Targeting Protein Kinase C: New Therapeutic Opportunities Against High-Grade Malignant Gliomas?

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ABSTRACT

A large body of evidence suggests that the abnormal phenotype of neoplastic astrocytes, including their excessive proliferation rate and high propensity to invade surrounding tissues, results from mutations in critical genes involved in key cellular events. These genetic alterations can affect cell-surface-associated receptors, elements of signaling pathways, or components of the cell cycle clock, conferring a gain or a loss of relevant metabolic functions of the cells. The understanding of such phenomena may allow the development of more efficacious forms of cancer treatment. Examples are therapies specifically directed against overexpressed epidermal growth factor receptor, hyperactive Ras, excessively stimulated Raf-1, overproduced ornithine decarboxylase, or aberrantly activated cyclin-dependent kinases. The applicability of some of these approaches is now being assessed in patients suffering from primary malignant central nervous system tumors that are not amenable to current therapeutic modalities.

Another potentially useful therapeutic strategy against such tumors involves the inhibition of hyperactive or overexpressed protein kinase C (PKC). This strategy is justified by the decrease in cell proliferation and invasion following inhibition of the activity of this enzyme observed in preclinical glioma models. Thus, interference with PKC activity may represent a novel form of experimental cancer treatment that may simultaneously restrain the hyperproliferative state and the invasive capacity of high-grade malignant gliomas without inducing the expected toxicity of classical cytotoxic agents. Of note, the experimental use of PKC-inhibiting agents in patients with refractory high-grade malignant gliomas has indeed led to some clinical responses. The present paper reviews the current status of the biochemistry and molecular biology of PKC, as well as the possibilities for developing novel anti-PKC-based therapies for central nervous system malignancies. The Oncologist 2002;7:17-33

INTRODUCTION

Primary tumors of the central nervous system (CNS) constitute less than 5% of human neoplasms [1]. Nevertheless, these malignancies are among the most prevalent childhood solid tumors, representing an important cause of cancer mortality in children under the age of 15 years [1]. Although less common in adolescents and adults, brain cancers are also responsible for a considerable number of deaths in these age...
groups [1]. The most frequently occurring primary CNS tumors are the gliomas, particularly those of astrocytic origin [1-2]. These malignancies account for approximately 30% of all cerebral cancers and are generally regarded as incurable in more aggressive histopathological types such as anaplastic astrocytoma or glioblastoma multiforme [2].

The poor prognosis of high-grade malignant gliomas can be attributed to various characteristics [1-3]. These tumors are incompatible with life at a relatively small burden, becoming symptomatic at a size between 30 and 60 grams (3 – 6 × 10^9 cells), and fatal when reaching about 100 grams (10^11 cells). In addition, the absence of lymphatic drainage impedes the removal of necrotic debris, which may result in the formation of life-threatening intracranial edema, respiratory failure, and death of the patient.

First-line treatment of brain tumors consists of surgery (mean survival, 14 weeks) associated with radiotherapy (increase in mean survival to 36 weeks), and this may be followed by chemotherapy (further increase in mean survival to 51 weeks). Chemotherapy, however, does not substantially change the prognosis; when employed as a precautionary measure, recurrence is only slightly delayed, and the mean duration of survival only slightly prolonged [1-5]. This is probably due to the low specificity of most chemotherapeutic agents against gliomas, the intrinsic chemoresistance of CNS neoplasms, the low tolerance of normal nervous tissue to the toxic side-effects of the treatment, and the low penetration of most antitumor agents in the CNS [3-6]. Consequently, current forms of treatment of high-grade gliomas are only partially effective with clinical responses being mostly palliative and of short duration.

For the above-mentioned reasons, research efforts have focused on the investigation of molecular aberrations in neoplastic glia, aiming at the identification of novel and more specific therapeutic targets. Such alterations often result from multiple mutations in proto-oncogenes and tumor-suppressor genes [7-8], which encode for proteins that play important roles in signal transduction. Irregularities in one or more signaling elements can cause cancer by affecting the pace of cell division, the completeness of cell differentiation, the threshold of apoptosis, the anchorage of cells to basement membranes and their interaction with extracellular matrix components, the degree of cell motility, as well as the formation of new blood vessels to support tumor growth [7-8].

Well-characterized oncogenic mutations that have been detected in a significant number of high-grade malignant gliomas [7-8] include: overexpression or amplification of genes encoding for growth factors and/or their receptors; loss-of-function mutations in the G1 checkpoint genes p53 and/or Rb; overexpression of the p53 counterpart, the mdm-2 gene; alterations in anti-apoptotic genes such as bcl-2, and deletions in genes encoding for certain cyclin-dependent kinase (cdk) inhibitors. Such alterations are generally believed to be the basis for the excessive proliferation rate, increased mitotic activity, invasive behavior, abundant vascularization, and resistance to cytotoxic drugs and ionizing radiation seen in many high-grade malignant gliomas [7-8] (Fig. 1).

