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Review paper

Protein kinase C: a worthwhile target for anticancer drugs?

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Protein kinase C (PKC) is an enzyme family with serine/threonine kinase function which is involved in the transduction of signals for cell proliferation and differentiation. The important role played in processes relevant to neoplastic transformation, carcinogenesis and tumor cell invasion renders PKC a potentially suitable target for anticancer therapy. Bryostatin 1, a macrocyclic lactone isolated from *Bugula nerutina*, is a partial PKC agonist, and has shown potent antineoplastic properties *in vitro* and *in vivo*. Staurosporine, an alkaloid isolated from microbial sources, is one of the most potent PKC inhibitors and has shown high antiproliferative activity *in vitro*, but poor selectivity. Staurosporine analogs have thus been synthesized with the aim of obtaining more selective PKC inhibition; among these, CGP 41251 has shown reduced PKC inhibitory activity, but a higher degree of selectivity when assayed for inhibition of different kinases. Several studies indicate a role for PKC in the regulation of the multidrug resistance (MDR) phenotype, since several PKC inhibitors are able to partially reverse MDR and inhibit P-glycoprotein (Pgp) phosphorylation. The MDR phenotype is also associated with variation in PKC isoenzyme content, in particular with PKC-α overexpression. While adequate PKC modulation might offer an attractive concept to modulate MDR, other potential mechanisms of PKC interaction with anticancer drugs exist and have been documented, such as the enhancement of chemotherapy-induced apoptosis by safingol, a specific PKC inhibitor. Three phase I clinical trials with bryostatin have been completed so far and have shown that myalgia is the dose-limiting toxicity, while some antitumor activity is evident. Safingol is presently undergoing a phase I clinical trial in combination with doxorubicin. While no definitive data are presently available, it appears that safingol plasma levels approach those associated with chemopotentiation in animals and no pharmacokinetic interaction between the two drugs exists. Drugs targeting PKC are well worth considering for clinical trials, particularly for their potential as modulators of currently available cytotoxic agents.

Key words: Drug resistance, protein kinase C, tumor growth.

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Introduction

Recent findings suggest that cancer can be considered a disease of altered intracellular signaling and that the components of signaling pathways provide attractive targets for the development of new types of anticancer drugs.[1,2]

Protein kinase C (PKC) is an isoenzyme family with serine/threonine kinase function which has been shown to play a key role in the signal transduction pathway elicited by a variety of extracellular stimuli, such as growth factors, hormones and neurotransmitters.[3]

PKC isoenzymes are closely related structurally and consist of a single polypeptide chain divided into two domains: a regulatory domain at the N-terminus and a catalytic domain at the C-terminus. The enzyme can be divided into four regions conserved across isoenzymes (C1–C4) and five variable regions (V1–V5).[4] At least 12 different PKC isoenzymes have been identified so far. They can be divided in at least two large subfamilies: one containing PKC-α, -β and -γ (calcium-dependent); the other containing PKC-δ, -ε, -η and -θ (calcium-independent). PKC-ξ constitutes what could be considered a separate branch.[5] It seems likely that the full range of PKC isotypes has not yet been uncovered and that even more members of the PKC family will be identified in the future. Such heterogeneity and tissue-selective distribution of the gene products that constitute the PKC family may explain the extraordinary diversity of responses to PKC activation.[4,6,7]

Role of PKC in tumor growth

Two major findings in the early 1980s established the importance of PKC in signal transduction and tumor promotion. In particular, Kishimoto *et al.*[8] discovered that the basal activity of PKC was

