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Targeting Protein Kinase C subtypes in pancreatic cancer

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Summary

In preclinical studies protein kinase C (PKC) enzymes have been implicated in regulating many aspects of pancreatic cancer development and progression. However, clinical phase I or phase II trials with compounds targeting classical PKC isoforms were not successful. Recent studies implicate that mainly atypical and novel PKC enzymes regulate oncogenic signaling pathways in pancreatic cancer. Members of these two subgroups converge signaling induced by mutant Kras, growth factors and inflammatory cytokines. Different approaches for development of inhibitors for aPKC and nPKC have been described; and new compounds include allosteric inhibitors and inhibitors that block ATP binding.

Keywords

PKC; protein kinase C; inhibitor; pancreatic cancer; therapy

Quote: “Novel and atypical Protein Kinase C isoenzymes may be valuable targets for therapy”

The protein kinase C (PKC) family comprises ten members and, based on their structural components and activation mechanisms, is subdivided into three groups (Fig. 1), the classical PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC). Predominant isoforms in pancreatic cancer are the classical PKCs PKC α and PKC β , the novel PKCs PKC ϵ and PKC δ , as well as the atypical isoforms PKC ζ and PKC ι (for a comprehensive analysis on PKC isoform expression in normal pancreas, chronic pancreatitis, pancreatic cancer tissue and stroma see [1]).

In pancreatic cancer PKCs seem to regulate all aspects of tumorigenesis including initiation, progression and metastasis. However, different isoforms are not compensatory, but rather fuel into similar signaling pathways downstream of different stimuli. For example tumor necrosis factor- α (TNF) or oncogenic Kras have been shown to activate aPKCs, whereas cPKCs are mainly activated by growth factors such as epidermal growth factor (EGF) and oxidative stress. Activation of both leads to similar downstream signaling resulting i.e. in the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and signal transducer

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and activator of transcription 3 (STAT3) that control survival and metastasis in pancreatic cancer [2–6].

Their role in converging different upstream signaling pathways that drive pancreatic cancer suggests PKCs as valuable targets for drug development. A challenge when developing strategies to selectively-target different PKCs or isoforms within subgroups is the high similarity between family members in their kinase domains and ATP binding sites. Therefore, the focus switched from development of ATP-competitive inhibitors towards development of allosteric inhibitors that target activation or downstream signaling by affecting protein-protein interactions.

Approaches to target classical Protein Kinase C (cPKC) subtypes in pancreatic cancer

The subgroup of classical PKCs consists of PKC α , PKC β I, PKC β II and PKC γ . Of these, only PKC β expression was increased in pancreatic ductal carcinoma, when compared to normal ductal tissue [1]. Little is known on the role of PKC γ in pancreatic cancer, although its expression is increased in stroma adjacent to ampullary carcinomas [1]. Moreover, the role of PKC α in pancreatic cancer is somewhat controversial. While Evans *et al.* showed that in patient samples its expression is unchanged to normal controls [1], others have shown that tumorigenicity of pancreatic cancer cell lines *in vivo* is related directly to overexpression of PKC α expression. Moreover, downregulation of PKC α conferred to a dramatic survival benefit of animals [7]. As a potential mechanism, PKC α has been shown to down-regulate PTEN and to promote proliferation and metastasis of pancreatic cancer cell lines [8]. In addition, PKC α has been attributed to induce drug resistance of pancreatic cancer cells [9]. In line with this, treatment with staurosporine blocked pancreatic cancer cell proliferation and reverted Ras-mediated transformation [10]. However, there are other reports showing that activation of PKC α inhibits the proliferation of pancreatic cancer cell lines [11] and increases the expression of pro-apoptotic proteins [12].

Quote: “Compounds that target classical PKCs failed in clinical trials”

Many inhibitors of cPKC are structurally similar to staurosporine and include UCN-01, Gö6976, Bisindolylmaleimide I (BIM 1) and PKC412 (midostaurin). Other inhibitors that show some selectivity in targeting cPKC and have been promising in blocking proliferation of pancreatic cancer cell lines either alone or in combination with gemcitabine are R0-32-0432 and bryostatin-1 [13]. Although a multitude of these pan-cPKC inhibitors have been clinically tested, their combination with drugs currently-used for pancreatic cancer did not result in any benefit for patients. For example, bryostatin-1 has been combined with paclitaxel in patients with advanced metastatic pancreatic carcinoma in a phase II study, but failed to be an effective therapy [14]. Similarly, clinical trials with UCN-01 were terminated at the phase II stage. PKC412, although not been tested for pancreatic cancer, failed in phase I and II studies for metastatic melanoma and non-small-cell lung cancer.

