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Protein kinase C isozymes as therapeutic targets for treatment of human cancers

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Introduction

Protein kinase C (PKC; EC 2.7.11.13) was originally identified as a calcium-activated serine/threonine kinase that was dependent upon phospholipid and diacylglycerol (DAG) for full activity. The PKC gene family comprises 10 structurally related phospholipid-dependent serine/threonine protein kinases whose catalytic activity can be regulated by phosphorylation, interaction with calcium and lipid-derived co-factors, and through specific protein-protein interactions. The PKC enzyme family is divided into three subgroups: the conventional, calcium-dependent cPKCs [α (α), β I (β I), β II (β II), and γ (γ)]; the novel nPKCs [δ (δ), ϵ (ϵ), η (η) and θ (θ)]; and the atypical aPKCs [ζ (ζ) and ι (ι), also known as λ (λ) in mice]. These groupings are based on the presence or absence of functional domains that confer isozyme-specific co-factor and activator requirements. Conventional PKCs are calcium, DAG and phosphatidyl-serine dependent due to the presence of conserved modular C1 and C2 domains within the regulatory region of the enzyme. Novel PKCs are DAG and phosphatidyl-serine dependent but do not require calcium. Due to the unique structure of their N-terminal regulatory region, the atypical PKCs do not require calcium, DAG or phosphatidylserine for activation. Instead, the N-terminal regulatory region of atypical PKCs contains a Phox/Bem1 (PB1) domain that mediates homo- and hetero-typic protein-protein interactions that are critical for atypical PKC activation and localization (Lamark *et al.*, 2003). All of the PKC isozymes are the products of independent genes, except PKC β I and PKC β II which are splice variants of the PKC β gene that result from the inclusion of alternative final exons encoding the last ~50 amino acids of the protein (Kubo *et al.*, 1987).

Biochemical and immunologic studies indicate that multiple PKC isozymes are expressed in virtually all cell and tissue types. The expression of individual PKC isozymes is developmentally regulated and is responsive to the differentiation state of the cell. For these reasons, PKC isozymes are thought to fulfill distinct, often non-redundant functions in cells.

Similar activator requirements and substrate specificities *in vitro* have made identification of physiologically relevant, isozyme-specific substrates and functions difficult. The ability to genetically manipulate the expression level of specific PKC isozymes and to express mutant forms of individual PKC isozymes with altered kinase activity have allowed greater success in identification of PKC isozyme-specific functions. Using these genetic techniques, many laboratories, including our own, have demonstrated isozyme- and cell type-specific roles for

PKC isozymes in cellular proliferation, differentiation, apoptosis and cell polarity. The discovery that PKC is a cellular receptor for the tumor-promoting phorbol esters led to an intense interest in the role of individual PKC isozymes in cancer development. Indeed, the expression level and cellular localization of individual PKC isozymes has been shown to be regulated during carcinogenesis in numerous tissue types. However, despite exhaustive investigation, no somatic or germline mutations have been found in the coding regions of any PKCs associated with human cancers or other diseases, and to date, only one PKC isozyme, atypical PKC ϵ , been shown to satisfy the criteria of a human oncogene. This report summarizes work from our laboratory to characterize PKC isozyme-specific functions in human cancer and puts this work in the context of the relevant literature. We have also included a discussion of the identification and characterization of two PKC isozyme-selective inhibitors that are currently being evaluated clinically for their therapeutic potential in the treatment of cancer.