stimulated by diacylglycerol (DAG), a product of the phosphatidylinositol cycle. Subsequently, evidence was provided that PKC is the major intracellular receptor for tumor-promoting phorbol esters,[9,10] as these compounds directly activate the enzyme both *in vitro* and *in vivo*, and a correlation exists between the ability of individual phorbol esters to promote tumors and to activate the enzyme;[3] furthermore, similar distribution patterns among tissues of the enzyme and phorbol ester binding sites were observed.[3] Phorbol esters activate PKC in a way similar to the endogenous activator DAG, with the difference that phorbol ester activation can be maintained for a long time due to their metabolic stability.[9] Thus, presently available evidence suggests that most, if not all, of the pleiotropic actions of tumor-promoting phorbol esters may be mediated through the prolonged stimulation of PKC. In addition, it has been suggested that other different tumor promoters may act via PKC. In fact, mezerein, which is classified as a second-stage tumor promoter, can activate PKC.[3] Teleocidin and aplysia toxin, which are tumor promoters structurally unrelated to phorbol esters, also activate PKC *in vitro* at high concentrations.[3] Although these compounds do not have a DAG-like structure, they are likely to cause similar changes in membranes.

PKC has also been implicated in the process of invasion and metastasis. Tumor cells that have increased PKC activity have an enhanced ability to invade and metastasize. Such association has been shown for mouse Lewis lung carcinoma cells,[11] mouse B16 melanoma cells,[12] mouse mammary adenocarcinoma cells,[13] murine fibrosarcoma cells,[14] human bladder carcinoma cells[15] and human gastric cancer cells.[16] In addition, pretreatment of tumor cells with phorbol esters considerably enhances the metastatic potential of the cells, with a mechanism that possibly involves the modulation of cellular adhesion to the extracellular matrix in response to PKC. In fact, many cell adhesion receptors are PKC substrates;[17] in addition, PKC can induce the expression of adhesion proteins.[18] A key issue is the difference, if any, between tumors and normal tissues in terms of PKC levels and/or isoenzyme distribution. PKC levels in surgical specimens of human breast tumors were significantly higher than the enzyme activities in normal breast tissue obtained from the same patients.[19] Several estrogen receptor negative (ER^-) human breast cancer cell lines express higher levels of PKC than ER^+ human breast cancer cell lines, indicating a negative correlation between PKC and estrogen receptor expression in the cells.[20]

Particulate PKC activity was elevated significantly in thyroid carcinomas compared with normal thyroid tissue and adenomas.[21] Malignant human gliomas express very high PKC activity when compared to non-transformed glial cells, and this high activity correlates strongly with the proliferation rates of these tumors *in vitro*.[22] In sharp contrast with these findings, in human colonic adenoma or carcinoma samples PKC activity was significantly reduced as compared to adjacent mucosa tissue and mucosa in control subjects.[23] Becker *et al.*[24] showed that normal human melanocytes did not express PKC-α, -β or -γ, whereas the α isoenzyme was expressed in primary and metastatic melanoma. However, in another study PKC-α, -β and -ε were found in primary melanocytes, but PKC isotype gene expression changed during the progression of melanocytes to metastatic melanoma.[25]

PKC modulators and growth arrest

Bryostatin 1 is the prototype of a novel class of structurally related macrocyclic lactones isolated from the marine bryozoan *Bugula neritina*.[26] These lactones have potent antineoplastic properties against human and murine tumor cell lines,[27,28] and murine B16 melanoma *in vivo*.[29] The bryostatins modulate PKC activity binding to and activating the enzyme; they show pharmacologic activity that is best described as a partial agonist.[30] In fact, bryostatin 1 can activate PKC, but, in the presence of activating ligands, acts mostly as an antagonist, possibly inhibiting tumor cell growth. In addition, bryostatin 1 can activate effector cells of the immune system and stimulate cytokine production.[31,32]

Several frank inhibitors of PKC have been detected and are capable of inhibiting PKC-mediated cellular responses. The entire group of drugs which target PKC can be grossly subdivided according to the interaction with PKC catalytic or regulatory domain (Table 1). The non-steroidal antiestrogen tamoxifen, used increasingly in the adjuvant setting and for the chemoprevention of breast cancer, is a PKC inhibitor ($IC_{50} = 40-100$ μmol), possibly through a specific binding site on the enzyme.[33,34] Since not all of tamoxifen action can be explained by its blockade of estrogen receptors,[35] it is likely that PKC inhibition is at least partially responsible for tamoxifen's activity against cancer cells.[36,37]