These insights led to the development of rational, potentially more effective and less toxic treatment approaches such as immune [9] and gene therapies [10], which can be used either alone or in conjunction with conventional treatment regimens. Such strategies may allow for the restoration of defective cancer-inhibitory genes such as p53 and Rb, or the transduction or transfection of antisense DNA sequences or suicide genes to cancer cells, enabling, for instance, control over overstimulatory epidermal growth factor (EGF) receptor or hyperactive cdks [11]. Also, several anti-angiogenic approaches, such as the administration of thalidomide, protamine, angiogenin, or monoclonal antibodies against basic fibroblast growth factor or vascular endothelial growth factor, have been developed [12, 13]. Other treatment possibilities include: modulation of drug resistance, for instance by P-glycoprotein antagonists [14] or O6-alkylguanine DNA transferase inhibitors [15]; inhibition of matrix metalloproteinases [16], and the administration of agents such as phenylbutyrate or valproic acid that have shown promising anti-proliferative effects in vitro [17].

Less explored, but potentially useful, targets include: the platelet-derived growth factor (PDGF) receptor; components of mitogen-activated protein kinase (MAPK) cascades such as Raf-1, MAPK/extracellular-signal-regulated kinases (MEKs), and extracellular-signal-regulated kinases (ERKs); cell adhesion molecules, such as integrins; antiapoptosis proteins such as bcl-2, mdm-2, and survivin, and the cell-life-span enzyme telomerase.
The preliminary results from these studies, both at the preclinical and early clinical level, are encouraging, but also indicate the necessity to overcome important problems in order to incorporate such approaches into everyday clinical practice. The inefficient delivery and tissue distribution of suicide genes and antisense oligonucleotides and the lack of adequate methodologies to evaluate response and toxicity in clinical trials with angiogenic drugs, matrix metalloproteinase inhibitors, therapeutic antibodies, and telomerase inhibitors, are examples.

**Protein Kinase C**

In addition to the above-mentioned molecular aberrations, overexpressed or hyperactive protein kinase C (PKC) is among the most distinguished characteristics of malignant CNS tumors [18, 19]. PKC constitutes a family of serine-threonine kinases that catalyze numerous biochemical reactions critical to the function of many cellular constituents [20, 21]. Examples are the activation of topoisomerase II [22] or P-glycoprotein [14-23], for which phosphorylation by PKC is required. As a result, alterations in PKC expression or activity may significantly affect the antitumor efficacy of drugs that target the nuclear enzyme or that are substrates for the transmembrane drug efflux pump. Of note, both phenomena are believed to be involved in the chemo- and radioresistance of primary high-grade malignant brain tumors.

Situated at the crossroads of many signal transduction pathways, PKCs are also crucial to the relay of a large diversity of signals from the cytoplasm to the nucleus [24, 25]. PKCs are reversibly activated by upstream signaling elements such as growth factor receptors [26-28], and are able to reversibly activate downstream signaling modules such as the Raf-1 [29-31] and the bcl-2 cascade [32]. The Raf-1 cascade is one of the main systems for the transduction of signals through the cytoplasm [25-33]. Its overstimulation by hyperactive PKC may therefore contribute to the erroneous expression of many genes, including those that participate in cell proliferation and invasion. The involvement of PKC in the activation of the bcl-2 protein is thought to represent an important cytoprotective device against lethal stimuli [34]. This mechanism, presumably in conjunction with a diminished availability of cell-death-mediating substances, such as neutral sphingomyelinase and ceramide [34, 35], may further contribute to the reduced propensity of neoplastic glia to undergo apoptosis in response to cytotoxic agents.

Currently, the PKC family incorporates 13 isoforms, which can be classified into four subgroups on the basis of their mode of activation [20, 21] (Fig. 2). The group of classical PKCs (cPKCs) consists of the α, β, δ, and γ isoforms, all of which depend on calcium, diacylglycerol (DAG), and phosphatidylserine (PS) for activation. The isoforms that are independent of calcium but require DAG and PS, are classified as the new PKCs (nPKCs), and include the subtypes δ, ε, η, and θ. The third group of isoforms is that of the atypical PKCs (aPKCs) ζ and ζ. Their activation does not require calcium or DAG, but only PS. More recently, a fourth group of structurally distinct PKCs has been identified. Similar to the aPKCs, these so-called PKC-related kinases (PRKs 1, 2, and 3) require only PS for activation.

The distribution of the different PKC isoforms shows considerable tissue- and cell-specificity. PKCγ expression is thought to be characteristic of normal CNS tissue, while the α, δ, and ζ isoforms are encountered more abundantly in non-neuronal tissues [36]. In the rat pancreas, the β cells contain only PKCα, while certain α cells contain only PKCγ, and the δ cells only PKCε [37]. Likewise, in the human retina, PKCζ is exclusively seen in the photoreceptors, and PKCε, preferentially, in the bipolar cells [38].