PKC Isozyme-specific Functions in Cancer

The Human Leukemia Model

In initial studies to evaluate PKC isozyme-specific functions, we utilized human leukemia cell lines as a model system. The human chronic myelogenous leukemia (K562) cell line expresses two major conventional PKCs, PKC α and PKC β II, that serve distinct functions in these cells (Hocevar *et al.*, 1992). K562 cells can be induced to cease dividing and to differentiate to megakaryocytes by treatment with PMA (Hocevar *et al.*, 1992). Expression of PKC α , but not PKC β II, correlates with PMA-induced cytostasis and megakaryocytic differentiation and over-expression of PKC α induced cytostasis in K562 cells (Murray *et al.*, 1993). Others groups have reported a similar role for PKC α in leukemia cell differentiation (Nagata *et al.*, 1996; Dieter and Schwende, 2000). Expression of chimeric PKC molecules in which the regulatory and catalytic domains of PKC α and PKC β II are exchanged established that the effects of PKC α on differentiation and cytostasis are mediated by isozyme-specific sequences within the catalytic domain of the PKC α (Walker *et al.*, 1995).

In contrast to PKC α , expression of PKC β II is required for K562 cell proliferation (Murray *et al.*, 1993). PKC β II selectively translocates to the nuclear membrane of these cells where it phosphorylates the nuclear envelope protein, lamin B (Hocevar and Fields, 1991). Further studies revealed that PKC β II is a physiologic mitotic lamin kinase that is required for nuclear envelope breakdown and G2/M phase transition (Goss *et al.*, 1994; Thompson and Fields, 1996). Nuclear translocation and lamin B phosphorylation is specific to the PKC β II isozyme in this cellular context, and selective nuclear translocation and activation of PKC β II is conferred by the extreme carboxyl-terminal 13 amino acids of PKC β II (Gokmen-Polar and Fields, 1998).

PKC ϵ , an atypical PKC, is also highly expressed in K562 cells (Murray and Fields, 1997). Despite a high level of homology with the other atypical PKC, PKC ζ (72% homology at the amino acid level), we and others have determined that these isozymes play distinct, non-overlapping roles in most, if not all systems studied (Selbie *et al.*, 1993; Murray and Fields, 1997; Leitges *et al.*, 2001; Soloff *et al.*, 2004). By genetically altering the expression and activity of PKC ϵ , we identified a critical role for PKC ϵ in human K562 leukemia cells (Murray and Fields, 1997; Jamieson *et al.*, 1999). K562 cells are highly resistant to chemotherapeutic drugs, such as taxol, that induce cell death by apoptosis. This resistance is mediated by the chimeric tyrosine kinase oncogene Bcr-Abl, the transforming activity that causes chronic myelogenous leukemia (Bedi *et al.*, 1995). Over-expression or inhibition of expression of PKC ϵ in K562 cells has no significant effect on the proliferative capacity or the sensitivity to PMA-induced cytostasis and megakaryocytic differentiation, indicating that PKC ϵ does not play a critical role in these processes (Murray and Fields, 1997). Rather, PKC ϵ serves to protect K562 cells against chemotherapeutic drug-induced apoptosis (Murray and Fields, 1997). Over-

expression of PKC ζ protects K562 cells against both okadaic acid- and taxol-induced apoptosis, whereas inhibition of PKC ζ expression sensitizes cells to induction of apoptosis, indicating that PKC ζ plays a general protective role against apoptotic stimuli (Murray and Fields, 1997). Overexpression of PKC ζ has no protective effect against apoptosis, demonstrating that the anti-apoptotic effect is isozyme-specific (Murray and Fields, 1997). Treatment of K562 cells with taxol leads to a strong, sustained activation of PKC ζ in K562 cells (Jamieson *et al.*, 1999). In contrast, Bcr-Abl-negative, myeloid leukemia (HL60) cells, which are sensitive to taxol-induced apoptosis, exhibit only weak and transient PKC ζ activation in response to taxol treatment (Jamieson *et al.*, 1999). Mutational analysis of the human PKC ζ promoter reveals that Bcr-Abl regulates PKC ζ expression through the MAPK/ERK kinase- (MEK; EC 2.7.12.2) dependent activation of an Elk1 element within the proximal PKC ζ promoter (Gustafson *et al.*, 2003). Expression of a constitutively active mutant of PKC ζ (caPKC ζ) protects K562 cells from taxol-induced apoptosis in the presence of a Bcr-Abl inhibitor (Jamieson *et al.*, 1999). Taken together, these results demonstrated that both Bcr-Abl and PKC ζ activity are necessary for apoptotic resistance in K562 cells and that PKC ζ is a critical downstream target of Bcr-Abl that is sufficient to mediate the anti-apoptotic effects of Bcr-Abl.

