Targeting the oncogenic protein kinase \( \zeta \) signalling pathway for the treatment of cancer

A.P. Fields, L.A. Frederick and R.P. Regala
Department of Cancer Biology, Mayo Clinic College of Medicine, Jacksonville, FL 32224, U.S.A.

Abstract
PKC (protein kinase C) isoenzymes are key signalling components involved in the regulation of normal cell proliferation, differentiation, polarity and survival. The aberrant regulation of PKC isoenzymes has been implicated in the development of many human diseases including cancer [Fields and Gustafson (2003) Methods Mol. Biol. 233, 519–537]. To date, however, only one PKC isoenzyme, the aPKC (atypical PKC), has been identified as a human oncogene [Regala, Weems, Jamieson, Khoor, Edell, Lohse and Fields (2005) Cancer Res. 65, 8905–8911]. PKCs has also proven to be a useful prognostic marker and legitimate target for the development of novel pharmacological agents for the treatment of cancer. The PKC gene resides at chromosome 3q26 and is a frequent target of tumour-specific gene amplification in multiple forms of human cancer. PKC gene amplification in turn drives PKC overexpression in these cancers. Genetic disruption of PKC expression blocks multiple aspects of the transformed phenotype of human cancer cells including transformed growth in soft agar, invasion through Matrigel and growth of subcutaneous tumours in nude mice. Genetic dissection of oncogenic PKC signalling mechanisms demonstrates that PKC drives transformed growth by activating a PKC \( \rightarrow \) Rac1 \( \rightarrow \) PAK (p21-activated kinase) \( \rightarrow \) MEK (mitogen-activated protein kinase) \( \rightarrow \) ERK (extracellular-signal-regulated kinase) kinase \( \rightarrow \) signalling pathway [Regala, Weems, Jamieson, Copland, Thompson and Fields (2005) J. Biol. Chem. 280, 31109–31115]. The transforming activity of PKC requires the N-terminal PB1 (Phox-Bem1) domain of PKC, which serves to couple PKC with downstream effector molecules. Hence, there exists a strong rationale for developing novel cancer therapeutics that target the PB1 domain of PKC and thereby disrupt its interactions with effector molecules. Using a novel high-throughput drug screen, we identified compounds that can disrupt PB1-PB1 domain interactions between PKC and the adaptor molecule Par6 [Stallings-Mann, Jamieson, Regala, Weems, Murray and Fields (2006) Cancer Res. 66, 1767–1774]. Our screen identified the gold compounds ATG (aurothioglucose) and ATM (aurothiomalate) as specific inhibitors of the PB1-PB1 domain interaction between PKC and Par6 that exhibit anti-tumour activity against NSCLC (non-small-cell lung cancer) both in vitro and in vivo. Structural analysis, site-directed mutagenesis and modelling indicate that ATM specifically targets the PB1 domain of PKC to mediate its anti-tumour activity [Erdogan, Lamark, Stallings-Mann, Lee, Pellechia, Thompson, Johansen and Fields (2006) J. Biol. Chem. 281, 28450–28459]. Taken together, our recent work demonstrates that PKC signalling is required for transformed growth of human tumours and is an attractive target for development of mechanism-based cancer therapies. ATM is currently in Phase I clinical trials for the treatment of NSCLC.

Introduction
PKC (protein kinase C) isoenzymes are a diverse family of serine/threonine kinases involved in multiple intracellular signalling pathways. The PKC isoenzymes are subdivided into three main classes: the cPKCs (conventional PKCs; PKC\(\alpha\), \(\beta\), \(\beta\)I, \(\beta\)II and \(\gamma\), the nPKCs (novel PKCs; PKC\(\beta\)I, \(\epsilon\), \(\eta\) and \(\theta\)) and the aPKCs (atypical PKCs; PKC\(\zeta\) and \(\iota\)). PKC isoenzymes share a high degree of structural and functional homology within the catalytic domain. However, structural differences in the N-terminal regulatory domain of PKCs confer isoenzyme-specific differences in regulation and function.