Another clinically used anticancer agent which inhibits PKC ($IC_{50} = 30$ μmol) is suramin.[38] This is a large and highly charged molecule which does not

Table 1. Main PKC modulators

At the catalytic domain	At the regulatory domain
Staurosporine	Calphostin C
Hydroxy-staurosporine (UCN-01)	Ether lipids
N-benzoyl-staurosporine (CGP 41251)	Sphingosine
H7	Safingol
Suramin	Bryostatins
	Tamoxifen

readily cross the cell membrane; therefore, the biological significance of suramin's ability to inhibit an intracellular enzyme such as PKC is unknown.

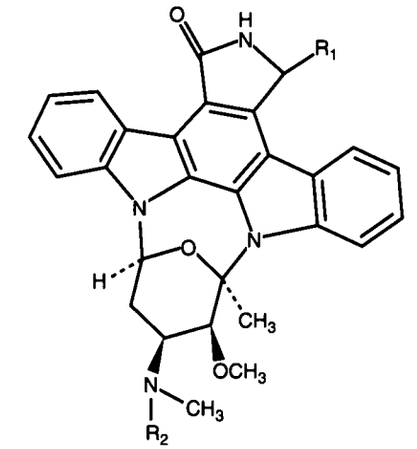
One of the most potent PKC inhibitors yet described is staurosporine, an alkaloid isolated from microbial sources (IC_{50} = 3 nmol).[39] Staurosporine has shown potent antiproliferative effect against various cell lines *in vitro*[39] and has been shown to inhibit, at non-toxic concentrations, the invasion both of the invasive human bladder carcinoma cell line EJ, through an artificial basement membrane,[15] and, more recently, of the invasive SK-GT-1 and SK-GT-5 gastric cancer cell lines.[16] Although potent, staurosporine demonstrates poor selectivity and is inhibitory towards tyrosine kinases and cAMP-dependent protein kinases at concentrations similar to those that inhibit PKC.[40] This finding seems to suggest that staurosporine interacts with a conserved region of these kinases. Staurosporine analogues have been synthesized with the aim of obtaining a more selective PKC inhibition which may result in a better therapeutic index *in vivo*. UCN-01 (7-hydroxy-staurosporine), an agent isolated from streptomyces, is equally potent, but more selective for PKC inhibition than staurosporine and possesses weak antitumor activity against murine lymphatic leukemia P388.[41] Meyer *et al.* have studied CGP 41251 (*N*-benzoyl-staurosporine) and compared it with its parent compound in terms of PKC inhibition and antiproliferative activity.[42] This drug showed reduced PKC activity (IC_{50} = 50 nmol), but a higher degree of selectivity when assayed for inhibition of cyclic AMP-dependent protein kinase, S6 kinase and tyrosine-kinase specific activity of epidermal growth factor receptor. When assayed for antiproliferative activity in three cell lines (human bladder carcinoma line T-24, human promyelocytic leukemia line HL-60 and bovine corneal endothelial cells) staurosporin was three to nine times more potent than CGP 41251. Data revealed good correlation between potency of PKC inhibition and antiproliferative activity of the compounds; the sensitivity of the cells to the kinase inhibitors was uniform, even though three totally different target cells were selected. The chemical structure of some of the most important PKC modulators is represented in Figure 1.

PKC modulators and drug resistance

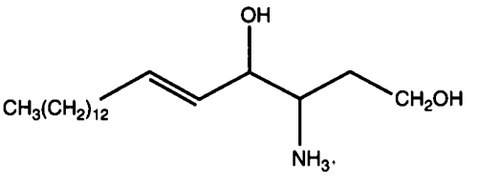
A potentially very exciting therapeutic use of PKC modulators could lie in their ability to interact with chemotherapeutic agents and potentiate their activity. The largest body of evidence in this setting implicates PKC in the regulation of the multidrug resistance (MDR) phenotype. This is a phenotype expressed by some tumor cell populations, in which a 170 kDa broad specificity drug efflux pump (P-glycoprotein, Pgp) is activated with consequent cross-resistance to major classes of anticancer drugs in clinical use (vinca alkaloids, anthracyclines, podophillotoxins, taxanes), because of reduced intracellular drug accumulation.[43]

