# Fine Tuning of Protein Kinase C (PKC) Isoforms in Cancer: Shortening the Distance from the Laboratory to the Bedside

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**Abstract:** The serine/threonine protein kinase C (PKC) family was first identified as intracellular receptor(s) for the tumor promoting agents phorbol esters. Thirty years after the discovery of PKC, the role of specific PKC isoforms has been described in relationship with an altered pattern of expression in different types of cancer and a good number of small molecule inhibitors (inhibitory peptides, antisense oligonucleotides or natural compounds) targeting PKC are now available. Despite all these achievements and a huge amount of basic research studies on the biochemical regulation of PKC, there has been a delay in clinical trials with drugs targeting PKC function. This delay is easily explained taking into account the extreme biological complexity of the PKC family of isoforms and the incomplete understanding of the specific role of each PKC isozyme in different types of cancers. Some of the difficulties in developing pharmacological compounds selectively tuning the different PKCs have started to be overcome. In this review, the growing evidences of the role of the PKC isoforms α, βII, δ, ε, ζ and ι is in promoting or counteracting tumor progression will be discussed in relationship with promising therapeutic perspectives.

**Keywords:** PKC, tumour cell biology, innovative drug compounds.

## INTRODUCTION

More than thirty years ago, Nishizuka *et al.* described for the first time an enzymatic activity, later called protein kinase C (PKC), working as intracellular receptor for the phorbol esters [1-3] and for the PKC lipidic ligand diacylglycerol (DAG) mimickers are present in the croton oil and used as tumor promoter agents in the "Berenblum-Mottram" skin carcinogenesis mouse model [4, 5]. At present ten PKC isozymes have been discovered and they have been classified in three subfamilies according to the functional domain composition: classic/conventional (cPKCs: PKC-α, PKC-βI, PKC-βII and PKC-γ), novel (nPKCs: PKC-δ, PKC-ε, PKC-η and PKC-θ) and atypical (aPKCs: PKC-ζ and PKC-ι). In addition, PKC-related kinases with similar regulatory properties have been described, but for clarity we are not going to consider them in this review: they are known as PKC-related kinases (PRK/PKN) [reviewed in 6] and PKD (including PKC-μ and PKC-ν) [reviewed in 7].

All PKCs family members share a common structure (Fig. (**1**)): a cell membrane targeting N-terminal regulatory domain linked by a flexible hinge segment to the C-terminal catalytic domain. The cPKCs regulatory domains C1 and C2 confer binding to the lipid second messenger DAG, phorbol esters and phosphatidylserine (PS) as well as to $Ca^{2+}$. Similarly, nPKCs contain the C1 domain and a novel C2 domain; they are regulated by DAG but not by $Ca^{2+}$. In contrast, the C1 domain of the aPKCs does not bind nor DAG or $Ca^{2+}$[8]. Major regulatory mechanism of the aPKCs is the protein-protein interaction mediated by the Phox/Bem1 domain (PB1) and the carboxy-terminal domain. HSP90 and mTORC2 are two examples of the regulatory proteins [9, 10]. The plasma membrane recruitment is regarded as a key event for the activation of all PKCs that already lost the inhibitory conformation due to the occupation of the active site by the pseudosubstrate sequence. Indeed, PKC phosphorylates its substrate only in the membrane bound, open, active conformation and this happens only after the interaction with $Ca^{2+}$/DAG for cPKCs/nPKCs or/and phosphorylation of the activation loop by PDK1 and eventually of the hydrophobic motif by mTORC2 for aPKCs [reviewed in 11].

PKC isozymes can redistribute differently inside the cell in response to apoptotic stimuli through intrinsic localization sequences or specific-scaffolding protein binding [12-15]. Spatial regulation of PKC isozymes seems to be functional to the phosphorylation of substrates present only in specific subcellular districts, i.e. lipid rafts, endosomes, nucleoli, nucleus, Golgi, mitochondrion. An example of the importance of the nature of the regulatory molecule(s) bound by PKC for the intracellular localization is given by PKC-δ which translocates differently to the Golgi or mitochondrion, the nucleus or the membrane accordingly as it binds respectively ceramide, DAG or RACKs (Receptor for Activated C Kinases) protein [reviewed in 16].

## INVOLVEMENT OF PKC ISOZYMES IN TUMORIGENESIS

A large body of evidences sustains unquestionably that the serine/threonine PKC isozymes are involved in tumor

