Biochimica et Biophysica Acta 1846 (2014) 547–559

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbacan

Review

Targeting protein kinase C in sarcoma

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ARTICLE INFO

Article history:
Received 17 June 2014
Received in revised form 19 September 2014
Accepted 8 October 2014
Available online 16 October 2014

Keywords:
Kinases
Protein kinase C
Sarcoma
Therapeutics

ABSTRACT

Protein kinase C (PKC) is a family of serine/threonine tyrosine kinases that regulate many cellular processes including division, proliferation, survival, anoikis and polarity. PKC is abundant in many human cancers and aberrant PKC signalling has been demonstrated in cancer models. On this basis, PKC has become an attractive target for small molecule inhibition within oncology drug development programmes. Sarcoma is a heterogeneous group of mesenchymal malignancies. Due to their relative insensitivity to conventional chemotherapies and the increasing recognition of the driving molecular events of sarcomagenesis, sarcoma provides an excellent platform to test novel therapeutics. In this review we provide a structure–function overview of the PKC family, the rationale for targeting these kinases in sarcoma and the state of play with regard to PKC inhibition in the clinic.

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1. Introduction

Sarcomas are a group of mesenchymal tumours that resemble connective tissues such as bone, muscle, fat, peripheral nerves, blood vessels and fibrous tissues [1]. Sarcomas account for about 1% of all

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http://dx.doi.org/10.1016/j.bbcan.2014.10.002
0304-419X/

malignant solid tumours in adults over 20% in children [2,3]. The mainstay of treatment is surgical resection, however approximately 20% of patients will present with advanced disease — inoperable or metastatic disease. Despite multimodality treatment including surgery, chemotherapy and radiotherapy, the 5-year survival for all patients diagnosed with sarcoma is about 55% in the UK, but when disease is advanced the median survival is only just over 1 year [2,4]. There is a clear clinical need for the identification of molecular targets for new and improved therapeutics for sarcoma.

Protein kinase C (PKC) comprises a group of serine/threonine protein kinases that are involved in a diverse array of cell signalling cascades which regulate cellular functions including proliferation, mitosis, differentiation, polarity, invasion, migration, survival and apoptosis [5–8]. Since the landmark discovery that tumour promoting phorbol esters bind to and activate PKC [9], there has been accumulating evidence implicating the PKC family in the initiation and/or progression of cancer (see [10,11] for reviews). This branch of the AGC kinases are being recognised as differentially expressed in sarcoma suggesting a possible role in this group of malignancies [12–14]. As sarcoma is a rare disease there are a limited number of studies looking at signal transduction mechanisms of sarcomagenesis and fewer still that have focused on PKC. However, with improved molecular technologies, cross-fertilisation of ideas from other tumour models and structure-based design of new inhibitors there is an emerging role for the PKC family as therapeutic targets in sarcoma. In this review we give an overview of the PKC family, discuss the roles of these kinases in sarcoma and the clinical experiences of anti-PKC strategies to date.

2. Structure and activation of PKC

The PKC family of Ser/Thr protein kinases initially came to wide attention through the work of Nishizuka who identified in a series of papers a calcium activated phospholipid dependent kinase, which was also the cellular receptor for diacylglycerol (DAG) and critically, from a cancer perspective, the tumour promoting phorbol ester, PMA [15]. This work essentially defined the conventional PKC family (cPKC) subfamily, expressed from three closely related genes (α, β and γ) [16]. Extensive homology screening eventually extended the repertoire to 12 PKC super family genes in mammals confirmed as the complete set with the sequencing of the human genome [17]. These kinases, which bear highly homologous C-terminal kinase domains, are subdivided into the conventional, novel, atypical and PKN subfamilies based on their lipid dependencies and/or regulatory domain structure. Here we will briefly cover the regulation of the PKC family members from generic mechanisms, through to subfamily and isotype specific examples.

Modular regulatory domains define PKC subfamily and function [17]. The PKC subfamilies are defined by divergence within their regulatory domains. cPKC kinases bear C-terminal kinase domain coupled to a N-terminal regulatory domain composed of a DAG/PMA binding C1 domain and the Ca^{2+}/PS responsive C2 domain. In the novel PKCs (nPKCs), the C1 and C2 domains have been transposed and the Ca^{2+} dependence has been lost; nPKCs retain PS and DAG/PMA responsiveness. Atypical PKC (aPKC) conversely has a truncated C1 domain which does not retain DAG/PMA regulation, coupled to a PB1 domain. The PKN kinases, in contrast to other family members bear three tandem coiled motifs, termed HR1 domains which confer Rho GTPase regulation [18,19]. While this has often led to classification of the PKNs as PKC 'related' kinases, the presence of HR1 domains and Rho coupling in the archetypal PKC of budding yeast, might suggest that PKN could be considered as mammalian Rho-responsive PKC [20].