PKC ζ mediates resistance to taxol-induced apoptosis downstream of Bcr-Abl through a mechanism involving nuclear factor- κ B (NF- κ B). Taxol weakly induces I κ B α proteolysis and NF- κ B nuclear translocation in K562 cells, but potently induces its transcriptional activity (Lu *et al.*, 2001). Inhibition of NF- κ B activity (by blocking I κ B α degradation) significantly sensitizes cells to taxol-induced apoptosis (Lu *et al.*, 2001). Likewise, expression of antisense PKC ζ mRNA or kdPKC ζ in K562 cells sensitizes cells to taxol, and this effect can be overcome by overexpression of NF- κ B (Lu *et al.*, 2001). Expression of caPKC ζ upregulates NF- κ B transactivation and rescued cells from apoptosis induced by inhibition of Bcr-Abl (Lu *et al.*, 2001). Taxol potently trans-activates RelA-dependent DNA binding and transcriptional activity (Lu *et al.*, 2001) and this trans-activation can be further upregulated by expression of caPKC ζ , and inhibited by expression of kdPKC ζ (Lu *et al.*, 2001). Taken together, these results demonstrate that RelA trans-activation is an important downstream target of the PKC ζ -mediated Bcr-Abl signaling pathway and is required for resistance to taxol-induced apoptosis in K562 cells.

Colon Cancer Models

Our laboratory has utilized complimentary cell-based and genetic animal models to investigate the functional role of PKC isozymes in intestinal epithelial cell homeostasis and colon carcinogenesis. PKC α mRNA abundance and protein expression is dramatically reduced in early preneoplastic lesions, aberrant crypt foci (ACF), induced by the colon-specific carcinogen azoxymethane (AOM) (Gokmen-Polar *et al.*, 2001). The reduction in PKC α expression is sustained and progressive throughout the carcinogenic process, such that PKC α expression is almost completely lost in AOM-induced colon tumors (Gokmen-Polar *et al.*, 2001). Others have observed a similarly dramatic downregulation of PKC α in intestinal tumors of *APC*^{Min/+} mice, a model for familial colon cancer (Oster and Leitges, 2006). In addition, genetic knockout of PKC α increases tumor formation and decreases survival of *APC*^{Min/+} mice (Oster and Leitges, 2006). These data demonstrate that, similar to its role in leukemia cells, PKC α does not promote tumorigenesis in the colon but rather functions as a tumor suppressor. This conclusion is further supported by *in vitro* studies demonstrating a role for PKC α in growth suppression and enhanced differentiation in intestinal epithelial and colon cancer cell lines (Scaglione-Sewell *et al.*, 1998; Guan *et al.*, 2007).

In contrast to PKC α , PKC β II mRNA abundance and protein expression is highly induced in ACF and subsequent colon tumors in AOM-treated mice (Gokmen-Polar *et al.*, 2001). PKC β expression is also induced in intestinal tumors in *APC*^{Min/+} mice (Oster and Leitges, 2006).

Transgenic mice that express elevated PKC β II in the colonic epithelium (transgenic PKC β II mice) are more susceptible to colon cancer than their non-transgenic littermates (Murray *et al.*, 1999). On the other hand, PKC β knockout mice are resistant to AOM-mediated colon carcinogenesis (Liu *et al.*, 2004). Bi-transgenic PKC β KO/PKC β II mice (which express PKC β II only in the colonic epithelium) exhibit sensitivity to AOM-mediated colon carcinogenesis indistinguishable from non-transgenic mice (Liu *et al.*, 2004) demonstrating that expression of PKC β II in the colonic epithelium is required for colon carcinogenesis.