The physiological basis for these observations is not clear, but may be related to tissue- and cell-specific roles of each isoform in gene expression during development and homeostasis, as well as tumorigenesis. For instance, PKCα activity levels seem to be increased in breast cancers [39] and malignant gliomas [40], but underexpressed in colon cancers [41], suggesting that this PKC isoform acts as an oncogene in the former, and as an anti-oncogene in the latter cases. However, PKCα overexpression in cultured MCF-10A nonmetastatic human breast cancer cells led to suppression of proliferation, while at the same time endowing the cells with properties consistent with a metastatic phenotype [42]. The overexpression of PKCα has also been associated with multidrug resistance [43].

On the other hand, in certain tumors, a reduction in PKCα expression merely results in growth inhibition, so that a meaningful cytotoxic effect requires initiation of the cell death program through activation of PKCδ [44, 45]. This observation, together with the cell differentiation-inducing potential of PKCδ [46], suggests that this isoform may exhibit tumor-suppressor function, at least in some cases. This assumption is consistent with the blockade of the tumor-promoting effect of phorbol myristate acetate (PMA) seen in cells depleted of PKCδ by exposure to bryostatin 1 [47].

PKCε is presumably involved in processes associated with cell differentiation and growth [48-50], as well as with mechanisms related to tumor cell invasion and metastasis [19, 51]. Also, reducing the levels of PKCε in breast cancer cells appeared to sensitize the cells to the death-promoting effects of tumor necrosis factor [52], pointing toward a cytoprotective role of this isoform. Such observations highlight not only the significance of the various
PKC isoforms, but also the complexity of the regulation of their activity.

**Structure and Activation of PKC**

All PKC isoforms contain a regulatory and a catalytic domain [21-24], each of which consists of four conserved regions (C1 to C4) and five variable regions (V1 to V5). Sequencing studies with kinases from mammals, nematodes, and yeast showed the location of the C1 and C2 regions within the regulatory domain, and of the C3 and C4 regions within the catalytic domain of the enzyme (Fig. 2).

The C1 domain of the cPKCs and the nPKCs contains two cysteine-rich zinc fingers referred to as C1a and C1b [53] (Fig. 2), the latter being involved in the binding of the cofactor DAG and phorbol esters [54]. The aPKCs contain a C1 domain with only one zinc finger, while the PRKs do not possess this structure at all [53] (Fig. 2). This explains why these isoforms do not require DAG for activation, and do not respond to treatment with PKC-activating DAG analogues such as PMA. The function of the zinc finger in the aPKCs is thus far unknown.

Close to the C1 region of the cPKCs, nPKCs, and aPKCs, a so-called pseudosubstrate is located [53] (Fig. 2). This structure can interact with the catalytic region maintaining the enzyme in its inactive form (Fig. 3). Conversion into the active form occurs when the affinity of the pseudosubstrate for the catalytic region decreases in response to the binding of DAG to the C1 region. The latter reaction takes place upon the interaction of calcium with the C2 domain, the introduction of critical conformational changes in the enzyme, and the association of membrane-bound PS with the C2 domain [55] (Fig. 3).

In addition to the binding sites for calcium and PS, the C2 domain contains portions for interaction with receptors for activated C kinase (RACKs) [56]. Together with the V5 region, RACKs probably play important roles in the translocation of activated PKCs from the cytosol to the plasma membrane [57]. RACKs can, furthermore, modulate the stability and activity of PKCs by

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Figure 2. The figure shows a comparison of the protein architecture of the various subgroups of the PKC family.
altering the degree of phosphorylation of the regulatory sites [27, 28].

It must be emphasized that the above-mentioned mechanism of activation—involving PS and calcium—only applies to the cPKCs. Whether these events also occur during activation of the non-calcium-dependent nPKCs, aPKCs, and PRKs, remains to be determined. Furthermore, following their activation and cytosol-to-membrane translocation, most PKCs bind to a membrane receptor, after which they are rapidly degraded and downregulated [58, 59]. However, certain PKC isoforms may be selectively localized to the nucleus [60], where they may mediate the transcription of genes participating in mitogenesis such as c-jun [61].

PARTICIPATION OF PKC IN SIGNAL TRANSDUCTION PATHWAYS

As mentioned above, PKCs participate in signal transduction by reversibly activating certain downstream proteins after having been activated by upstream signaling elements [26-28]. Well-studied examples of the latter are plasma-membrane-associated lipid cascades incorporating, among others, phospholipases C (PI-PLCs), phosphoinosotide 3-kinases (PI3Ks), and/or phospholipase D (PLD) [26] (Fig. 4). Their activation serves

Figure 3. Mechanism of cPKC activation. The pseudosubstrate interacts with the catalytic region, maintaining the enzyme in its inactive form. The binding of DAG to the C1 region decreases the affinity of the pseudosubstrate for the catalytic region leading to the conversion into the active form. The latter reaction takes place upon the interaction of calcium with the C2 domain, the introduction of critical conformational changes in the enzyme, and the association of membrane-bound PS with the C2 domain.