PKCs are at a critical nexus in many signal transduction pathways and play key regulatory roles in a diverse number of cellular processes including proliferation, cell cycle control, differentiation, survival and polarity [1,2]. Deregulation of these processes mediated by distinct changes in tissue-specific expression patterns and activity of PKC isoenzymes has been linked to several types of diseases [3]. Since the discovery that PKCs are cellular receptors for the tumour-promoting phorbol esters [4,5], PKCs have been implicated in various aspects of cancer [6]. Direct evidence implicating PKC in carcinogenesis first came with the studies showing that rat fibroblasts overexpressing PKC exhibit enhanced morphological changes in response to phorbol esters. PKC-overexpressing cells exhibited anchorage-independent growth [7] and

Key words: atypical protein kinase C (aPKC); aurothioglucose; lung cancer; Park; Phox-Bem1 domain (PB1 domain); Rac1; targeted therapeutics.

Abbreviations used: ATG, aurothioglucose; ATM, aurothiomalate; CML, chronic myelogenous leukaemia; ERK, extracellular-signal-regulated kinase; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; NF-wB, nuclear factor wB; NSCLC non-small-cell lung cancer; PKC, protein kinase C; cPKC, nPKC and aPKC, conventional, novel and atypical PKC respectively; PB1 domain, Phox-Bem1 domain; SCC, squamous cell carcinoma.

To whom correspondence should be addressed (email fields.alan@mayo.edu).
enhanced tumorigenicity when inoculated into nude mice [8]. Additionally, transgenic mice overexpressing PKCβII show hyperproliferation of the colonic epithelium and are highly sensitive to AOM (azoxymethane)-induced colon cancer, and mice deficient in PKCβ are resistant to colon cancer [9]. Antisense depletion of PKCa or PKCβI in human gastric cancer blocks anchorage-independent growth and reduced tumour formation in mouse xenografts [10]. The role of PKC in cancer was solidified when the PKCs were shown as key cellular components in major oncogenic signalling pathways involving Ras, Myc and Foxs [11]. However, despite intense study, it was only in the past year that the first PKC isoenzyme was shown to be a human oncogene [12]. It is ironic that this distinction is held by an aPKC isoenzyme, PKCi, which does not bind tumour-promoting phorbol esters, the property that originally suggested a causative role for PKC in cancer.

Unlike the cPKCs and nPKCs, the aPKCs are not activated by phorbol esters, diacylglycerol, calcium or phosphatidylyserine [13,14]. Instead, aPKC activity can be regulated by 3-phosphoinositides, PDK1 (phosphoinositide-dependent protein kinase 1), tyrosine phosphorylation and specific protein–protein interactions. Direct interactions between aPKC and effector molecules are mediated through a motif within the N-terminal regulatory domain of aPKCs termed the PB1 domain (Phox-Bem1 domain), a structurally conserved domain that mediates specific homo- and hetero-interactions between PB1 domain-containing proteins [15]. The PB1 domain-containing proteins ZIP/p62 [16,17], Par6 [18–21] and MEK5 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase 5] [17,22] specifically bind aPKC via PB1–PB1 domain interactions [23,24]. These interactions have proven to be critical for the ability of aPKCs to perform their biological functions in several contexts, including oncogenesis.

**PKCi is an oncogene**

Cancer genes are defined by virtue of the fact that they (i) play a critical, indispensable role in cancer cell biology; (ii) are activated, often by overexpression, in primary tumours; and (iii) are targets for tumour-specific genetic alterations that activate the gene. PKCi is the first PKC isoenzyme shown to possess each of these properties of a human oncogene [12]. Overexpression of PKCi is found in NSCLC (non-small-cell lung cancer) cell lines, primary NSCLC tumours [12] and ovarian cancer [25,26], suggesting a role in human cancer. Interestingly, both NSCLC tumours and matched normal lung tissues express low levels of the related aPKC isoenzyme, PKCc, indicating that PKCi is the predominant aPKC in normal and malignant lung tissue and is selectively targeted during oncogenesis [12].

Like other oncogenes, PKCi has been identified as a critical target of frequent, tumour-specific somatic genetic alteration by gene amplification [12,25,26], and PKCi gene amplification is an important mechanism that drives PKCi expression in these tumours [12,25,26]. The PKCi gene resides at chromosome 3q26. In NSCLC, amplification of chromosome 3q26 is among the most frequent cytogenetic changes, occurring in approx. 20% of all NSCLCs [27,28], predominantly in SCC (squamous cell carcinoma). Tumour-specific PKCi gene amplification has also been observed in ovarian tumours [25,26]. PKCi expression and gene copy number correlate with chromosome 3q26 gains in these tumours, indicating that, as in the lung, PKCi is a relevant gene target for tumour-specific chromosome 3q26 amplification in the ovary [25,26]. The finding of PKCi gene amplification in these tumour types takes on more significance given the fact that chromosome 3q26 amplification has been shown to occur in SCC of the head and neck [29], oesophagus [30,31] and cervix [32]. Thus PKCi will probably emerge as a key oncogene in other forms of human cancer.