The cancer-prone phenotype of transgenic PKC β II mice results, at least in part, from PKC β II-mediated hyper-proliferation of the colonic epithelium, as characterized by an increased proliferative index and resultant hypercellularity of the colonic crypts (Murray *et al.*, 1999; Murray *et al.*, 2002). This hyper-proliferative phenotype is caused by PKC β II-mediated activation of several pro-carcinogenic/proliferative signaling pathways in the intestinal epithelium. The Wnt/APC/ β -catenin proliferative signaling pathway is a key regulator of colon carcinogenesis in mice and humans (Su *et al.*, 1992; Pennisi, 1998; Takahashi *et al.*, 2000). Transgenic PKC β II mice exhibit reduced GSK-3 β (EC 2.7.11.26) activity and increased β -catenin protein expression in the colonic epithelium (Murray *et al.*, 1999). These data indicate that PKC β II induces colonic epithelial hyper-proliferation via activation of the Wnt/APC/ β -catenin signaling pathway.

To further investigate the molecular mechanisms underlying PKC β II-mediated carcinogenesis, we generated and analyzed non-transformed rat intestinal epithelial (RIE) cell lines that over-express PKC β II (RIE/PKC β II) (Yu *et al.*, 2003). Unlike parental RIE cells, RIE/PKC β II cells exhibit a highly invasive phenotype (Zhang *et al.*, 2004). PKC β II-induced invasion in RIE cells is blocked by the PKC β -selective inhibitor LY379196, as well as by expression of a kinase-deficient allele of PKC β II, demonstrating the requirement for PKC β II activity for invasion (Zhang *et al.*, 2004). PKC β II-induced invasion is mediated through a Ras $\rightarrow$ PKC α /Rac1 $\rightarrow$ MEK signaling pathway, indicating that elevated expression of PKC β II early in colon carcinogenesis may represent a phenocopy of an activated *Ras* allele (Zhang *et al.*, 2004) and can stimulate the invasive properties associated with Ras-transformed cells.

In addition, PKC β II represses transforming growth factor beta receptor type II (TGF- β RII) expression in transgenic PKC β II mice *in vivo* and in intestinal epithelial cells *in vitro*, resulting in reduced sensitivity to TGF- β -mediated growth inhibition (Murray *et al.*, 2002). In contrast, PKC β II induces the expression of pro-carcinogenic cyclooxygenase type 2 (Cox-2) in RIE cells *in vitro* and in the colonic epithelium of transgenic PKC β II mice *in vivo* (Yu *et al.*, 2003). Celecoxib, a selective Cox-2 inhibitor, and inhibitor of colon carcinogenesis, restores expression of TGF- β RII in RIE/PKC β II cells *in vitro* and in the colonic epithelium of transgenic PKC β II mice *in vivo* (Yu *et al.*, 2003). Thus PKC β II promotes colon cancer, in part, through induction of Cox-2, suppression of TGF- β signaling, and establishment of a TGF- β -resistant, hyper-proliferative state in the colonic epithelium. These studies defined a pro-carcinogenic PKC β II $\rightarrow$ Cox-2 $\rightarrow$ TGF- β signaling axis within the colonic epithelium, and provided a plausible molecular mechanism by which non-steroidal anti-inflammatory agents such as Celecoxib suppress colon carcinogenesis.