One may argue that many of the pan-cPKC inhibitors failed in clinical trials, because they lack specificity for one of the PKC subtypes of this family. However, approaches to more selectively-inhibit individual members of the cPKC family also were not successful. For

example, aprinocarsen (LY900003), an antisense oligonucleotide inhibitor that selectively-targets the mRNA encoding PKC α failed in phase III cancer trials [15]. Similarly, enzastaurin (LY317615), a potent inhibitor with a high selectivity for PKC β (IC₅₀ of 6 nM), although it demonstrated anti-tumor activity on freshly-explanted primary pancreatic cancer specimen, was ineffective in clinical trials [16]. For example, in a clinical phase II study combination of enzastaurin with gemcitabine in locally advanced or metastatic pancreatic cancer did not improve clinical outcomes as compared to gemcitabine alone [17].

Targeting PKC ϵ , PKC δ or downstream signaling in pancreatic cancer

Novel PKCs comprise PKC ϵ , PKC δ , PKC η and PKC θ . Relatively little is known on the roles of PKC η and PKC θ in development or progression of pancreatic cancer, whereas PKC ϵ and PKC δ have been implicated in both. Both seem to regulate responses to pancreatic acinar cell injury including activation of NF- κ B and acinar cell dedifferentiation [5,6,18]. Acinar cell dedifferentiation, in context of oncogenic Kras mutations can lead to formation of PanIN lesions. In pancreatic cancer cell lines expressing mutant Kras, PKC δ and PKC ϵ regulate anchorage-independent growth [19]. Particularly, PKC δ overexpression was described for human pancreatic adenocarcinoma. Moreover, Panc-1 cells overexpressing PKC δ exhibited a high growth rate of tumors and lung metastasis in animal models [20]. PKC δ has been implicated in regulating basal autophagy which is essential for growth and survival of tumors with constitutively-active Kras including pancreatic cancer (for an overview [2]). In addition PKC δ may have a role in tumor repopulation. Dying pancreatic cancer cells after radiotherapy can act as feeder cells for living tumor cells. It was shown that this is mediated by a Caspase 3/PKC δ signaling pathway that is active in dying tumor cells [21].

The most commonly used inhibitor for PKC δ is rottlerin, a relatively unspecific compound. In pancreatic cancer cells rottlerin stimulates apoptosis and in an orthotopic model of PDA leads to a decrease in primary tumor size [22]. Recent efforts involved pharmacophore modelling and investigation of structure-activity relationships (SAR) to develop isoform specific inhibitors that prevent ATP binding to PKC δ . The inhibitor KAM1 was designed by combining structural elements of the broad spectrum protein kinase inhibitor staurosporine and domains from the rottlerin scaffold. KAM1 showed increased PKC δ -specificity, potent activity against human cancer cells expressing mutant Kras, and also was effective in *in vivo* animal models [23]. Another inhibitor, BJE6-106, with further increased potency and isoform specificity was designed on the basis of KAM1 [24]. BJE6-106 inhibits PKC δ with an IC₅₀ of 50 nM and is approximately 1000-fold selective versus PKC α . To date no ATP competitive inhibitor that is selective for PKC ϵ has been characterized. Of the more broad inhibitors of PKC, sotrastaurin may be a good option with a K_i of 3.2 nM for PKC ϵ .

In order to obtain high isoform-specific selectivity for novel PKCs, peptide antagonists may be best options. Receptors for activated C-kinase (RACK) proteins serve as specific anchoring molecules to different areas of the cell, and peptides that mimic the RACK binding site on PKC can function as selective isoform-specific inhibitors of translocation and activity *in vivo* [25]. Based on this strategy, selective antagonists for PKC δ and PKC ϵ have been developed and employed for investigating NF- κ B signaling in pancreatic cells

[5,26]. Of note, neither these peptide regulators nor other above discussed inhibitors so far have been tested in clinical trials for pancreatic cancer.