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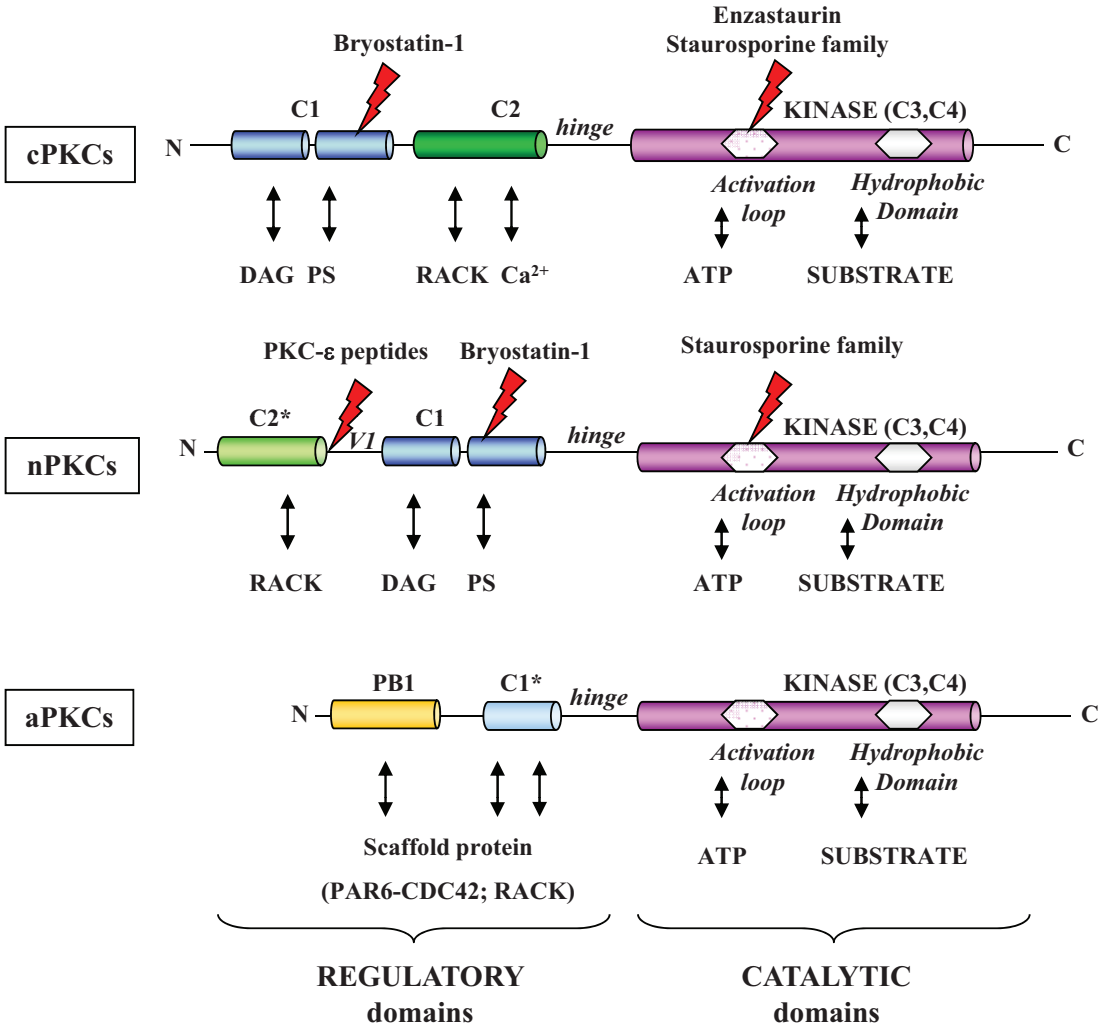


**Fig. (1). Schematic representation of the functional domain composition of the three PKC subfamilies: classic (cPKCs), novel (nPKCs), atypical (aPKCs).** PKCs family members share a common structure: a cell membrane targeting N-terminal regulatory domain linked by a flexible hinge segment to the C-terminal catalytic domain. The cPKCs regulatory domains C1 and C2 confer binding to the lipid second messenger DAG, phorbol esters and phosphatidylserine (PS) and $Ca^{2+}$. The nPKCs contain the C1 domain and a novel C2 domain (C2*) regulated mostly by DAG but not by $Ca^{2+}$. RACK (Receptor for Activated C Kinases) protein can bind C2 domain of activated cPKCs and nPKCs. Major regulatory mechanism of the aPKCs is the protein-protein interaction mediated by the PB1 and the carboxy-terminal domain. The C1 domain of the aPKCs (C1*) does not bind DAG. Drugs inhibiting selectively PKC isozymes and their target domains are also indicated.

progression and metastasis. Each PKC isoform is unique in its contribution to cancer development and progression. The study of the altered expression in different human cancers, in *in vivo* and *in vitro* models of overexpression or downregulation of the different isozymes resulted in noteworthy achievements for some PKCs and in particular for the cPKCs α, βII, the nPKCs δ and ε and both the aPKCs ζ and ι [reviewed in 17]. PKCs play either as oncogenes or tumor suppressors in human cancer development (Table **1**). There is evidence of aberrant expression of PKC isoforms in different types of cancer [18-44] but an important limitation of these histopathological studies is represented by the low number of the examined samples, which affects the statistical significance of the association between the levels of PKCs total protein/trascripts and the risk of cancer development/progression.

In this respect, the role of different PKCs mostly comes from studies in cancer cell lines or animal models, and two major examples are offered by PKC-ε and PKC-δ. *In vitro* studies reported that PKC-ε promotes the survival and proliferation of different tumour cell models [45, 46], and *in vivo* confers a tumorigenic and metastatic invasiveness phenotype in nude mice [47, 48]. Differently, PKC-δ seems to have possible tumor-inhibitory characteristics except in the brain and pancreatic cancer, where it is more pro-oncogenic [36]. This supposition is strongly corroborated by *in vitro* and *in vivo* studies where PKC-δ knockdown promotes a trasformed phenotype by loss of its pro-apoptotic influence [49-54]. Of note, *in vivo*, a potential mechanism by which phorbol esters can promote malignant transformation is by inducing the depletion of PKC-δ [52]. Animal models have provided important information on the role of PKC-ε and