The MDR phenotype is accompanied by changes in PKC activity and many observations indicate a role for PKC in the regulation of this phenotype. First of all, drug-resistant lines have altered levels of PKC;[44,45] in particular, the expression of PKC in primary cell cultures of human renal cell carcinomas significantly correlates with both resistance to doxorubicin and high Pgp expression;[46] the same correlation has been found in human non-small cell lung cancer when the above parameters were assessed histochemically.[47] Secondly, while activators of PKC are able to induce the MDR phenotype via increased Pgp phosphorylation,[45,48] several PKC inhibitors have been shown able to partially reverse MDR and inhibit Pgp phosphorylation.[37,49,50,51]

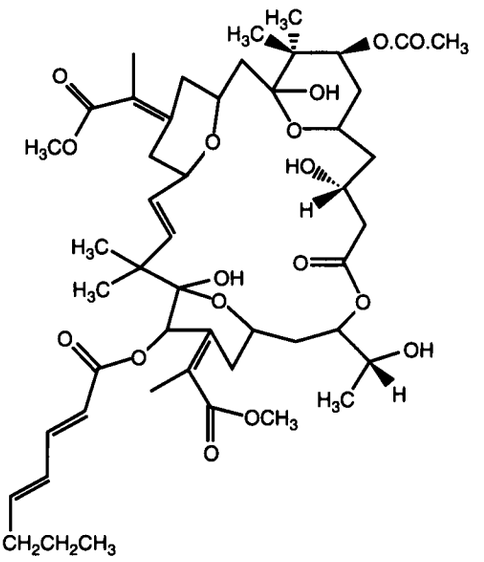
In particular, Utz *et al.*[52] have shown that CGP 41251 sensitizes multidrug-resistant CCRF-VCR 1000 and KB-8511 cells upon treatment with adriamycin or vinblastine. In fact, after treatment of the multidrug-resistant human lymphoblastoid cell line CCRF-VCR 1000 with 500 nM adriamycin, cell proliferation was reduced to 81% of untreated controls. A combination of 500 nM adriamycin with a non-toxic concentration of 150 nM CGP41251 inhibited cell proliferation of CCRF-VCR 1000 cells to 29% of untreated controls. In sensitive CCRF-CEM cells no enhancement of adriamycin-induced cytotoxicity was observed upon addition of 150 nM CGP 41251. Strong synergism of the inhibition of cell proliferation was also observed after concomitant treatment of KB-8511 cells with CGP 41251 and vinblastine



R1, R2, = H: Staurosporine
R1 = OH, R2 = H: UCN-01
R1 = H, R2 = benzoyl group: CGP 41251



Sphingosine



Bryostatin 1

Figure 1. Chemical structures of PKC modulators.

or adriamycin. Drug-sensitive KB-31 cells could not be further sensitized to adriamycin or vinblastine with CGP 41251 doses above 100 nM. These data show that concentrations of drug which exert only marginal antiproliferative effects by themselves are able to potentiate the antiproliferative activity of adriamycin and vinblastine in multidrug-resistant, but not in sensitive cells and are consistent with a modulation of the MDR phenotype by the PKC inhibitor. Recently, Budworth *et al.*[53] have compared staurosporine and four PKC-selective cogeners, CGP 41251, UCN-01, RO 318220 and GF 109203X, in terms of their MDR-reversing properties and susceptibility towards Pgp-mediated drug efflux from the

human breast cancer-derived multidrug-resistant MCF-7/Adr cell line. Among the analogs studied, staurosporine and CGP 41251 were the most potent modulators of the Pgp probe rhodamine 123 efflux and of vinblastine binding to its specific binding sites, probably Pgp. However, their cytostatic potential was affected only a little by the presence of Pgp in the resistant cells, suggesting that although both agents have a high binding affinity for Pgp, they are inefficiently transported by it. This property renders staurosporine and CGP 41251 attractive candidates for clinical development as MDR-reversing agents.