Besides direct inhibition of nPKC, another option is to inhibit downstream effector kinases. These include members of the protein kinase D (PKD) family. nPKC directly phosphorylates PKD at its activation loop leading to increased kinase activity. PKD has been shown to regulate pancreatic cancer cell proliferation and protection from apoptosis [27]. With CRT0066101, a selective inhibitor for this kinase family is available that can be orally administered and has been shown to decrease primary tumor growth in an orthotopic animal model for pancreatic cancer [28].

Quote: “Selective inhibitors for atypical and novel Protein Kinase C isoenzymes are available for clinical testing”

Roles of atypical PKCs in pancreatic cancer and approaches for targeted inhibition

Atypical PKCs are structurally and functionally distinct from other PKCs. The two members of this group, PKC ζ and PKC ι show 84% amino-acid sequence homology in their kinase domains, but are less conserved in their regulatory domains. Although both have been implicated in regulating cell polarity, cell proliferation and survival, they are not functionally redundant and cannot compensate for each other. In pancreatic cancer both PKC ζ and PKC ι are directly linked to oncogenic Kras signaling [29,30].

Of the two isoforms PKC ζ seems to be the main activator of the canonical NF- κ B pathway, and activation of NF- κ B is impaired in PKC ζ knockout mice [31]. PKC ζ activates NF- κ B downstream of TNF or lipopolysaccharide (LPS) [32]. PKC ζ expression and localization is similar in normal pancreas, pancreatic ductal adenocarcinoma cells and carcinoma tissue [33]. In pancreatic cancers PKC ζ is required for transformed growth and invasion and this is mediated by PKC ζ -induced activation of STAT3 [34]. Similar to PKC ζ , PKC ι also is required for pancreatic cancer cell transformed growth and tumorigenesis, but acts through the Rac1-MAPK pathway [30]. A comparison of PKC ι expression in two types of pancreatic neoplasia, pancreatic ductal adenocarcinomas (PDAs) and intraductal papillary mucinous neoplasms (IPMNs) indicate that high expression levels can be correlated with advanced stage of tumors and this may have prognostic value [35].

Because both aPKCs target different pathways of equal importance for tumorigenesis of PDA, pharmacological targeting with pan inhibitors may be of benefit for patients. Many of the ATP competitive aPKC inhibitors such as hydroxyphenyl-1-benzopyran-4-ones [36] and PKC ζ I257.3 [N-(4-((dimethylamino)methyl)-benzyl)-1H-pyrrole-2-carboxamide] [37] target both PKC ζ and PKC ι , but also other molecules unrelated to PKC. Other inhibitors show some isoform specificity include the thieno[2,3-d]pyrimidine-based compound CRT0066854 which was reported 4-fold more potent against PKC ι than PKC ζ [38]; and a 2-(6-phenylindazolyl)-benzimidazole derivative that shows increased inhibitory activity for PKC ζ over PKC ι [39].

A high selectivity for aPKC can be achieved by targeting protein-protein interactions. For PKC ζ highly-selective and potent allosteric inhibitors that bind to the hydrophobic motif pocket in the kinase domain have been described. These include 4-benzimidazolyl-3-phenylbutanoic acids [40] and 1,3,5-trisubstituted or 1,3,4,5-tetrasubstituted pyrazolines [41]. As a mechanism of action they block the phosphorylation of PKC ζ in its activation loop at Thr410. Other approaches use a specific inhibitory peptide that is a cell-permeable fraction of the PKC ζ pseudosubstrate domain. Another allosteric inhibitor for aPKC is the thio-gold compound aurothiomalate (ATM). ATM blocks the interaction of PKC ι with the polarity protein Par6. This is mediated by the formation of gold-cystein adducts with Cys69 on PKC ι . Since ATM also targets a similar residue in PKC ζ it also blocks interaction with p62 and activation of NF- κ B (work on ATM is summarized in [42]). A Phase I dose escalation study for ATM has been successfully-conducted for Kras-dependent cancers including pancreatic cancer [43].