Figure 4. Participation of PKC in signal transduction pathways.
to amplify signals generated upon the binding of an agonist, such as a growth factor, to its cognate cell-surface-associated receptor [26].

Activated PI-PLCs (β, γ, and δ) can catalyze the formation of DAG and inositol 1,4,5-trisphosphate (IP3) from phosphatidyl inositol-3,4-bisphosphate (PIP2). This process promotes the release of calcium from the endoplasmic reticulum, which, together with DAG, stimulates the activation of the cPKCs and nPKCs [26, 62]. Activated PI3Ks can produce PIP2 and phosphatidyl inositol-trisphosphate (PIP3), which can contribute to signal transduction by activating the nPKCs δ, ε, and η [26, 63]. PLD can produce DAG through hydrolysis of phosphatidylcholine [64], providing a source of DAG, and thus of PKC, additional to that produced via PIP2 [65] (Fig. 4).

Once activated, PKC can transmit signals to the nucleus via one or more MAPK cascades, which may incorporate Raf-1, MEKs, and ERKs [25, 29, 30, 66]. Raf-1 can also be activated by Ras [33, 67], in some cases after crosstalk with PKC [25-30, 33, 67]. Activated ERKs can activate transcription factors such as myc, myb, max, fos, and jun [25, 33, 66], enabling the expression of genes encoding for enzymes required for key metabolic functions such as cell proliferation and invasion. Among these is the gene for ornithine decarboxylase (ODC) [68], the rate-limiting enzyme in the biosynthesis of spermine, spermidine, and putrescine [69]. These polyamines have been implicated in cell transformation [70, 71], the accelerated G1-to-S phase passage of cancer cells [72], as well as processes related to excessive extracellular matrix degradation [68, 73, 74].

ODC mRNA expression can be induced through EGF-receptor-mediated pathways which may or may not incorporate PKC. This could account for the increased ODC mRNA expression and enzyme activity, as well as the progression to malignancy, unchecked S phase transition, growth factor independence, protection against apoptosis, and invasive behavior seen in both non-glial- and glioma-derived cell lines containing amplified EGF receptor [73-75], constitutively active Ras [72], hyperactivated ERK [74, 75], and/or overexpressed PKC [69, 76, 77]. These observations are in accordance with a significant contribution of PKC in well-established signaling pathways for glioma cell proliferation and invasion.

**PKC-Inhibiting Agents: Preclinical and Clinical Studies**

Total PKC expression and activity levels in normal nervous tissue are significantly higher when compared with non-neuronal tissue [18, 78], suggesting that this enzyme system plays a fundamental role in normal CNS physiology. Indeed, PKCs have been implicated in, among other things, the regulation of astrocyte growth, the differentiation of oligodendrocytes, the prolonged potentiation of neurons, the outgrowth of axons, and the release of neurotransmitters [79, 80]. Furthermore, PKC activity levels have been found to be much higher in neoplastic astrocytes when compared with normal glia [18, 19, 78].

These observations led to the implication of inappropriately activated PKC in the development of a variety of diseases of the CNS including cancer [18, 78, 81], and to the suggestion that the use of selective inhibitors of PKC may have wide-ranging therapeutic potential. For these reasons, a large variety of structurally and mechanistically distinct anti-PKC agents have been identified or developed, some of which are briefly addressed here.

Bryostatin 1, a macrocyclic lactone originally isolated from the marine bryophyte Bugula neritina [82], is a potent PKC activator that lacks tumor-promoting activity, and that antagonizes the tumor-promoting effects of phorbol esters [83]. The mechanism of action of bryostatin 1 involves the initial activation of PKC, followed by its rapid downregulation [84]. In several murine and human tumor models, this agent displayed antitumor, and in some cases, differentiation-inducing activity also, and is now undergoing early clinical evaluation. In these studies, myalgia appeared to be the dose-limiting toxicity, and a few partial responses were documented in patients suffering from refractory malignant melanoma, non-Hodgkin’s lymphoma, or ovarian carcinoma [85-88].

The triphenylethylene, tamoxifen, is an antiestrogen that is widely used for the palliative treatment of estrogen-receptor-positive breast carcinoma [89]. Its reported efficacy against estrogen-receptor-negative cancers is thought to be due to interference with the negative growth factor, transforming growth factor β (TGF-β) [90]. Subsequent studies showed that tamoxifen was also able to inhibit the activity of many kinases including PKC [91, 92]. Following its partitioning into the plasma membrane, tamoxifen is thought to elicit the membrane association, irreversible activation, and downregulation of PKC, which, in part, may lead to cell growth inhibition [59]. The tamoxifen-mediated inhibition of PKC activity and proliferation in cultured glioma cell lines occurred at comparable kinetics [93-95]. Importantly, administration of this agent to patients with refractory high-grade malignant glioma led to some objective clinical responses [96-105] (Table 1).