The variation in PKC isoenzyme content associated with the MDR phenotype may also be a key factor in its reversal. The most comprehensive analysis of PKC isoenzymes has been performed in the human breast cancer cell line MCF-7, where the MDR phenotype has been found associated with a 30-fold increase in PKC-α expression and a 10-fold increase in calcium-dependent PKC activity, but also with a decrease in PKC-δ and -ε protein levels with a 10-fold decrease in calcium-independent activity.[54] In another study, transfection of the Pgp expressing MCF-7 with PKC-α enhances multidrug resistance.[55]

The association between the MDR phenotype and PKC-α overexpression has been observed also in other cell lines[49,54,56] and the reduction of PKC-α expression by antisense has been shown to attenuate the MDR phenotype.[57]

Adequate PKC modulation might therefore offer an attractive concept to modulate established multidrug resistance.

Chaudhary and Roninson[58] have utilized highly sensitive assays to determine if transient exposure to chemotherapeutic drugs would have an effect on multidrug resistance gene (*MDR1*) expression in human cells and to assess if PKC inhibitors would influence such an effect. Transient exposure to chemotherapeutic drugs induced Pgp and *MDR1* mRNA expression in most of the tested cell lines. Drug-induced *MDR1* induction was blocked by non-specific protein kinase inhibitors that are active against PKC, but not by a protein kinase inhibitor ineffective against PKC. This finding lends support to the hypothesis that PKC plays a central role in cellular response to different types of cellular damage and indicate that protein kinase inhibitors active against PKC provide a potentially useful pharmacologic approach also to preventing the emergence of multidrug resistance during cancer chemotherapy.

Modulation of MDR is probably not the only mechanism of PKC interaction with anticancer drugs. Safingol, an optical isomer of dihydrosphingosine, is a new specific inhibitor of PKC, which has been shown to be non-toxic at doses that achieve serum levels sufficient to inhibit PKC enzyme activity.[59]

Preclinical studies have shown that the combination of safingol with conventional therapeutic agents, such as doxorubicin and cisplatin, substantially potentiates the antitumor effects of these drugs.[60] While the synergism with doxorubicin can be explained by inhibition of Pgp phosphorylation and consequent reversal of the MDR phenotype, the synergism reported for combinations of safingol and drugs that are not believed to produce resistance by the MDR mechanism (e.g. cisplatin) is harder to explain.

Therefore, pathways other than Pgp inhibition are likely to be involved in the safingol-mediated enhancement of chemotherapy. It is now widely accepted that the antitumor activity of many chemotherapeutic agents is a consequence of their induction of apoptosis.[61] In this context, it has been proposed that activation of PKC serves as an antagonist to apoptosis, whereas inhibition of PKC promotes apoptosis.[62] Thus, safingol-mediated potentiation of chemotherapy might be attributed to its PKC inhibitory effect, subsequently leading to increased apoptosis after drug-induced damage. Further insights to this concept have been recently brought by Schwartz *et al.*,[63] who have shown a safingol-induced enhancement of the cytotoxic activity of the chemotherapeutic agent mitomycin C in gastric cancer cells. This effect is due to a sensitization to drug-induced apoptosis which occurs regardless of the p53 status or the drug resistance status of the cells. Simultaneous exposure of cells to safingol and the PKC activator phorbol myristate acetate, which competes with safingol for the regulatory binding site of PKC, effectively abrogates the safingol effect of potentiating mitomycin C-induced apoptosis, thus supporting the hypothesis that this process is a PKC-dependent event. Potential enhancement of chemotherapy-induced apoptosis could thus represent an additional mechanism of potentiation of the cytotoxic effects of anticancer chemotherapy by PKC modulators.

Clinical trials with PKC modulators

Among the drugs whose main mechanism of antitumor action is PKC modulation, bryostatin 1 is the most extensively evaluated in clinical trials so far.