Expert commentary

So far, efforts to target PKC signaling in clinical trials for pancreatic cancer have failed. Most inhibitors used in such studies target the classical PKC isoforms PKC α and PKC β . However, laboratory data of recent years suggest that mainly members of the nPKC and aPKC groups regulate signaling pathways that have been identified as drivers of pancreatic cancer. Future key targets for drug development are PKC δ , PKC ϵ , PKC ζ and PKC ι . Although the exact roles of these isoforms in pancreatic cancer development and progression as well as surrounding stroma and inflammatory environment are still ill-defined, enough preclinical data exists that would justify testing their inhibition in clinical trials. At this point it is unclear if the approach to target individual isoforms will be more successful over using compounds that inhibit multiple members of a subgroup or even both subgroups. More investigation is also needed to determine if such inhibitors can act additive or synergistically when combined with currently-used drugs or radiation therapy.

Compounds that target PKC enzymes often successfully worked in pancreatic cancer cell lines or xenograft animal models, but failed in patients. This is because of a strong desmoplastic reaction leading to a fibrotic and inflammatory environment, which blocks drugs from reaching tumor cells [44]. Different animal models have been developed to test penetration of drugs or methods to deplete the tumor stroma. In a genetic model, KPC mice express an oncogenic version of Kras (Kras^{G12D/+}) as well as a mutant form of p53 (p53^{R172H/+}) under a pancreas-specific promoter (Pdx1^{CreTG/+}). Mice resemble human disease and develop metastatic pancreatic cancers within weeks [45]. Another strategy to resemble human disease is orthotopic xenografting of patient-derived tumor tissue (patient-derived orthotopic xenograft; PDOX) into immuno-compromised mice [46,47]. This model can be enhanced by passaging tumors orthotopically in transgenic nude mice that ubiquitously express fluorescent proteins, which leads to acquisition of fluorescent stroma [48–50]. Both models have their advantages. While the KPC animal model is more defined and ideal for initial testing of new drugs, patient-derived orthotopic xenografts resemble the heterogeneity of human tumors and therefore also allow testing an individualized treatment approach tailored specific to the patient.

Five-year view

A main focus within the next five years will be on inhibiting PKC ϵ , PKC δ , PKC ζ and PKC ι in pancreatic cancer. Meanwhile, several ATP-competitive or allosteric inhibitors are available to inhibit these PKC isoforms. For most of them promising preclinical data exists and it now needs testing in clinical trials if they have the potential to halt or regress metastatic tumors in patients, either alone or in combination with currently-used drugs.

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Key-issues

- Different PKC isoforms have been implicated in pancreatic cancer
- Clinical trials targeting classical PKC isoforms were not successful
- Preclinical data indicate important functions for aPKC and nPKC in pancreatic cancer
- aPKC and nPKC drive activation of NF- κ B and STAT3
- Inhibitors for aPKC and nPKC have been tested in preclinical studies
- Inhibitors for aPKC and nPKC now need testing in clinical trials
- Combination therapy with currently-used chemotherapeutic drugs may be effective

Fig. 1. Schematic overview of the three groups of PKC enzymes

Based on their structural elements and homology, members of the PKC kinase family are subdivided into three groups. The classical PKC group (cPKC) consists of the four isoforms, PKC α , PKC β I, PKC β II, PKC γ ; the novel PKC group (nPKC) of the four isoforms, PKC η , PKC ϵ , PKC δ and PKC θ ; and the atypical PKC group (aPKC) of the two isoforms PKC ζ and PKC λ/ι . While the c-terminal catalytic domain (C3 and C4 domains) shows the highest homology between PKC isoforms, the different groups distinguish in their n-terminal regulatory domains. Classical and novel PKCs are responsive to phorbol esters (PMA) and diacylglycerol (DAG), which bind to the C1a and C1b domains. Atypical PKC enzymes have an altered C1 domain (C1') and are irresponsive to these lipid activators. Classical PKCs also have the unique feature to bind Ca²⁺ via the C2 domain, while novel PKCs have an altered C2 domain (C2') that is not responsive to Ca²⁺, but required for protein-protein interactions. Atypical PKCs lack a C2 domain and express a PB1 domain that is important for protein-protein interactions. In addition all PKC subtypes have a pseudosubstrate motif (PS) in their regulatory domain.