Gene amplification is not the only mechanism by which PKCi is overexpressed in human tumours. In LAC (lung adenocarcinoma), PKCi protein expression is elevated, despite the fact that PKCi gene amplification is rare in this tumour type [12]. PKCi is also frequently overexpressed in other tumour types that rarely harbour chromosome 3q26 amplification, including colon cancers [33], pancreatic cancers [34] and CML (chronic myelogenous leukaemia) [35]. We recently demonstrated that Bcr-Abl transcriptionally activates PKCi through a specific Elk1 element within the proximal PKCi promoter in CML cells [35]. Whether this transcriptional mechanism plays a role in the control of PKCi expression in other tumour types remains an important area of active investigation. Other potential mechanisms of PKCi regulation in tumours include post-transcriptional regulation, post-translational modifications and/or somatic mutations. In this regard, there have been no reports to date of somatic mutations within the PKCi gene in human cancers.

**PKCi is a prognostic marker in human cancer**

Elevated levels of PKCi expression correlate with poor clinical outcome in NSCLC patients [12]. Patients with high PKCi in early stage lung cancer are ten times more likely to die from their disease than those with low PKCi [12]. Interestingly, PKCi expression does not correlate with tumour stage, which is currently the best prognostic marker in NSCLC [36]. PKCi levels are consistently high in patients in early and late stages of the disease [12]. Therefore PKCi expression profiling holds particular promise as a prognostic marker to identify NSCLC patients with early stage disease who are at high risk for relapse.

Unfortunately, lung cancer is often not detected until later stages of the disease where treatments are less effective and survival rates are dramatically decreased [36]. In light of these findings, high-risk patients could be identified for more aggressive treatments, including possibly specific PKCi-targeted therapies that may prevent cancer progression and recurrence. Elevated PKCi is also frequently observed in ovarian cancer patients where it too is prognostic of poor clinical outcome [25,26,37]. In ovarian cancer, PKCi expression correlates more closely with tumour stage, suggesting that PKCi overexpression may be a later event
PKCι resides in several major signalling pathways implicated in human cancer. Many components of these pathways are mutated, often by multiple mechanisms (i.e. gene amplification and somatic mutation), in human tumours (indicated by grey boxes). Arrows indicate flow through signalling pathways; touching boxes indicate direct binding of signalling components. Phosphorylation events are indicated by circled Ps. AOM, azoxymethane; cIAP, cellular inhibitor of apoptosis protein; EGF, epidermal growth factor; EGFR, EGF receptor; HGF, hepatocyte growth factor; IκB, inhibitor of NF-κB; IκKβ, IκB kinase β; PAK, p21-activated kinase; PI3K, phosphoinositide 3-kinase.

Oncogenic PKCι signalling mechanisms
PKCι is at the hub of several major oncogenic signalling pathways (Figure 1). Herein, we discuss two key molecular aspects of oncogenic PKCι signalling.

PKCι in Ras-mediated signalling
Activating Ras mutations occur in approx. 30% of all human cancers, making it the most frequently targeted oncogene in humans [38]. Ras can activate aPKCs [39,40] and PKCι has been directly implicated in Ras-mediated signalling [41–43]. As mentioned above, the Bcr-Abl oncogene regulates PKCι expression through a Ras/MEK-dependent activation of an Elk1 element within the proximal PKCι promoter [35]. Transcriptional activation of PKCι is necessary in turn for Bcr-Abl-mediated chemoresistance in CML cells [35]. In CML cells, PKCι mediates survival through activation of the NF-κB (nuclear factor κB) pathway downstream of Ras [44]. Two downstream effector molecules of aPKCs, Rac1 and NF-κB, have been shown to be critical for oncogenic Ras-mediated transformation [45,46], suggesting that aPKC itself is required for Ras-mediated transformation. Indeed, PKCι activity is required for Ras-mediated transformation, invasion and anchorage-independent growth of intestinal epithelial cells, NSCLC cells harbouring mutant Ras, and is also critical for Ras- and carcinogen-mediated colon carcinogenesis in vivo [33,47,48]. Thus PKCι is necessary for oncogenic Ras-mediated carcinogenesis and is also critical for transformed growth of tumour cells that rely on cellular Ras.