Diet is a major risk factor for colon cancer development both in humans and mice. We and others have shown that a diet high in colon cancer-promotive ω -6 fatty acids increases membrane-associated PKC β II (Davidson *et al.*, 2000; Murray *et al.*, 2002; Jalili *et al.*, 2003). In contrast, chemopreventive ω -3 fatty acids, which inhibit AOM-induced colon carcinogenesis *in vivo* (Reddy and Maeura, 1984; Reddy and Maruyama, 1986; Nelson *et al.*, 1988; Reddy and Sugie, 1988; Deschner *et al.*, 1990; Reddy *et al.*, 1991), can directly inhibit PKC β II activity *in vitro* (Holian and Nelson, 1992; Davidson *et al.*, 2000; Seung Kim *et al.*, 2001). Furthermore, we have shown that chemopreventive ω -3 fatty acids inhibit colonic

PKC β II activity *in vivo* and block PKC β II-mediated hyper-proliferation and enhanced colon carcinogenesis in transgenic PKC β II mice (Murray *et al.*, 2002). Chemopreventive ω -3 fatty acids also inhibit PKC β II-mediated induction of Cox-2 protein expression and repression of TGF- β RII expression in RIE/PKC β II cells (Murray *et al.*, 2002). Thus, our studies demonstrate that PKC β II is not only an important colon cancer gene, but also an important target for cancer risk modulation by chemopreventive dietary fatty acids *in vivo* (Murray *et al.*, 2002). PKC β II drives key aspects of colon carcinogenesis through activation of several major signaling pathways involved in proliferation, invasion and resistance to TGF β -mediated growth inhibition (Figure 1). PKC β II is an important mediator of environmental, particularly dietary, effects on colon cancer and is therefore, is an ideal target for chemopreventive drug therapy.

Like PKC β II, PKC ι expression is elevated in human colon cancer and AOM-induced colon tumors in mice (Murray *et al.*, 2004). PKC ι expression is also induced in intestinal tumors in *APC^{Min/+}* mice compared to normal intestinal epithelium (Oster and Leitges, 2006), suggesting that PKC ι may play an important role in colon carcinogenesis. To test this hypothesis, we generated transgenic mice that express either a constitutively active PKC ι allele (caPKC ι) or a dominant negative, kinase-deficient PKC ι allele (kdPKC ι) in the colonic epithelium. Expression of either PKC ι mutant has no demonstrable effect on proliferation or differentiation of the colonic epithelium (Murray *et al.*, 2004). However, expression of caPKC ι in the colon increases susceptibility to AOM-mediated ACF, while expression of kdPKC ι significantly inhibits AOM-induced ACF formation (Murray *et al.*, 2004). caPKC ι mice also exhibit an increase in the number of AOM-induced colon tumors, and an increase in progression of these tumors from benign adenoma to malignant intramucosal carcinoma (Murray *et al.*, 2004).

Ras signaling is thought to play an important role in colon carcinogenesis. Oncogenic *Ras* mutations are detected in ~30% of colon cancers (Slattery *et al.*, 2001; Takayama *et al.*, 2001) and atypical PKCs have been implicated in oncogenic *Ras* signaling (Uberall *et al.*, 1999; Kampfer *et al.*, 2001). Therefore, we investigated the role of PKC ι in oncogenic *Ras*-mediated transformation of RIE cells. We found that oncogenic *Ras* activates PKC ι activity in RIE cells and that expression of kdPKC ι inhibits oncogenic *Ras*-mediated invasion and anchorage-independent growth (Murray *et al.*, 2004). Rac1 is activated by oncogenic *Ras* and is required for *Ras*-mediated transformation (Qiu *et al.*, 1995; Murray *et al.*, 2004). kdPKC ι blocks oncogenic *Ras*-mediated Rac1 activation, and expression of a constitutively active Rac1 allele, RacV12, overcomes dnPKC ι -mediated inhibition of invasion (Murray *et al.*, 2004). Thus, oncogenic *Ras*-mediated cellular invasion is dependent on both Rac1 and PKC ι . Interestingly, expression of either wildtype PKC ι or caPKC ι in the absence of oncogenic *Ras* fails to induce invasion or alter anchorage-dependent or independent growth, indicating that PKC ι is necessary for *Ras*-mediated invasion, but is not sufficient to induce transformation in the absence of oncogenic *Ras* (Murray *et al.*, 2004). Finally, in an *in vivo* model of *K-ras*-mediated colon carcinogenesis, the *K-ras^{LA2}* mouse, expression of kdPKC ι in the colonic epithelium inhibits *K-ras*-mediated ACF formation (Murray *et al.*, 2004). Thus, PKC ι is required for oncogenic *Ras*-mediated transformation of the intestinal epithelium *in vitro* and *in vivo*.

