve the bioavailability of curcumin, numerous approaches have been undertaken: (i) the use of adjuvant like piperine that inter-

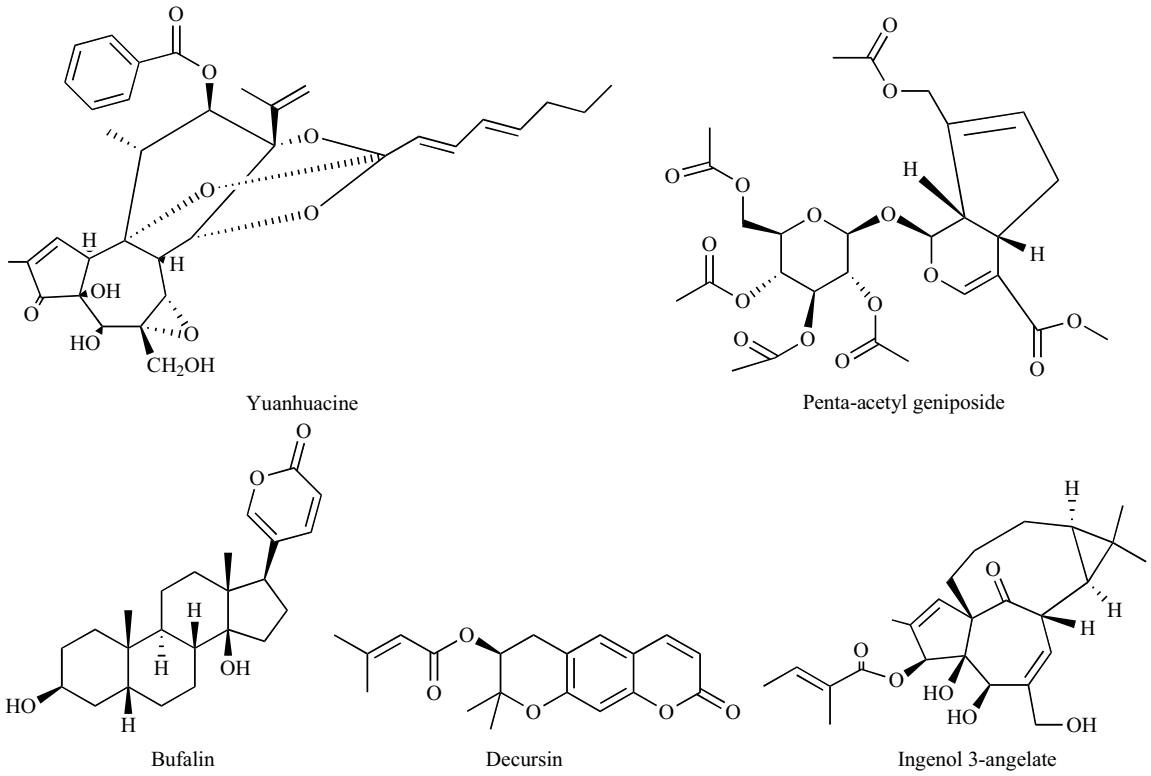

Fig. (2). Structures of emerging natural molecules with PKC modulatory effects.

feres with glucuronidation; (ii) the use of liposomal curcumin; (iii) the use of curcumin phospholipid complex; (iv) the use of structural analogues of curcumin (e.g., EF-24). The latter has been reported to have a rapid absorption with a peak plasma half-life. Despite the lower bioavailability, therapeutic efficacy of curcumin against various human diseases, including cancer, cardiovascular diseases, diabetes, arthritis, neurological diseases and Crohn's disease, has been documented [reviewed in 102]. Moreover, its combination with some cytotoxic drugs has demonstrated synergistic effects [103]. Low systemic bioavailability after oral dosing limited the application of curcumin in treating cancers outside the gastrointestinal tract. A phase III clinical trial to test the effects of gemcitabine, curcumin and celebrex in patients with metastatic colon cancer will start soon. In addition, a phase III clinical trial will be launched soon to test the effects of gemcitabine, curcumin and celebrex in patients with advance or inoperable pancreatic cancer [www.clinicaltrials.gov, accessed December 29 2008].

Peplin or ingenol 3-angelate is a selective small molecule activator of PKC-δ, extracted from the plant *Euphorbia peplus*, which displays potent antileukemic activity, inducing apoptosis in both myeloid leukemia cell lines and primary acute myeloid leukemia blasts at nanomolar concentrations through a PKC-δ dependent mechanism [104]. Of note, peplin did not induce apoptosis in normal CD34+ cord blood hematopoietic progenitor cells at up to 2-log concentrations higher than those required to induce cell death in primary leukemic blasts [104].

Another natural drug affecting PKC activity is decursin, a pyranocoumarin isolated from *Angelica gigas* that exhibits cytotoxic effects on various human cancer cell lines. Decursin competed the binding of phorbol esters to PKC-α and -βII isozymes in leukemia cells and down-regulates the activities of these PKC isoforms [105, 106]. Decursin treatment in mice determined a significant increase in life span and a significant decrease in the tumor weight and volume of animals inoculated with sarcoma cells [106]. Several chemically synthesized decursin analogues containing the coumarin structure showed antiproliferative effects on human leukemia cell lines, and the compounds 1-3 and 4-6 inhibited the cell proliferation in a PKC-βII-independent and –dependent manner, respectively, indicating that the side chain of compounds determines its selectivity for the PKC-βII isoform [107].