The PKC-specific inhibitor safingol (L-threo-dihydrosphingosine) and the PKC modulator, tissue plasminogen activator (TPA), have shown promising effects in phase I trials. First, safingol potentiated the effect of doxorubicin in a pilot phase I study in solid tumors. Although signs of clinical activity were observed in this study, it was not possible to definitively state whether this effect was from doxorubicin or from the combination therapy [106]. In addition, TPA...
treatment increased WBC and neutrophil counts toward the normal range in previously treated patients with solid tumors and depressed WBC [107].

Staurosporine is an alkaloid produced by *Streptomyces* bacteria, and is, like tamoxifen, a broad-spectrum kinase inhibitor [108], but with greater potency [95]. In fact, staurosporine is one of the most powerful PKC inhibitors in in vitro models [109]. However, its poor kinase selectivity hampered its further development, prompting efforts to synthesize more PKC-selective analogues.

Among these are 7-hydroxystaurosporine or UCN-01 [110] and N-benzoyl-staurosporine or CGP 41251 [111], which have less PKC-inhibitory activity than the parent compound, but a higher degree of PKC selectivity when assayed for inhibition of different kinases [112, 113]. These agents are not specific for particular PKC isoforms, but they inhibit the cPKCs more potently than the nPKCs, aPKCs, and PRKs [114, 115]. Due to considerable homology among the catalytic domains of different classes of kinases, UCN-01 and CGP 41251 are also able to inhibit the activity of certain cdk5s and their partner cyclins, inducing G1 arrest and apoptosis [116]. In fact, UCN-01 can be considered primarily an inhibitor of Chk1 protein kinase [117]. These agents can also reverse the multidrug-resistance phenotype of cancer cells [14, 23] and enhance the cytotoxicity of other antitumor drugs such as DNA-damaging agents and antimetabolites [118, 119]. They displayed encouraging preclinical antitumor activity and are currently under clinical evaluation, showing promising activity against malignant melanoma and several hematological malignancies [120].

Bisindolylmaleimides such as GF 109203X represent another series of staurosporine derivatives that also showed a relatively high degree of selectivity for PKC over closely related kinases [121]. Their mechanism of action probably involves competition with ATP for interaction with PKC [122]. GF 109203X has been selected to further characterize this group of inhibitors, and this compound appeared to preferentially act on the α, βI, βII, γ, δ, and ε PKC isoforms [122]. Bisindolylmaleimides are not in clinical use, but are extensively employed as tools in the laboratory to investigate the role of PKC in signal transduction. Interestingly, GF 109203X has been reported to influence multidrug resistance in vitro mainly through the direct binding to P-glycoprotein, rather than by interfering with PKC [123].

Calphostins are perylenequinones extracted from the fungus *Cladosporium cladosporoides* and are somewhat less potent than staurosporine, but much more selective in inhibiting PKC [124]. Calphostin C (UCN-1028C) is a well-known representative of this class of inhibitors that, in contrast to GF 109203X, affects most PKC isoforms [122]. Bisindolylmaleimides are not in clinical use, but are extensively employed as tools in the laboratory to investigate the role of PKC in signal transduction. Interestingly, GF 109203X has been reported to influence multidrug resistance in vitro mainly through the direct binding to P-glycoprotein, rather than by interfering with PKC [123].

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<th>Table 1. Tamoxifen in glioma therapy</th>
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<td><strong>Cancer use</strong></td>
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<td>Refractory malignant gliomas in children [97]</td>
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<td>Anaplastic astrocytoma [98]</td>
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<td>High-grade glioma (irradiation+tamoxifen+carboplatin) [101]</td>
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<td>Glioblastoma (irradiation+tamoxifen+BCNU) [102]</td>
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<td>Brainstem gliomas (irradiation+tamoxifen) [103]</td>
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<td>Recurrent high-grade glioma [104]</td>
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<td>Recurrent glioma (tamoxifen+interferon α2a) [105]</td>
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Pts = patients; TS = mean time of survival; NS = data not shown; CR = complete remission; PR = partial remission; SD = disease stabilization; GBM = glioblastoma multiforme; AA = anaplastic astrocytoma
transport, growth inhibition, and the rapid stimulation of apoptosis [128]. Calphostin C cytotoxicity appeared to be strictly light dependent [126, 129, 130], which, together with its potent in vitro antiglioma effect [129], suggested its usefulness in photodynamic therapy of gliomas.

A novel, more specific approach to inhibit certain PKC isoforms involves the use of antisense oligonucleotides or peptide fragments to either inhibit or promote translocation of PKC isoforms to specific anchoring proteins [131-133]. Indeed, the antisense oligonucleotide ISIS 3521, an oligonucleotide of 20-mer that impedes the expression of PKCα, markedly reduced tumor proliferation and invasion in laboratory animals [134, 135]. ISIS 3521 is now undergoing clinical testing [136, 137].

PKC AND CELL PROLIFERATION

One of the hallmarks of neoplasms is their excessively high proliferation rate. This characteristic probably results from the aberrant expression of elements of signaling pathways for mitogenesis as well as those involved in mechanisms that regulate apoptosis. Since PKC is thought to play a fundamental role in both pathways [18, 34, 35, 78, 138], its overexpression may promote hyperpersistence and oppose cell death, while its inhibition, downregulation, or depletion may restrict tumorous growth and lower the apoptotic threshold for cytotoxic stimuli [139, 140].