PKC ι is a Human Lung Cancer Oncogene

In addition to its promotive role in colon cancer, PKC ι has also been implicated as a downstream effector of oncogenic *K-ras* in lung cancer (Regala *et al.*, 2005a). PKC ι , but not PKC ζ , is highly expressed in non-small cell lung cancer (NSCLC) cells (Regala *et al.*, 2005a). Consistent with our observations in human leukemic and intestinal epithelial cell lines, expression of kdPKC ι has no effect on adherent growth or survival in NSCLC cells (Regala *et al.*, 2005a). However, expression of kdPKC ι in NSCLC cells blocks transformed (anchorage-independent) growth *in vitro* and tumorigenicity *in vivo* (Regala *et al.*, 2005a). Likewise, we have shown

that Rac1 is a critical downstream target of PKC ϵ -dependent transformation of NSCLC cells *in vitro* and *in vivo* (Regala *et al.*, 2005a). Expression of the PB1 domain of PKC ϵ blocks PKC ϵ -dependent Rac1 activation and inhibits the transformed growth of NSCLC cells *in vitro* (Regala *et al.*, 2005a) demonstrating the importance of the PB1 domain in PKC ϵ -dependent transformation of NSCLC cells. Thus, PKC ϵ plays a critical role in the transformed growth, invasion and survival of cancer cells through several oncogenic signaling pathways (Figure 2).

Our finding that PKC ϵ signaling is required for transformed growth of human lung cancer cells led us to investigate the functional significance of PKC ϵ in human lung cancers. We observed that PKC ϵ (but not PKC ζ) is highly over-expressed at both the mRNA and protein level in the majority (~70%) of primary NSCLC tumors (Regala *et al.*, 2005b). Furthermore, the level of PKC ϵ expression in tumors is highly predictive of poor patient survival independent of tumor stage (Regala *et al.*, 2005b). Thus, patients with early stage lung cancer expressing high PKC ϵ levels are significantly more likely to die from their disease than those with low PKC ϵ expression. Given the prevalence of PKC ϵ over-expression in NSCLC tumors, we investigated the mechanisms responsible for elevated PKC ϵ expression in NSCLC. The PKC ϵ gene resides at chromosome 3q26, one of the most frequently amplified chromosomal regions in NSCLC, particularly in lung squamous cell carcinoma (LSCC) (Brass *et al.*, 1996). Therefore, we assessed PKC ϵ gene copy number in LSCC tumors. We determined that the PKC ϵ gene is frequently amplified in LSCC tumors (~70%) and that gene amplification drives PKC ϵ expression in these tumors (Regala *et al.*, 2005b). Disruption of PKC ϵ signaling by expression of dnPKC ϵ inhibits the transformed phenotype in human NSCLC cells with PKC ϵ gene amplification demonstrating that PKC ϵ is a critical target of amplification of chromosomal region 3q26 in lung cancer (Regala *et al.*, 2005b). These data demonstrate that PKC ϵ is a human oncogene in NSCLC. More recently, PKC ϵ has also been identified as a potential oncogene in ovarian cancer indicating that chromosomal instability and genomic amplification of PKC ϵ may be a common feature of numerous tumor types (Zhang *et al.*, 2006). Genomic amplification is not the only mechanism by which PKC ϵ is over-expressed in NSCLC, however, since PKC ϵ is also highly expressed in lung adenocarcinoma tumors which do not harbor PKC ϵ gene amplification (Regala *et al.*, 2005b). We are currently investigating mechanisms of oncogenic PKC ϵ activation of PKC ϵ in lung adenocarcinomas. These data are the first to demonstrate that any PKC isozyme is a bonafide human oncogene that is a specific target of tumor-specific genetic alteration and activation.