**Table 1. Expression of the Different PKC Isoforms in Human Tumours**

| Cancer | PKCs isoforms with Increased Expression | PKCs isoforms with Decreased Expression |
|---|---|---|
| Skin | | α, β, δ [17,42] |
| Colon | βII,δ, ι [17,18] | α, β, βII, δ, η [17,43] |
| Liver | α [19] | |
| Esophagus | δ [20] | |
| GIST | θ [17] | |
| Pancreas | β, ι, ζ [22] | ε, γ [21,22] |
| Bladder | α, ε [23] | β, δ [17,23] |
| Kidney | δ, η, ζ [24] | α [24] |
| Prostate | α, δ, ε [25] | β [17] |
| Ovary | ι [26,27] | α [17,44] |
| Endometrium | α [29] | |
| Breast | ε, α [30-32] | α, η [17] |
| Lung | ι, η [33,34] | |
| Head-Neck | ε [35] | |
| Thyroid | | ε [17] |
| Brain | γ, δ, ε [17,36] | α [17] |
| DLBCL | βII [37] | α [17] |
| CML | ι [38] | |
| B-CLL | βII, γ, δ, ζ [17,39-41] | |
| T-Cell Leukemia | | α, β [17] |

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 [55-57].

## ROLES OF PKC-ε AND PKC-δ IN CELL CYCLE REGULATION, APOPTOSIS AND AUTOPHAGY

The anti-cancer strategy targeting PKC underwent to critical review after the early dogma, that PKCs were mitogenic kinases, was proved to be only partially correct. Indeed, it is true that tumorigenic effects of PKC (Table **2**) are strictly dependent on individual isozyme, cell-type and subcellular localization. It is still controversial and the overview of the mechanisms with which the specific PKC isozymes control cell-cycle events. Generally they are considered as key negative regulators of the cell cycle at two stages, G1 phase and at G2/M transition (Cdk1-cdc2) [reviewed in 58]. Attenuation of G1/S transition through p21/Cip1 induction has been demonstrated for PKC-ε in non-small lung cancer cells [34]. More complex is the role of PKC-δ, which down-regulates the expression of cyclins A, D1 and E or up-regulates p27 and p21 in correlation to its cleavage status and related nuclear traslocation [58].

Regarding the role of PKC in apoptosis and survival, PKC-ε and PKC-δ offer one of the clearest examples of the isozyme-specificity of the downstream effects (Table **2**). Early studies in hematopoietic cells showed the 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 [59]. The major role of protecting normal hematopoietic cells from deprivation of serum and/or growth factors was independently confirmed by our group in a factor-dependent hematopoietic cell line [60] and, more recently, by a different group of investigators on primary hematopoietic cells [61]. A strong link between PKC-ε and suppression of apoptosis has been confirmed by many studies on a variety of different tumour cells types [34, 36, 62-65] as a key event in the context of cancer. PKC-ε silencing induces apoptosis in glioma cells and primary glioma cultures, basally overexpressing this kinase [62]. At variance to factor-dependent hematopoietic cells [59], 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 [62] and that PKC-ε over-expression prevents apoptosis induced by tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a

**Table 2. Examples of Opposite Effects of PKC-ε and PKC-δ in Different Mammalian Cell Types**

| Biological Effect | Cell Type | References |
|---|---|---|
| **PKC-ε:** | | |
| Anti-proliferative | Non-Small Cell Lung Cancer | [34] |
| | Vascular Endothelial Cells | [58] |
| | Vascular Smooth Muscle Cells | [58] |
| | Intestinal Epithelial Cells | [58] |
| | Keratinocytes | [58] |
| | Fibroblasts | [58] |
| Anti-apoptotic/pro-metastatic | Head And Neck Squamous Cell Carcinoma | [47] |
| | Breast Cancer | [31] |
| Anti-apoptotic | Skin Cancer | [56, 57] |
| | Brain Tumors | [36] |
| | Thyroid Cancer | [17] |
| | Melanomas | [63] |
| | Lung Cancer | [34] |
| | Ovarian Carcinomas | [66] |
| Pro-apoptotic | Glioma Cells | [36, 62] |
| Pro-autophagic | Leukemic Cells | [71, 72] |
| PKC-δ: | | |
| Anti-proliferative | Intestinal Epithelial Cells [Crypts] | [58] |
| | Leukemic Cells | [58] |
| | Fibroblasts | [58] |
| | Vascular Smooth Muscle Cells | [58] |
| | Microvascular Endothelial Cells | [58] |
| Pro-apoptotic | Skin Cancer | [56, 57] |
| | Prostate Cancer | [69] |
| Pro-apoptotic/Anti-autophagic | Fibrosarcoma Cells | [74] |
| Anti-autophagic | Pancreatic Cancer Cells | [73] |

death inducing ligand belonging to the TNF superfamily of cytokines [63, 64]. 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 agents [66, 67]. Therefore, 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 [68]. Further evidences of the PKC and TRAIL interplay consists in the existence of an autocrine apoptotic loop through the PKC-δ-triggered secretion of TRAIL in prostate cancer cells [69].