The involvement of PKC in processes related to glioma cell proliferation is supported by the results from numerous in vitro studies. PKC activity levels correlated well with the growth rate of nontransformed murine astrocytes stimulated with serum or growth factors, fluctuated in accordance with slow, exponential, and plateau-phase cell growth, and showed increased activities in relation with increasing degree of tumor malignancy [18, 19, 54, 141-143]. Such observations led to the hypothesis that inhibition of hyperactive PKC could represent a potential therapeutic strategy against high-grade CNS malignancies. Indeed, reduction of PKC activity in cultured glioma cells using staurosporine, tamoxifen, CGP 41251, calphostin C, or specific anti-PKC antisense oligonucleotides, led to an equivalent decrease in their growth rate [91, 93-95, 109, 129-133, 144, 145]. In addition, the stimulation of cells with PMA, mitogens, or serum had the opposite effects [141-143]. These changes correlated well with corresponding alterations in cell cycle passage and abnormalities in mitogenesis [146].

These and other laboratory data prompted the initiation of clinical trials to evaluate PKC-inhibiting agents for their efficacy against refractory high-grade malignant gliomas [96-99]. Most of these studies were carried out with tamoxifen that, due to its low toxicity [89] and its availability as an oral formulation, can be given at relatively high doses (up to 400 mg daily) for various consecutive weeks [96-99]. Although tamoxifen at such schedules appeared to be of benefit in some patients, no accurate assessment could be made as to whether the observed clinical responses were actually caused by inhibition of PKC activity in neoplastic glia. It was also not possible to determine exactly whether interference with other kinases [91, 92], the estrogen receptor [89], and/or TGF-β production [90] had also contributed to the antiproliferative effect of tamoxifen.

These questions were addressed in an in vitro study with human glioma cell lines [93] where tamoxifen was found to inhibit both cell proliferation and PKC activity in a dose-dependent fashion and according to comparable kinetics without a significant involvement of estrogen-receptor- or TGF-β-associated phenomena. Moreover, the use of structurally and mechanistically distinct PKC inhibitors such as staurosporine, calphostin C, or GF 109203X instead of tamoxifen, also revealed a direct correlation between the degrees of PKC reduction and cell growth inhibition [147]. These findings emphasize the role of PKC in glioma cell growth, and support therapeutic strategies to control the proliferation of these malignancies by blocking hyperactive PKC.

Apart from restraining glioma cell proliferation, reduction of PKC activity levels may induce apoptosis in certain cell lines [34, 139, 140]. Such observations may be explained by the apparent involvement of PKC in cytoprotective mechanisms that continuously oppose default pathways leading to cell death [34, 148]. The use of PKC-inhibiting, PKC-downregulating, or PKC-depleting agents may further render cancer cells more susceptible to apoptotic stimuli, restoring their sensitivity to chemotherapeutic drugs and ionizing radiation. Noteworthy is the result that the use of staurosporine or its analogues bryo-trans-statins, calphostin C, tamoxifen, or other PKC-inhibiting agents, appeared to sensitize various nonglioma- and glioma-derived cell lines to cytotoxic drugs such as cytarabine, cisplatin, vincristine, melphalan, mitomycin C, ACNU, BCNU, as well as γ-radiation [93, 148, 149]. Notably, the concurrent administration of tamoxifen and BCNU has been found to produce durable responses in patients with recurrent grade 3 or 4 astrocytoma [150].

The cytoprotective signaling systems affected by the anti-PKC treatments have not been definitely characterized, but may be associated with loss of the MAPK response. This could be derived from the results of recent preclinical studies, demonstrating that pharmacological disruption of the MEK-ERK module can mimic the chemo- and radiopotentiating actions of PKC inhibition or downregulation [148, 151]. Efforts to translate these findings to the clinic are currently under way.
**PKC AND CELL INVASION**

The capacity to trespass biological barriers and to invade normal, surrounding tissues, is one of the most devastating characteristics of high-grade malignant gliomas. Cell invasion, including that of glioma cells, is a multistep process that involves: the degradation of basement membranes and the extracellular matrix; the entrance of cancer cells into the blood circulation; survival of the cells in the bloodstream from assaults by the immune system; extravasation of the cells, and cell invasion into, proliferation in, and neovascularization of the new tissue of residence [152, 153]. Critical players in these phenomena are various diffusible growth factors, cytokines, and enzymes produced by the tumoral and stromal cells, which, among other things, can render the tumor cells more motile, destroy surrounding tissue, and promote neangiogenesis. The extracellular matrix and the newly formed microvasculature, in their turn, produce factors that can directly stimulate tumor growth and spread [152, 153].