in ovarian carcinogenesis and contribute to ovarian tumour progression [25,26,37].
PKCι PB1 domain interactions contribute to oncogenic PKCι activity
Adaptor proteins containing PB1 domains interact with the PB1 domain of PKCι and play a key role in oncogenic PKCι signalling. In NSCLC cell lines, ectopic expression of the PKCι PB1 domain blocks anchorage-independent growth in soft agar [48], indicating that the PB1 domain of PKCι is required for its oncogenic activity. Expression of the PB1 domain of PKCι disrupts transformed growth by uncoupling PKCι from Rac1 activation [48]. We have shown that Rac1 is a critical effector of oncogenic PKCι that is required for the transformed phenotype of NSCLC cells both in vitro and in vivo at least in part by activating the MEK→ERK signalling axis [48]. Par6 is a PB1 domain protein that links aPKCs to Rac1 [18–21]. Our current results suggest that Par6 may be a critical mediator of oncogenic PKCι signalling in NSCLC cells (L.A. Frederick and A.P. Fields, unpublished work). These properties suggest that targeting the PB1 domain of PKCι represents an effective strategy for disrupting oncogenic PKCι signalling.

Inhibition of PKCι signalling in cancer therapy
Given the role of the PB1 domain of PKCι in oncogenic signalling, we hypothesized that small molecules that target this domain could be of therapeutic benefit. To identify such molecules, we developed a FRET (fluorescence resonance energy transfer)-based assay to identify chemical inhibitors of PB1–PB1 domain interactions between PKCι and Par6 [49]. The drug screen identified several high-affinity compounds that block PB1 domain interactions between PKCι and Par6 including the gold compounds ATG (aurothioglucose) and ATM (aurothiomalate) [49]. Both ATG and ATM disrupt PKCι-dependent signalling to Rac1 and inhibit transformed growth of NSCLC cells in vitro and tumour formation in vivo [49]. Structural analysis and site-directed mutagenesis reveal that ATM inhibits PKCι-mediated transformation by targeting a unique cysteine residue, Cys69, within the PKCι PB1 domain [50] and that ATM is a highly selective inhibitor of PB1 domain interactions involving PKCι but not other PB1 domain-containing proteins [5]. Molecular modelling predicts that ATM interacts with Cys69 on PKCι to form a cysteinyl-ATM adduct that extends into the Par6-binding cleft within the OPCA (OPR/PC/AID) motif of PKCι leading to steric hindrance of Par6 binding (Figure 2).

Our studies to date have shown that ATM mediates its anti-tumour effects through inhibition of the PKCι–Par6–Rac1 pathway. However, the molecular mechanism of ATM inhibition predicts that ATM could also inhibit PKCι–p62 interactions in vivo [50]. Thus the possibility exists that in some tumour types, such as CML [44], the PKCι–p62–NF-κB pathway may contribute to PKCι-mediated transformation and be a target of ATM-mediated inhibition. The role of the PKCι–p62–NF-κB pathway in lung tumorigenesis is currently being investigated. ATM is currently being evaluated in Phase I clinical trials for the treatment of NSCLC.

Conclusion
Compelling experimental evidence over the past few years has shown that PKCι is a human oncogene and that the oncogenic PKCι signalling axis is a ‘druggable’ target for novel mechanism-based cancer therapy. The PKCι inhibitors ATG and ATM have shown efficacy in relevant preclinical models, including human lung cancer cell lines in vitro and human tumour xenograft models in immunodeficient mice in vivo [49]. Ongoing preclinical studies with ATM have shown that the compound is an effective anti-tumour agent
against virtually all lung cancer cell lines tested, but that these lines exhibit widely divergent sensitivity to the drug. Interestingly, increased ATM-sensitivity of human lung cancer cell lines correlates with PKC protein expression in the cell lines (R.P. Regala and A.P. Fields, unpublished work). These results suggest that tumours with highly elevated PKC expression, either through PKC gene amplification or transcriptional activation, are addicted to oncogenic PKC signalling, which may confer sensitivity to PKC-targeted therapy. These results indicate that tumour PKC expression profiling may be useful not only as a prognostic marker of disease outcome, but also as both a surrogate and a surrogate marker of response to PKC-targeted therapy such as ATM. These results will probably inform the design of Phase II clinical trials with ATM.

References


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