Recent findings support the thesis of a role for PKCs in promoting/inhibiting autophagy in a cell-type dependent way (Table **2**). Studies reported autophagy induction after PKC-α activation in phorbol-treated rat hepatocytes [70] as well as after PKC-ε activation in human leukemic cells treated with anti-neoplastic drugs [71, 72]. On the other hand, PKC-δ inhibits autophagy in pancreatic cancer cells [73]. In keeping with these observations, some groups have reported induction of autophagy upon treatment with specific PKC-δ inhibitors [74]. Coward *et al.* reported autophagy post-treatment with non-inducing apoptosis doses of safingol, a drug inhibiting PKC-ε but probably also PKC-δ [75]. A hypothetical involvement of PKCs in autophagy promo-

tion/inhibition is important since established tumours depend on this mechanism to survive under starvation, oxidative stress and hypoxia [reviewed in 76]. On the other hand, it is also true that some anti-cancer drugs induce selective type of autophagy to kill tumour cells or to prevent malignant trasformation of cells accumulating stressed mithocondria and misfolded proteins. Of interest, Chen *et al.* reported evidences of a time-regulated role of PKC-δ in autophagy-apoptosis crosstalk [77]: under hypoxic-condition, PKC-δ activates "functional selective autophagy" by promoting JNK1-mediated Bcl-2 phosphorylation and dissociation of the Bcl-2/Beclin 1 complex; after prolonged hypoxic stress, instead, PKC-δ is cleaved by caspase-3, and translocates as constitutively active catalytic fragment of PKC-δ (PKC-δCF) to the nucleus where it serves a feed-forward function for the reciprocal PKC-δ and caspase-3 proteolytic activation. Thus, PKC mostly induces autophagy, as observed for PKC-ε , PKC-α and PKC-β and only PKC-δ seems to be able to counteract this cellular mechanism.

## FIRST GENERATION OF PKC MODULATORS ENROLLED IN CLINICAL TRIALS OF HUMAN CANCERS: ENZASTAURIN AND BRYOSTATIN-1

Since PKC oncogenic activity has been demonstrated in human cancer, newly characterized drugs inhibiting selectively PKC isozymes (Fig. (**1**)) caught great interest for possible clinical trials. Among the miriad of PKC inhibitors, enzastaurin and bryostatin-1 are the most considered (Table **3**).

### Enzastaurin

Enzastaurin is an analogue of staurosporine, the best-known pan-PKC inhibitor in *in vitro* models [78]. Staurosporine is an alkaloid produced by *Streptomyces* bacteria, which is served as lead compound for the synthesis of more PKC-selective analogues, i.e. 7-hydroxystaurosporine/ UCN-01 [79], ruboxistaurin/LY333531 [80] sotrastaurine/ AEB071 [81], N-benzoyl-staurosporine/midostaurin/PKC412/ CGP41251 [82-83] and enzastaurin/LY317615 [84]. Enzastaurin is a specific PKC-β inhibitor only at low concentration, while at higher concentrations it also inhibits other isoforms i.e. PKC-ε [84]. Initially developed as possible anti-angiogenetic drug, enzastaurin was considered for anti-cancer therapy after preclinical evidences of therapeutical effects against both solid and hematological malignancies [84-94]. Enzastaurin is metabolyzed by cytochrome 450-3A in major and minor metabolites, comparably active in preventing substrate phosphorylation as competitors for PKC-β ATP binding site. For the first clinical trial in 47 patients with advanced cancer, Carducci *et al.* [86] pre-selected a target mean steady state concentration based on the free fraction of drug that produced 90% PKC-β inhibition (IC90) *in vitro*, considering 95% of plasma protein binding, equivalent to a dose of 525 mg/die, necessary to yield a plasma concentration of approximately 2μM. Despite all the following trials adopted this recommended dose as estimated to be effective *in vivo*, there are no published results that confirm a real PKC-β inhibition in cancer cells of patients treated with this dose of enzastaurin. Most of the clinical trials agree in attributing to this drug a favourable toxicity profile up to 900 mg/die, sometimes even with less side effects, both in monotherapy and in combination with conventional chemotherapeutic drugs [86, 95-103]. This is very important for patients with advanced cancers who are unable to receive chemotherapy because of comorbidities or debilitation. Recently, Kreisl *et al.* [104, 105] reported controversial data regarding unacceptable toxicities in patients treated for recurrent gliomas. Since it is unlikely that enzastaurin will enter into the clinical practice as single pharmacological agent, it is particularly noteworthy that this molecule displays 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 (MM) cell models and retains cytotoxicity when MM cell lines were co-cultured with multipotent mesenchymal stromal cells [90, 91]. Besides being active in MM, some preliminary evidences indicate that enzastaurin also shows significant *in vitro* and *in vivo* anti-tumoral activity in Waldenstrom's macroglobulinemia, a low-grade lymphoplasmocytic lymphoma [92]. The observations that enzastaurin enhances the *in vitro* antitumor activity of bortezomib, rituximab, fludarabine and dexamethasone strongly supports the potential therapeutic value of using enzastaurin in combination with these agents [93, 94]. Overall, the results of the clinical trials testing enzastaurin suggests that therapeutical strategies including this drug, increases progression-free survival rate in diffuse large B-cell lymphoma (DLBCL), recurrent high grade gliomas, non-chemonaive non-small cell lung cancer, relapsed/refractory mantle cell lymphoma (MCL [89,99,105]. The results of two clinical trials enrolling patients for innovative therapeutical treatment combining or not enzastaurin with conventional therapy, revealed that these combinations do not have superior clinical efficacy compared with, respectively, lomustine against recurrent intracranial gliomas [101] or pemetrexed against advanced non-small cell lung cancers [103]. Although no direct evaluation of PKC-β inhibition has been performed in the patient samples enrolled in the mentioned clinical trials, GSK-3β inhibition has been tested as potential biomarkers [105].