Notably, such processes are not restricted to neoplastic cells, but are physiological events inherent to normal cells, occurring, for instance, during trophoblast implantation, involution of the mammary gland, embryological morphogenesis, as well as tissue remodeling [154, 155]. Tumor cell invasion is a pathological process that results from loss of control over these functions.

Degradation of the basement membrane is accomplished by the actions of a variety of proteolytic enzymes [156-158] (Fig. 5). Among these are the matrix metalloproteinases (MMPs), a family of at least 15 secreted and membrane-bound endopeptidases, which are probably of major relevance to the invasive capacity of glioma cells [156-158]. MMPs possess a zinc ion at their active site, and contain domains for the regulation of their secretion, latency state, substrate recognition, and association with specific inhibitors. MMP-2 (type IV collagenase or gelatinase A) and its activator, membrane-type 1 MMP (MT1-MMP), in particular, are presumed to be involved in glioma cell invasion [157-160]. In addition, MMPs, including MMP-2, seem to be essential for the outgrowth of new blood vessels into the expanding tumor [157-160].

Normally, the powerful tissue-degrading capacities of MMPs are strictly controlled at three levels. First, MMP-encoding genes are only transcribed upon stimulation by signals that recognize special ‘tag,’ represented by AP-1 or PEA-3 promotor sites situated adjacent from these genes [161]. Such a mechanism helps to prevent the expression of these genes in response to nonspecific stimuli, as is the case with certain housekeeping genes. Furthermore, MMPs are produced as proenzymes or zymogens, which must be proteolytically processed to generate the active proteases. This can be accomplished by other MMPs, such as MT1-MMP that converts latent, 72-kDa pro-MMP-2 into active, 59/62-kDa MMP-2 [156, 157, 159, 162]. Notably, MT1-MMP itself also has important proteolytic activity [159, 160]. A third mechanism to control the tissue-degrading capacity of MMPs involves their affinity for more or less specific tissue inhibitors of MMPs (TIMPs), which can render activated MMPs inactive [163, 164].

Perturbation of any of these control mechanisms favors an excess of MMPs and MT-MMPs, resulting in increased proteolytic activity of the tumor and excessive degradation.

![Figure 5. Cell invasion.](http://theoncologist.alphamedpress.org/)
of extracellular matrix components, contributing to glioma cell invasion and angiogenesis. Indeed, numerous studies have shown a close relationship between MMP mRNA expression and activity in a tumor, and its invasive and metastatic behavior and potential [156, 158, 162]. In some human cancers, a positive correlation has also been demonstrated between the degree of neoangiogenesis and the likelihood of developing metastases [165, 166]. Thus, control of MMP activity in these two different contexts is of considerable interest as a possible therapeutic target in high-grade malignant gliomas.

The administration of TIMPs is probably not suitable for such purposes because of their short half-life in vivo [163, 164]. Currently, many efforts are dedicated to the exploitation of the synthetic, low-molecular weight MMP inhibitors batimastat and marimastat as anti-invasion and angiostatic drugs [165]. These compounds have a collagen-mimicking hydroxamate structure that facilitates chelation of the zinc ion at the active site of MMPs and are able to inhibit MMPs potently and specifically. Batimastat was the first synthetic MMP inhibitor studied in humans with advanced malignancies, but its usefulness has been limited by extremely poor water solubility, which required its intraperitoneal administration as a detergent emulsion. Marimastat is a second-generation MMP inhibitor that, in contrast to batimastat, is orally available. Both these agents are in clinical trials in the U.S., Canada, and Europe.

Accumulating evidence suggests that PKC is also involved in the invasiveness of glioma cells. For instance, exposure of normal or transformed astrocytes or glioma cells to PKC-activating agents such as PMA, led to a more pronounced invasive phenotype, including an increased production of MT1-MMP and MMP-2, enhanced expression of MT1-MMP mRNA, as well as a greater capacity to penetrate through an artificial basement membrane [74, 157, 167, 168]. Inhibition of PKC activity, for instance, by staurosporine, CGP 41251, calphostin C, or GF 109203X elicited the opposite effects [74, 167, 168].

Moreover, treatment of cultured human glioblastoma cells with PMA induced the secretion of MMP-9, led to the activation of MMP-2, downregulated TIMP-1 and TIMP-2 secretion, and increased MT1-MMP on the cell surface [169]. All these effects of PMA were reversed by the addition of the PKC inhibitor Go 6983 [169]. Comparable effects were reported upon downregulation of PKC by bryostatin 1, which led to the stimulation of TIMP-1 activity, as well as a reduction in MMP production [166]. These studies suggest that interference with PKC may represent a meaningful strategy to restrict glioma cell invasion. However, no clinical trials specifically aimed at addressing this topic have been carried out thus far.