PKC Isozymes as Targets for Chemotherapy

Multiple PKC isozymes have been the target for therapeutic drug development for treatment of cancer. The first isozyme-selective PKC inhibitor to reach the clinic as a potential cancer therapeutic targeted PKC α . A 20-base antisense oligonucleotide directed against PKC α (LY900003/Aprinocarsen) inhibits PKC α expression and exhibits anti-tumor activity in various human cancer cell xenografts in nude mice (Dean *et al.*, 1996; Geiger *et al.*, 1998). However, no clinical benefit was observed in phase II clinical trials using Aprinocarsen as a single agent against ovarian cancer (Advani *et al.*, 2004) or high grade glioma (Grossman *et al.*, 2005). Likewise, a large phase III clinical trial using Aprinocarsen in combination with gemcitabine and cisplatin to treat advanced stage non-small cell lung cancer observed no survival benefit or difference in response rate (Paz-Ares *et al.*, 2006). Therefore, despite encouraging pre-clinical data, PKC α has not yet been shown to be a successful target of cancer therapeutics. It is unclear whether the failure of Aprinocarsen in the clinical setting is due to an inability of the drug to sufficiently inhibit PKC α *in vivo* or relates to the relative importance of PKC α as a tumor promoting gene in the target cancers. In this regard, our studies and others in leukemia and colon cancer indicate that PKC α suppresses tumorigenesis, rather than promoting it, calling into question the rationale for PKC α inhibitors in cancer therapy.

A Small Molecule PKC β Inhibitor Shows Promise in Some Forms of Human Cancer

Enzastaurin (LY317615) is a macrocyclic bisindolylmaleimide compound that exhibits PKC β -selective inhibition in an ATP-competitive manner (Ishii *et al.*, 1996; Jirousek *et al.*, 1996). Enzastaurin is one of a series of PKC β -selective inhibitors produced and characterized by Lilly Research Laboratories. LY379196 (Jirousek *et al.*, 1996), a closely related compound to Enzastaurin, blocks PKC β II-mediated cellular invasion (Zhang *et al.*, 2004) and inhibition of TGF- β RII expression (Murray *et al.*, 2002) in rat intestinal epithelial cells and similar results have been obtained with Enzastaurin (unpublished observations). In addition, Enzastaurin blocks PKC β II-mediated gene expression in human colon cancer cells (Liu *et al.*, 2004). Preclinical studies in human tumor xenografts in nude mice have shown that oral administration of Enzastaurin inhibits tumor cell proliferation, induces apoptosis, and decreases plasma VEGF levels and intratumoral vessel formation (Keyes *et al.*, 2004; Graff *et al.*, 2005). A phase I clinical trial demonstrated that Enzastaurin is well tolerated with few toxic side effects (Carducci *et al.*, 2006). Phase II clinical trials of Enzastaurin have shown promising results in treatment of diffuse large B-cell lymphomas and in high grade gliomas (Fine, 2005; Robertson *et al.*, 2007). A phase III clinical trial of Enzastaurin for treatment of recurrent glioblastoma is currently ongoing.