In the next future, several data coming from ongoing/just completed phase I/II clinical trials for the treatment of hematological malignancies, brain tumours and metastatic solid cancer could offer new insights on the enzastaurin anticancer activity. Great interest is also for the results of a phase III-advanced clinical trial testing relapse-prevention by enzastaurin in a small subset of patients who achieved remission after R-CHOP chemotherapy (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone) [www.clinicaltrials.gov, accessed August 25, 2010]. The European Medicines Agency already recognized to enzastaurin the orphan drug status for the treatment of patients with DLBCL where PKC-βII mRNA levels demonstrated prognostic for the relapse of the disease after R-CHOP treatment [37].

### Bryostatin-1

Differently from enzastaurin, bryostatin-1 is a natural compound, a macrocyclic lactone derived from the symbiontic proteobacterium *Candidatus Endobugula sertula*, which potently binds the regulatory domain of novel and classical PKC isoforms in a tightly time-regulated way [106-114].

**Table 3. Significant Clinical Trials on the Anti-Cancer Activity of Enzastaurin and Bryostatin-1**

| Drug | Clinical Trial, (Phase) | Cancer (n Pts evaluable for response) | Drug dose, administration + combined drug(s) | Biomarker Assays to assess drug activity or find prognostic factor | Outcome | Ref. |
|---|---|---|---|---|---|---|
| Enzastaurin: | I | Advanced solid (n=47) | (525-700) mg/die | Phosphorylation of PKC-substrates (FACS) | 45% stable disease | [86] |
| | II | DLBCL (n=55) | 500 mg/die | PKC-β total level (IHC) | 56% PFS/complete remission | [89] |
| | I | Advanced solid (n=27) | 500 mg/die<br>+ capecitabine 1000 mg/m$^2$ | None | 18% stable disease | [95] |
| | I | Advanced solid (n=33) | (350-500) mg/die<br>+ gemcitabine (1000-1250) mg/m$^2$<br>+ cisplatin (60-75) mg/m$^2$ | Circulating Endothelial Cells CD146$^+$CD133$^+$ (FACS/RT-PCR ) | 39,4% stable disease<br>9,1% PR | [96] |
| | Ib | Advanced/metastatic (n=68) | (250-500) mg/die<br>+ pemetrexed 500 mg/m$^2$ | None | Preliminary evidences of anti-thyroid cancer activity | [98] |
| | II | Relapsed/refractory MCL (n=60) | 500 mg/die | PKC-β total level (IHC) | No Obj. response;<br>37% PFS~ 3mo, 27% PFS >6mo | [99] |
| | III | Recurrent intracranial gliomas (n=266) | 500 mg/die | None | = lomustine alone | [101] |
| | II | Metastatic NSCLC, II/II line therapy (n=55) | 500 mg/die | PlasmaticVEGF (ELISA)<br>PKC-βII, GSK-3β, EGFR (IHC) | 13% PFS>6mo | [102] |
| | II | Advanced NSCLC (n=160) | 500mg/die<br>+ pemetrexed 500 mg/m$^2$/3weeks/i.v. | None | = pemetrexed alone | [103] |
| | I | Recurrent gliomas (n=19) | (500-1000) mg/die in 2 doses | GSK-3β phosphorylation in PBMCs, (WB) | 11% Obj. radiographic response | [104] |
| | I/II | Recurrent high-grade gliomas (n=84) | (500-525) mg/die | GSK-3β phosphorylation in PBMCs | 25% Obj. radiographic response<br>7-16% PFS>6mo | [105] |
| Bryostatin-1: | II | Advanced sarcoma (n=12),head - neck(n=12) | 120 mcg/m$^2$/2weeks/72h i.v. | None | NO disease response | [115] |
| | II | Metastatic/recurrent SCC head -neck<br>(n= 14) | 25 mcg/m$^2$/die/24h i.v. | Tumor Metabolism (PET) and<br>Apoptosis (PARP, WB) | NO disease response<br>1 of 14 Pts Stable disease <4mo | [116] |
| | II | Metastatic melanoma (n=37) | 25 mcg/m$^2$/die/24h i.v. OR<br>40 mcg/m$^2$/2 weeks/72h i.v. | None | NO disease response<br>1 of 12 Pts[72h ] PR<7mo | [117] |