The herbal product penta-acetyl geniposide (PAG) induces apoptosis through activation of specific PKC isoforms. In C6 glioma cells, PAG-induced apoptosis of tumor cells occurred through the activation of PKC-δ and the upregulation of Bax protein [108].

Bufalin, a bufadienolide type steroid that is one of the major components of the toad venom-prepared traditional chinese medicine called Ch'an Su or Senso, has potent antiproliferative and differentiation activity in several human leukemic cell lines [109,110] at nanomolar concentration. The apoptotic effects of bufalin in human leukemia cells involved selective PKC isoforms, as demonstrated by the strong resistance to the bufalin-induced apoptosis shown by PKC-δ-defective leukemic cells [111].

Yuanhuacine is a diterpene ester isolated from the flower buds of *Daphne genkwa*, which induces apoptosis in human leukemic HL-60 cells [112]. *In vitro* assay demonstrated that it is a selective antagonist of the phorbol ester receptor in PKC. This compound strongly inhibited the binding of 3H-phorbol-12, 13-dibutyrate (PdBu) to PKC with an IC_{50} value in the nanomolar range. Yuanhuacine inhibited the PdBu-stimulated PKC activity in the catalysis of the phosphorylation of Histone III-S with an IC_{50} of 2.82 nmol/L (PdBu=10 micromol/L), while it had no effect on the basal and Ca^{2+}-stimulated PKC activity in the same assay system [112]. In addition, yuanhuacine suppressed tumour growth in Lewis lung carcinoma-inoculated mouse model [113]. In fact, the intraperitoneal administration of yuanhuacine in Lewis lung carcinoma-inoculated mice evidenced a significant inhibition of tumour size, with reductions of 24.2% and 45.8% at concentrations of 0.1 mg/kg and 0.5 mg/kg, respectively, as compared with the control mice, indicating that yuanhuacine is a potent antitumoral agent [113].

PKC-ε INHIBITORY PEPTIDES AND CROSS-TALK BETWEEN PKC AND p53

In recent years, a series of peptides derived from PKC have been shown to modulate its activity by interfering with critical protein-protein interactions within PKC and between PKC and PKC-binding proteins [114]. Focusing on PKC-ε isoenzyme and using a rational approach, one C2-derived peptide that acts as an isozyme-selective activator [115] and another that acts as a selective inhibitor [116] of PKC-ε have been identified. Taking into account the accumulating experimental data linking an abnormal PKC-ε activation to an increased survival and metastatic activity in different kinds of cancers, rationally designed PKC-ε inhibitory peptides are particularly promising for anti-cancer therapy. In this context, however, a cautionary note is represented by evidences showing that PKC-ε might play a cardio-protective role, at least in *in vitro* as well as in animal models of myocardial infarct [117]. In fact, it has been reported that activation of PKC-ε before ischemia protects the heart from subsequent prolonged ischemia and have a cardio-protective effect. An additional concern by using PKC-ε inhibitory peptide in anticancer therapy derives from the demonstration that normal immature erythroblasts, which are susceptible to TRAIL mediated cytotoxicity [118] become protected from TRAIL as they become mature erythroblasts by the increased levels of intracellular PKC-ε [29]. These findings predict that an association of PKC-ε inhibitory peptides plus recombinant TRAIL might induce anemia, which obviously would represent a serious side effect in patients affected by cancer.

In a therapeutic perspective, it is of particularly interest a recent study that has demonstrated that a target of PKC-ε activation is the murine double minute 2 gene product (MDM2, HDM2 in humans) [119], an E3 ubiquitin ligase for p53 and itself, that is over-expressed in many human tumors [120-122]. Although the MDM2 gene represents one of the major transcription targets of p53, MDM2 protein binds the p53 N-terminal trans-activation domain and negatively regulates tumor suppressor function by compromising transcriptional regulation and controls p53 half-life *via* ubiquitin-dependent degradation [123,124]. Any pharmacological