### Involvement of PKCs α and ε in Glioma Cell Proliferation and Invasion

An important issue that needs to be addressed concerns which specific PKC isoforms may be of particular relevance to glioma cell proliferation and invasion. Available data point toward the involvement of PKCs α and ε, in particular, in these phenomena. For instance, in cultured human glioma cells, PKCα appeared to be both necessary and sufficient to promote cell cycle progression when stimulated with PMA, whereas a decrease in its expression resulted in a marked reduction in cell proliferation [170]. Notably, a reduction in activity levels of this PKC isoform led to an increase in the expression of the cdk inhibitor p21WAF1/CIP1, the depletion of which by a specific antisense oligonucleotide attenuated the PKC-induced cell cycle progression [170]. Also, the malignant behavior of C6 rat glioma cells correlated well with the degree of overexpression of PKCα [143]. Furthermore, the use of the PKCε-specific antisense oligonucleotide ISIS 3521 considerably reduced the proliferation and invasion of tumors implanted in laboratory animals [83, 84].

Regarding PKCε, its expression and activity levels were consistently higher in biopsies from patients with primary malignant gliomas when compared with samples from normal glia, correlating well with the degree of malignancy of the tumor [19]. These observations are in accordance with the apparently high oncogenic potential of this PKC isoform at even moderately enhanced levels [171].

Very recent data from our laboratory provide further support for a pivotal role of PKCs α and ε in glioma cell proliferation [172]. Thus, inhibition of the in vitro growth of the U-87 MG, U-138 MG, and U-373 human glioma cell lines (which preferentially express PKCs α, ε, γ, and ζ [173]) by tamoxifen led mainly to a reduction in the activity levels of the two former isoforms, while leaving those of the latter two essentially unaffected. Of note, treatment of serum-starved cells with antisense oligonucleotides against PKCs α or ε, but not with antisense oligonucleotides against PKCs γ or ζ, abolished the stimulatory effects of exogenous EGF or PMA on the expression of the ODC gene, which, as mentioned above, probably plays an important role in glioma cell proliferation and invasion.

Combining these data with those presented in the preceding sections, the following model can be proposed for the involvement of PKCs α and ε in glioma cell proliferation and invasion (Fig. 6). Constitutively activated cell surface receptors (such as the EGF receptor or the PDGF receptor [17, 18]) that signal through PI-PLCγ/DAG, PI3K/PIP3 [26, 174, 175], or Ras [114], may cause hyperactivation of PKC as well as subsequent MAPK cascades [29, 31, 33, 74, 75, 176, 177], leading to the excessive...
Figure 6. Schematic representation of PKCs α and ε involvement in glioma cell proliferation and invasion.

formation of ODC mRNA transcripts [68, 69]. The resulting overproduction of ODC and polyamines may promote accelerated G1-S progression, unchecked DNA synthesis, and higher cell proliferation, following perturbation of checkpoint mechanisms [56-68].

Furthermore, other mechanisms acting partly through the same signaling pathways, may facilitate tumor cell invasion by stimulating MT1-MMP expression and MMP-2 activation [68, 178] and cell motility [179, 180], as well as the loss of cell-cell and cell-matrix adhesions [181]. Concomitantly, the PKCs may contribute to the increased expression of bcl-2 and bcl-xL [182, 183], as well as the overproduction of autocrine growth factors [31, 48, 174], further promoting the malignant phenotype.

Although incomplete, such a model can help to outline the involvement of these PKCs in signaling for the maintenance of various cellular functions and can help explain how their hyperactivation can contribute to tumorigenesis. The model can also contribute to our understanding of the mechanistic steps by which interference with PKCs α and ε can affect the processes in neoplastic astrocytes.

PROSPECTS

The data mentioned in this review clearly implicate PKC isoenzymes, particularly the α and ε isoforms, in glioma cell proliferation and invasion, as well as in the resistance of these cells to apoptosis-inducing stimuli. These findings provide the rationale for attempts to exploit PKC as a target for novel forms of treatment of malignant gliomas. The development of small molecules specifically directed against certain PKC isoforms, represents an important step toward such strategies. However, our incomplete understanding of the cell- and tissue-specificity of the different PKC isoforms, including the α and ε variants, may lead to unexpected and or undesired results in clinical practice. As an example, inhibition of PKC may lead to incomplete phosphorylation and activation of topoisomerase II, compromising the efficacy of concomitantly administered topoisomerase II-targeting drugs such as etoposide or anthracycline. For these reasons, the definite establishment as to whether modulation of PKC can be used for the improvement of glioma therapy represents a major challenge in the future.

It should also be taken into account that human tumors often contain multiple oncogenic mutations at the time of
diagnosis. These may occur in upstream signaling elements such as the EGF receptor and Ras, and/or in downstream elements such as Raf-1, the MAPK cascade, and ODC. In addition, neoplastic cells can activate various alternative signaling routes upon blockage of PKC. Therefore, it may be too much to expect that PKC therapies alone can be successful against high-grade malignant gliomas in the clinic. Perhaps such therapies may be studied in combination with other treatment modalities such as radiation and/or chemotherapy, or applied after surgical tumor detaching. Such approaches may help further refine our understanding of the biology and biochemistry of brain tumors, enabling us to develop new therapeutic opportunities against this disease.

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30 PKC in Primary Malignant Brain Tumors


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