**(Table 3). Contd…..**

| Drug | Clinical Trial, (Phase) | Cancer (n Pts evaluable for response) | Drug dose, administration + combined drug(s) | Biomarker Assays to assess drug activity or find prognostic factor | Outcome | Ref. |
|---|---|---|---|---|---|---|
| | II | Metastatic Colorectal (n=25) | (25-40) mcg/$m^2$/weekly/24h i.v. | None | NO disease response | [118] |
| | II | Relapsed MM (n=9) | 120 mcg/$m^2$/2weeks/72h i.v. | None | NO disease response | [119] |
| | II | NHL (n=14) | 25 mcg/$m^2$ /weekly/24h i.v. | None | NO disease response | [120] |
| | II | Recurrent epithelial ovarian Ka. (n=14) | 25 mcg/$m^2$/weekly/24h i.v. | None | NO disease response | [121] |
| | II | Metastatic RCC (n=32) | (25-40) mcg/$m^2$/weekly/1h i.v. | Plasmatic IL6, TNF-α, CRP (ELISA) | 25% stable disease/PR | [122] |
| | II | IL2-naive advanced kidney (n=35) | (5-35) mcg/$m^2$/weekly/ i.v. +IL2 (11x$10^6$IU) subcutaneously | Number/Activation/ IL2 ,IL4, TNF-α Lymphocytes (FACS,ELISA) | 3,2% overall response 1 Obj. PR; | [123] |
| | II | Locally adv/metastatic pancreatic adenoKa .(n=19) | 25 mcg/$m^2$/weekly/1h i.v. + paclitaxel 90mg/$m^2$/1h i.v. | None | NO confirmed obj. disease response | [124] |
| | I | Refractory non-hematologic, mostly melanomas (n=53) | 30 mcg/$m^2$ /1-24h i.v. + cisplatin (50-75)mg/$m^2$/1h i.v. | Phosphorylation PKC-η in PBMCs (WB) | 7,5% Obj. response | [125] |
| | II | Aggressive B-cell NHL (n=13) | 50 mcg/$m^2$/2 weeks/24h i.v. + vincristine 1.4mg/$m^2$ /24h i.v. | $CD5^+$Lymphocytes Apoptosis (AnnexinV, FACS) | 31% overall response | [126] |
| | I | Refractory non-hematological (n=36) | (25-45) mcg/$m^2$ + gemcitabine(0,6-1) mg/$m^2$ | None | 5,6% PR >6mo 22,2% stable disease | [127] |
| | II | Advanced gastric/GE junction (n=35) | (25-40) mcg/$m^2$/weekly/1h i.v + paclitaxel80mg/$m^2$/weekly/2h i.v. | None | 29% PR | [129] |
| | II | Advanced esophageal/GE junction (n=22) | (25-40) mcg/$m^2$/weekly/1h i.v. + paclitaxel (80-90)mg/$m^2$/2h i.v. | None | 27% PR, 10 of 40 pts HD | [130] |
| | I | Indolent NHL and CLL(n=59) | 50 mcg/$m^2$/monthly/24h i.v. + fludarabine 25 mg/$m^2$/die | Ex-vivo total PKC Activity and Apoptosis (Annexin V, FACS) Assays on PBMCs | Not definitively assessed | [131] |
| | II | Advanced/recurrent cervix (n=10) | (50-65) mcg/$m^2$/weekly/1h i.v. + cisplatin50mg/$m^2$ | None | NO disease response 20% stable disease | [132] |

Phase I/II clinical trials report that bryostatin-1 has a quite favourable toxicity profile but minimal single-agent activity during the treatment of solid cancer as well as hematological malignancies [115-121]. The best anti-cancer response has been obtained in metastatic renal cell carcinoma where weekly bryostatin-1 monotherapy produced partial remission in 25% of patients [122]. This is the reason why the most recent clinical trials concentrate on bryostatin-1 as part of an anti-cancer polychemotherapy, generally associated with dose-dependent non-hematological toxicities [123-132]. Moderately active bryostatin-1 combinations have been reported, such as: with vincristine for the treatment of Non-Hodgkin lymphoma (NHL) relapsing after autologous stem cell transplant achieving 31% (2 of 13 patients) of overall response [126]; with full dose of fludarabine against indolent lymphomas and CLL [131]; with gemcitabine versus refractory non-hematological cancers [127]. Therapeutical approaches, revealed as not effective, combined bryostatin-1 with paclitaxel for the treatment of advanced pancreatic carcinoma [130], with IL2 against renal cell carcinoma [123], with cisplatin versus advanced/recurrent cervix cancers [132].

### Bryostatin-1 Analogues: An Example of Function-Oriented Synthesis of Natural Compound Analogues for PKC Modulation

Two major problems delaying clinical advancement of bryostatin-1 are the low yields from the natural source and the difficulties in selectively modifying this molecule to obtain analogues possibly with superior effects in containing cancer cells growth [133]. Thus, Wender *et al.* [134] chose a function-oriented synthesis approach obtaining bryologs, tetrahydropyranyl analogues keeping oxycarbocyclic ring of bryostatin-1, *via* a highly efficient, functional-group-tolerant, and stereoselective prins-driven macrocyclization. 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 [135]. Bryologs, exhibiting nanomolar and picomolar activities in PKC affinity assays as well as in cellular antiproliferation assays [136], represent the possibility that bryostatin-1-like modulators of novel and classic PKC isoforms meet finally the clinical need. Currently clinical trials of these compounds are not yet ongoing.