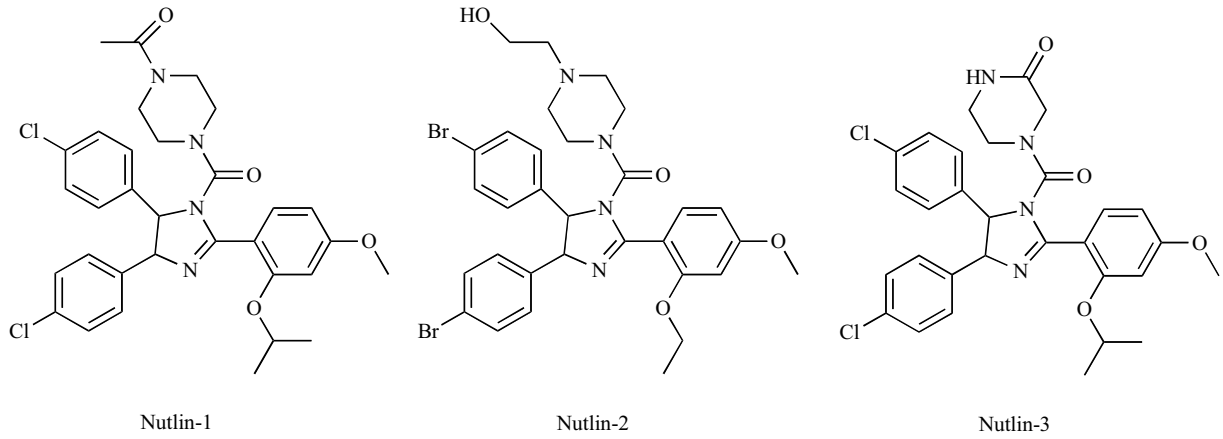

Fig. (3). Structures of small-molecule inhibitors of the p53/MDM2 interaction.

strategy that disrupts the p53/MDM2 interactions tends to have transient effects and is counterbalanced by the strong feed-back loop regulating the reciprocal interactions of these proteins [125].

The interplay between the PKC-δ and p53 intracellular signal transduction pathways has been demonstrated in previous studies [126-128]. In particular, it has been shown that PKC-δ is cleaved by caspase-3 to a kinase-active catalytic fragment (PKC-δCF) in the apoptotic response of cells to DNA damage. Expression of PKC-δCF contributes to the induction of apoptosis through the association with the p53 family member p73beta, a structural and functional homologue of the p53 tumour suppressor. The results show that PKC-δCF phosphorylates the p73beta transactivation and DNA-binding domains [126]. In a different study [127], it has been shown that PKC-δ transactivates TP53 expression at the transcriptional level. Reporter gene assays demonstrated that PKC-δ induces the promoter activity of TP53 through the TP53 core promoter element (CPE-TP53) and that such induction is enhanced in response to DNA damage. The results also demonstrate that, upon exposure to genotoxic stress, PKC-δ activates and interacts with the death-promoting transcription factor Btf to co-occupy CPE-TP53. These findings provide evidence that activation of TP53 gene transcription by PKC-δ triggers TP53-dependent apoptosis in response to DNA damage.

NUTLINS: SMALL-MOLECULE INHIBITORS OF THE p53/MDM2 INTERACTION

Since MDM2 represents the main regulator of p53 function and MDM2 is aberrantly expressed in tumors [129], an important therapeutic strategy followed by different research groups in the recent years has been focused on the possibility to activate the p53 pathway by disrupting the protein interaction(s) between p53 and MDM2. The Nutlins were identified from a class of cis-imidazoline compounds, by the group of Vassilev [130], as the first potent and selective small-molecule MDM2 antagonists among various compounds showing great variability in their cellular potency and selectivity for the molecular targets (Fig. (**3**)). The Nutlins have the ability to displace p53 from MDM2 *in vitro* with nanomolar potency (IC50 = 90 nM for Nutlin-3a, the active enantiomer of Nutlin-3). Importantly, Nutlins are able to enter multiple types of cultured cells, both continuous cell lines and primary tumor cells and show the remarkable property to inhibit the p53/MDM2 interaction in the cellular context, leading to stabilization of p53 and activation of the p53 pathway. Although originally tested on a variety of solid tumors [130], other studies have demonstrated that Nutlins show cytostatic/cytotoxic activity also in hematological malignancies, and in particular in B-chronic lymphocytic leukemia (B-CLL) cells [131-133] and block angiogenesis, at least *in vitro* [134]. Importantly, the original study performed by the group of Vassilev demonstrated that Nutlin-3a can be administrated orally to nude mice that bear established human solid tumor xenografts for up to 3 weeks without systemic toxic effects [135]. Apparently, Nutlin-3a was responsible for effective tumor-growth inhibition, providing the first *in vivo* demonstration that activation of wild-type p53 by pharmacological inhibitors of the p53/MDM2 interaction is feasible and might be an effective approach in cancer therapy [135].

CONCLUSIONS

Although it is clear that PKC is implicated in modulating almost all aspects of carcinogenesis, many areas in the field remain to be explored. Despite years of research, the relative role of individual isozymes in cancer are just beginning to emerge, a task that has been confounded by the heterogenicity in functional responses conferred by distinct PKC isozymes and the cell-type dependency of these effects. Furthermore, there is a surprisingly limited amount of information on the mechanisms that drive isozyme-specific compartmentalisation to access these substrates. Whereas PKCs have been extensively studied as phorbol-ester receptors, there is a general assumption that tyrosine-kinase receptors equally translocate and activate PKC isozymes through PLC mediated DAG generation. A considerable amount of efforts have been devoted to develop PKC modulators with anti-cancer therapeutic value, several of which are currently used in clinical trials. However the functional antagonism of PKC isozymes provides an ambiguous scenario for therapeutic opportunities, as PKC activators (of PKC-δ) and inhibitors (of PKC-ε) could both potentially be used as anti-cancer drugs.

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