Therapeutic Targeting of Oncogenic PKC ι Signaling

The fact that PKC ι is a bonafide oncogene in human lung cancer led us to explore whether PKC ι is a tractable drug target. Rather than targeting the catalytic domain of PKC ι , we chose to target the PB1 domain, which plays an important role in oncogenic PKC ι signaling (Regala *et al.*, 2005a). A novel fluorescence resonance energy transfer (FRET) based assay was developed and used to identify compounds in a commercial compound library that disrupt the PB1-PB1 domain interaction between PKC ι and its binding partner Par6 (Stallings-Mann *et al.*, 2006). The most potent compounds identified in this screen were the gold salts aurothioglucose (ATG) and aurothiomalate (ATM). These compounds are FDA approved for clinical use for the treatment of rheumatoid arthritis, and ATM is still clinically available for this indication. These compounds effectively inhibit PKC ι /Par6 interactions *in vitro* with an IC₅₀ of $\sim 1\mu\text{M}$, block PKC ι -dependent signaling to Rac1, and inhibit the transformed growth of NSCLC cells (Stallings-Mann *et al.*, 2006). Inhibition of transformed growth is relieved by expression of RacV12, consistent with a mechanism of action at the level of the interaction between PKC ι and Par6, which couples PKC ι to Rac1 (Stallings-Mann *et al.*, 2006). ATM is a highly selective inhibitor of PB1-PB1 domain interactions between PKC ι and the two adaptor molecules Par6 and p62, having no appreciable inhibitory effect on other PB1-PB1 domain interactions including p62-p62, p62-NBR1, and MEKK3-MEK5 interactions (Erdogan *et al.*, 2006). Structural analysis, site-directed mutagenesis and molecular modeling studies demonstrate that ATM targets a unique cysteine residue, Cys69, within the PB1 domain of PKC ι to exert its inhibitory effect on Par6 binding (Erdogan *et al.*, 2006). Cys69 resides within the OPCA motif of the PKC ι PB1 domain within the binding interface between PKC ι and Par6 where it interacts with Arg28 on Par6. Molecular modeling predicts that a cysteinyl-aurothiomalate adduct at C69 protrudes into the binding cleft normally occupied by Par6, providing a plausible structural explanation for ATM-mediated inhibition of Par6 binding. Mutation of Cys69 of PKC ι to isoleucine or valine, residues frequently found at this position in other PB1 domains, has little or no effect on the affinity of the resultant PKC ι mutant for Par6 but confers resistance to ATM-mediated inhibition of Par6 binding. Finally, expression of the PKC ι C69I mutant NSCLC cells has no effect on transformed growth, but confers resistance to the inhibitory effects of ATM on transformed growth (Erdogan *et al.*, 2006). Thus, ATM inhibits cellular transformation by selectively targeting Cys69 within the PB1 domain

of PKC ϵ and is a potent and effective anti-tumor agent in preclinical models of NSCLC. We are currently conducting a phase I dose escalation clinical trial of ATM in patients with advanced stage NSCLC to establish a tolerated dosing regimen of this compound.

Summary

PKC isozymes play specific, non-redundant functional roles in numerous cellular processes, including proliferation, differentiation, cellular invasion and apoptosis. We have determined that PKC β II and PKC ϵ are both critical pro-carcinogenic genes involved in multiple human cancers. PKC ϵ is a bonafide human oncogene, the first PKC isozyme that can be so classified. Both PKC β II and PKC ϵ are legitimate therapeutic targets to which novel targeted therapy has been successfully developed and is being evaluated clinically.

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Figure 1. PKC β II activates multiple pro-carcinogenic signal transduction pathways

PKC β II activates the Wnt/APC/ β -catenin proliferative signaling pathway by inactivating GSK-3 β and increasing the levels of β -catenin (Murray *et al.*, 1999). PKC β II also induces resistance to the growth inhibitory effects of TGF β via a PKC β II $\rightarrow$ Cox-2 $\rightarrow$ TGF β RII signaling pathway *in vitro* and *in vivo* (Murray *et al.*, 2002; Yu *et al.*, 2003). Finally, PKC β II induces Ras activation thereby inducing an invasive phenotype *in vitro* (Zhang *et al.*, 2004).

Figure 2. PKC ι is an oncogene that activates multiple signaling pathways critical for transformed growth, invasion and survival of human cancer cells

PKC ι resides in several major signaling pathways implicated in human cancer. Many components of these pathways, including PKC ι itself are oncogenically activated, often by multiple mechanisms (i.e. gene amplification and somatic mutation), in human tumors (indicated by shaded boxes). Arrows indicate flow through signaling pathways; touching boxes indicate direct binding of signaling components. Phosphorylation events are indicated by circled Ps.