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

Increasing literature reports the effects of natural products, or derivatives, as activator or inhibitor of PKC-dependent pathways. 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. Resveratrol is an example of these compounds already in oncological clinical trials. Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a compound primarily found in the skin of grapes as well as in other fruits and plants, known for the free radical scavenging and anti-tumour effects. Cancer chemopreventive activity of resveratrol was first published in 1997 [137]. Extensive *in vitro* studies revealed multiple intracellular targets of resveratrol, which affects cell growth, inflammation, apoptosis, angiogenesis, and invasion and metastasis [reviewed in 138]. Baur *et al.* summarized the numerous evidences supporting the cancer-preventing and anti-cancer properties of resveratrol in various murine models of different human cancers [reviewed in 139]. Moreover, analogues of this natural molecule are already available [140].

Resveratrol induces cell cycle block, apoptosis and autophagy targeting cell cycle regulators, cyclins and checkpoint kinases (p21WAF1, p27KIP, INK, ATM/ATR) tumor suppressors (p53 and Rb) as well as apoptotic and survival regulators (Bax, Bak, PUMA, Noxa, TRAIL, APAF, survivin, Akt, Bcl-2 and Bcl-XL). Resveratrol also has nuclear targets that are the transcription factors NF-kB, AP-1, c-Jun, and c-Fos. For the purpose of this review, it is particularly interesting, that Atten MJ reported that resveratrol-treated gastric cancer cells show loss of membrane-associated PKC-δ protein and a concomitant increase in cytosolic PKC-α, undergoing cell cycle arrest at the G2/M transition with accumulation of p21 and p53 [141]. Similarly, treated prostate cancer cells undergo p53-induced apoptosis after PKC-α activation [142]. PKC modulation by resveratrol seems to be restricted to PKC-α, PKC-δ and PKC-βI inhibition [143]. In addition, treatment with this PKC inhibitor sensitizes to TRAIL cytotoxic activity in cancer cells [144, 145]. Remarkably, resveratrol showed *in vitro* inhibition of invasiveness of cancer cells [146] and has great influence on angiogenesis by targeting VEGF, MMP-9 signalling [138]. Recently, some clinical trials started in order to assess chemopreventive activity of SRT501/resveratrol. The unique trial to test efficacy of resveratrol in combination with bortezomib for the treatment of MM has been suspended for unexpected safety events [www.clinicaltrials.gov, accessed August 25, **2010**]. A phase I clinical trial in 11 healthy volunteers reported that consumption of resveratrol did not cause serious adverse events and anticipated that even high-doses might be insufficient to elicit systemic levels commensurate with cancer chemopreventive efficacy [147]. A phase I clinical trial enrolling patients with colorectal cancer and hepatic metastases has just been completed and another phase I/II in the same oncologic setting is recruiting in patients. To conclude, other results should be published from a phase I biomarker study of dietary grape-derived low dose resveratrol for colon cancer prevention.

## PKC-ε INHIBITORY/TRANSLOCATION ACTIVATORS PEPTIDES

Great efforts, in the last years, have been focused in the identification of specific functional mechanisms attributing to single PKC isozymes oncogenic or anti-cancer activity. Moreover, considering the very high homology between different PKC isoforms and substrates condivision, it has been difficult to identify chemical compounds selective as aimed. In the recent years, the Mochly-Rosen's group approached this issue in an innovative way: the new generation of PKCs modulators should have been short peptides (6-8 aminoacids long) derived from interaction sites between each PKC isozyme and its receptor [148]. This drug design project is based on the theory that PKC isozymes translocate from one cell compartment to another when activated by the appropri-

ate signal with each isozyme translocating to a unique subcellular site. They suggest that this unique localization is mediated by binding of each of the activated isozymes to their corresponding isozyme-specific anchoring proteins, termed RACKs (Fig. (**1**)). The newly generated PKC modulators work selectively by inhibiting translocation and function of their corresponding isozymes at intracellular concentrations of 3-10 nM. Mochly-Rosen's group identified among a pool of peptides mimicking the variable region V1 containing the interaction sites for εRACKs, an activating (ψεRACK peptide, PKC-ε aa85-92) [149] and an inhibitor (εV1-2, PKC-ε aa14-21) PKC-ε drug [150]. Some concerns about the PKC-ε inhibitory peptide in anti-cancer therapy derives from the controversial data regarding variation of sensitivity to TRAIL-induced apoptosis. An increase of sensitivity has been reported in HL-60 [68], whereas in immature erythroblasts, basally susceptible to TRAIL mediated cytotoxicity [151], treatment with PKC-ε inhibitory peptide induces TRAIL resistance and intracellular PKC-ε accumulation [61]. 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.

## CROSS-TALK BETWEEN PKC AND P53

In a therapeutic perspective, it is of particular interest that a recent study demonstrating that murine double minute 2 gene product (MDM2, HDM2 in humans) is a target of activated PKC-ε [152]. MDM2 is an E3 ubiquitin ligase for p53 and itself, that is over-expressed in many human tumors [153-155]. Although the MDM2 genes represent 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 [156, 157]. Any pharmacological 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 [158].