The first phase I clinical trial of bryostatin 1[64]

involved the administration of the drug over 1 h every 2 weeks for three cycles. Nineteen patients entered the trial and the maximum tolerated dose was limited to 50 $\mu g/m^2$ by cumulative myalgia. In the second phase I trial[65] bryostatin 1 was administered over 1 h weekly for 3 weeks every month. Again, myalgia was the dose-limiting toxicity and the maximum tolerated dose was 25 $\mu g/m^2$. In this study two patients with metastatic malignant melanoma had partial remission which lasted 6 weeks and 10 months, respectively. Furthermore, 50% of the patients treated at the highest dose level (50 $\mu g/m^2$) developed significant increases in plasma tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations at 2 and 24 h after treatment, respectively. Jayson *et al.*[66] have recently completed the third phase I study of bryostatin in which the drug was given as a 24 h i.v. infusion, weekly, for 8 weeks. Myalgia was the dose-limiting toxicity and the maximum tolerated dose was 25 $\mu g/m^2$. Total or activated PKC concentration measurement in the peripheral blood mononuclear cells of three patients for the first 4 h of treatment and during the last hour of the infusion showed that the enzyme activity was significantly modulated during the infusion. While the previously reported rise in serum levels of IL-6 and TNF-α was not confirmed in this study, an increased IL-2-induced proliferative response in peripheral blood lymphocytes and enhanced LAK activity were shown in seven patients during the first three cycles of treatment. Responses were seen in four patients, including two partial responses of 4 month duration (ovarian carcinoma and low-grade non Hodgkin's lymphoma) and two minor responses.

Schwartz *et al.* have recently presented the preliminary results of a phase I trial of safingol in combination with doxorubicin.[67] Safingol has been administered as a 1 h infusion (starting dose, 15 mg/m^2) on day 1 and repeated on day 14 with doxorubicin 1 h later (cycles every 21 days). Safingol doses have been escalated to 120 mg/m^2 with a fixed doxorubicin dose of 45 mg/m^2. At these doses safingol plasma levels approached those associated with chemopotentiation in animals; however, no pharmacokinetic interaction between the two drugs has been recorded. Two minor responses have been observed so far, while dose-limiting toxicity has not yet been reached.

CGP 41251, administered orally, is presently undergoing a phase I clinical trial. It is planned to incorporate into this study a regular indirect measure of PKC inhibition as an indicator of biological activity; this represents an extension of a classical phase I trial design, aimed at identifying the optimal dose for further study with this compound.

Conclusions

Because of its pivotal importance for signal transduction mechanisms PKC is undoubtedly a logical target for drug intervention. There is no doubt that the complicated interaction between different second messenger systems which involve cascade of kinases, one of which is PKC, will render selective drug action using PKC modulators difficult. Meanwhile, there is preclinical evidence of a meaningful antiproliferative activity of PKC modulators, which correlates well with enzyme modulation; however, the most exciting results are probably to be expected with the combined administration of PKC modulators and other cytotoxic drugs, including those involved in the MDR phenotype. In fact, the agents which are currently used for MDR reversal, such as verapamil, cyclosporine and quinidine, act mainly by interaction with the drug-binding site of Pgp and subsequent inhibition of the drug-efflux pump. However, the use of these agents is frequently hampered by their toxic effects at the doses required to achieve relevant MDR reversal.[43,68] Pharmacokinetic interaction with anticancer drugs may further complicate the interpretation of clinical results.[69,70] PKC modulators, on the other hand, seem to act through a different mechanism, and, in the only clinical trial of a drug belonging to this class used in combination with doxorubicin, serum levels approximating those that potentiate the effects of chemotherapy in tumor-bearing animals have been achieved without significant toxicity, while no pharmacokinetic interaction has been recorded.[66]

We therefore believe that drugs targeting PKC might possibly become, in the future, part of the anticancer therapeutic armamentarium mainly as a means to counteract drug resistance during cancer chemotherapy and are well worth considering for clinical trials in this setting.

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