The interplay between the PKC and p53 intracellular signal transduction pathways is particularly noteworthy since several groups of investigators followed the important therapeutic strategy of restoring the p53 pathway targeting its main regulator MDM2 [159]. The first potent and selective small-molecule MDM2 antagonists, among a group of cis-imidazoline compounds, was identified by Vassilev *et al.* [160]. The Nutlins have the ability to displace p53 from MDM2 *in vitro* with nanomolar potency (IC50=90nM for Nutlin-3a, the active enantiomer of Nutlin-3) [161]. Importantly, with this effect, Nutlins 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 [153], other studies have demonstrated that Nutlins show cytostatic/cytotoxic activity in hematological malignancies too, including acute myeloid leukemias [162-165] and block angiogenesis, at least *in vitro* [166]. The original study performed by the group of Vassilev demonstrated that Nutlin-3a can be administered orally to nude mice bearing established human solid tumor xenografts for up to 3 weeks without systemic toxic effects [167], 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. RO5045337/RG7112, an oral formulation of Nutlin-3, is currently administered in phase I clinical trials enrolling both pediatric patients with acute lymphoblastic leukemia and adults with advanced solid cancer, hematological neoplasms and liposarcomas [www.clinicaltrials.gov, accessed August 25, 2010].

## CONCLUDING REMARKS

Despite years of research, the relative role of individual PKC isozymes in cancer are just beginning to emerge, a task that has been confounded by the heterogeneity in functional responses conferred by isozymes-specific regulation and the cell-type dependency of these effects. Still few research groups are focusing their efforts in studying the mechanisms that drives isozyme-specific compartmentalisation to access different substrates. It is fundamental that clinical investigators will study PKCs functions in cancer patient samples as well. The information about the overexpression or down-regulation of PKC isozymes, provided with immunohistochemical studies, are still insufficient in order to understand the real alteration of these kinase in human cancer. Currently integrative methodological approaches, based on techniques (i.e. real time PCR, immunofluorescence) which better quantify the levels of expression of PKCs at mRNA level, or describe subcellular localization of the active kinases, are ongoing. In general, an integrative methodological approach is recommended [reviewed in 168]. A successful example of this is the characterization of the oncogenic role of PKC-ι in human ovarian cancer, first highlighted with array-based comparative genomic hybridization and then validated with data regarding both RNA and protein expressions (RT-PCR, tissue array and WB) [27]. Proceeding from cancer cell lines and then studying patient biopsies, Nazarenko *et al*. investigated with great detail PKC-ζ role in ovarian cancer [26]. When such approach is not feasible, at least combining a study of the phosphorylation status to the immunohistochemistry could be already informative of the role of a PKC isoform for the development of a specific cancer [32].

Basic research provided selective PKC modulators with anti-cancer therapeutic value, several of which are currently used in clinical trials, i.e. enzastaurin and bryostatin-1. 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 act as anti-neoplastic drugs. Beside this, we should consider other factors limiting the success of first generation PKC modulators in anti-cancer therapeutical approaches. First of all, they are often not completely specific for single PKC isozymes, i.e. bryostatin-1. Second remark, clinical trials have offered limited information about the real PKC modulation in the treated patients. For this purpopose, several efforts are ongoing in order to identify surrogate markers that at least can monitor the efficacy of the administered dose of PKC modulator. At present GSK-3β inhibition, VEGF plasma levels, release of IL6 and TNF-α by patients PBMCs stimulated or not *in vitro* with phytohemoagglutinin, are the biomarkers that performed best in monitoring enzastaurin or bryostatin-1 activity [168-169]. Furthermore, the

clinical trials provided limited information about the patients who have benefited from PKC inhibitor. We need a critical review of the positive and negative achievements of all the completed clinical trials, in order to reach a fine tuning of the several functions of PKCs in cancer progression, metastasis and neoangiogenesis. In addition, 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. Therefore the combination of an "upstream-downstream" therapeutical approach might reveal noteworthyly effective.

In conclusion, we need a better knowledge of the spatial-regulated mechanisms underlying the PKC isozymes functions. Only in this way, a function-oriented synthesis approach of new PKC modulators, potentially derived even from natural compounds, will provide tools for a fine PKC tunability in cancer patients enrolled in clinical trials. First generation of PKC inhibitors already provided encouraging results when combined with other anti-neoplastic drugs, hence combinations with TRAIL/p53-restoring pathway agents/tyrosine kinase receptor small molecules inhibitors could be explored in the next future. Together with a more systematic clinical investigation of cancer patients enrolled in clinical trials for PKC isozymes modulating drugs, a future anti-cancer therapy including PKC isozyme targeting looks much closer.

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## ABBREVIATIONS

Adv = Advanced

aPKCs = Atypical PKCs

CLL = Chronic Lymphocytic Leukemia

CML = Chronic Myeloid Leukemia

cPKCs = Classic/conventional PKCs

DAG = Diacylglycerol

DLBCL = Diffuse Large B-Cell Lymphoma

ELISA = Enzyme Linked ImmunoSorbent Assay

FACS = Flow cytometry analysis

GE = Gastro-Esophageal

GIST = Gastro-Intestinal Stromal Tumour

HD = Higher Dose

IHC = Immunohistochemistry

IU = International Units

Ka = Carcinoma

mcg = Micrograms

MCL = Mantle Cell Lymphoma

MM = Multiple Myeloma,

Mo = Months

NHL = Non-Hodgkin Lymphoma

nPKCs = Novel PKCs

NSCLC = Non-Small Cell Lung Cancer

Obj = Objective

PB1 = Phox/Bem1 domain

PFS = Progression Free Survival

PKC = Protein kinase C

PR = Partial Response

Pts = Patients

RCC = Renal Cell Carcinoma

RT-PCR = Reverse Trascription Polymerase Chain Reaction

SCC = Squamous Cell Carcinoma

WB = Western blot

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