Thus in higher organisms, where the PKC family has dramatically expanded, the kinase domains have remained highly homologous, while the juggled regulatory domains have driven divergence and separation of inputs and localisation. This has allowed the same kinase module to regulate extremely diverse cellular functions and perhaps the challenge therefore around targeting PKC is that it is not 'what' activity must we block but more the 'where' and 'how' we must block it [17,21].

Despite this extensive regulatory domain shuffling conferring distinct wiring and localisation, the PKC family appears to retain some basic themes for regulating expression of their kinase activity. All PKC isoforms bear a regulatory domain pseudo-substrate site, which is understood to occupy the active site of the kinase domain to define a latent activity competent auto-inhibited conformer (Fig. 1) [22,23]. For the PKN kinases, controversy remains over identification of a pseudo-substrate motif within the HR1 repeat region [24]; alternative auto-inhibitory dimerization has also been proposed for PKN2 [25]. Interaction with membranes, specific lipid activators and/or protein partners serves to disengage the pseudo-substrate from the active site to allow target substrate access and trans-phosphorylation. Likewise, regulation of intrinsic kinase domain activity through priming phosphorylation is highly conserved in the PKC family [26]. This requires phosphorylation of the generic activation loop by the AGC master regulator PDK1 [27]. Additionally, phosphorylation (or charged residues) at the turn and hydrophobic sites within the AGC specific C-terminal tail, generally by mTORC2 [28–31], are required in all cases for activity; the phosphorylated C-terminal tail folds back into a hydrophobic pocket on the surface of the kinase domain to lock the active conformation.

Some debate surrounds the acute regulation of these priming phosphorylations and the kinase activities responsible for their occupation; in most cases priming site phosphorylation stoichiometry is high giving a constitutively primed kinase domain. Acute PKC regulation is thus dependent on the engagement or disengagement of the inhibitory pseudo-substrate motif (see above). Recent work has also revealed the key importance of nucleotide pocket occupation in the regulation of PKC priming phosphorylations [32]. Occupation of the nucleotide binding pocket by either ATP or ATP competitive PKC inhibitors drives adoption of a fully phosphorylated active conformer with implications for the therapeutic targeting of this family [33]; inhibitors locking the kinases in an activated state can drive kinase activity independent allosteric PKC functions [32,34]. Indeed, many ATP competitive inhibitors cause re-localisation of PKC to membranes and adoption of a pseudo-active state, possibly through disruption of active site-pseudo-substrate interactions [35,36].

The constitutively active conformer adopted by PKC kinase domains is perhaps also reflected in the absence of frequent PKC 'activating' mutations in cancer. Indeed, the rare cancer associated point mutations reported for PKC appear to affect either localisation or substrate targeting. The recurrent D294G in the PKCα regulatory domain in endocrine tumours for example has been reported to prevent correct membrane targeting of PKCα and thus limit its anti-tumourigenic function in these cell types [37–39]. Likewise, the cancer associated R471C mutation in oncogenic PKCι affects selective substrate targeting rather than activity; R471C mutation within the kinase domain disrupts a novel substrate recruitment motif identified through structural studies of the kinase domain [40].

These loss or change-of function mutations also highlight the often antagonistic roles of PKC family members with respect to tumourigenic behaviours. Pro-survival and pro-apoptotic pathways are widely reported to be regulated by PKCα, PKCδ and PKCε contingent on the cellular context. Similarly, both increases and decreases in aPKC activity/expression can lead to dysregulation of cell polarity, a phenotype associated with malignancy [41]. Therefore, in the context of developing PKC inhibitors it is critical to understand the PKC signalling mechanisms in specific disease states and to recognise the possible limitations of drugging this target.

3. PKC signalling in sarcoma

3.1. GIST

Gastrointestinal stromal tumours (GIST) are the most common sarcoma of the GI tract with an incidence of at least 15/million/year [42].

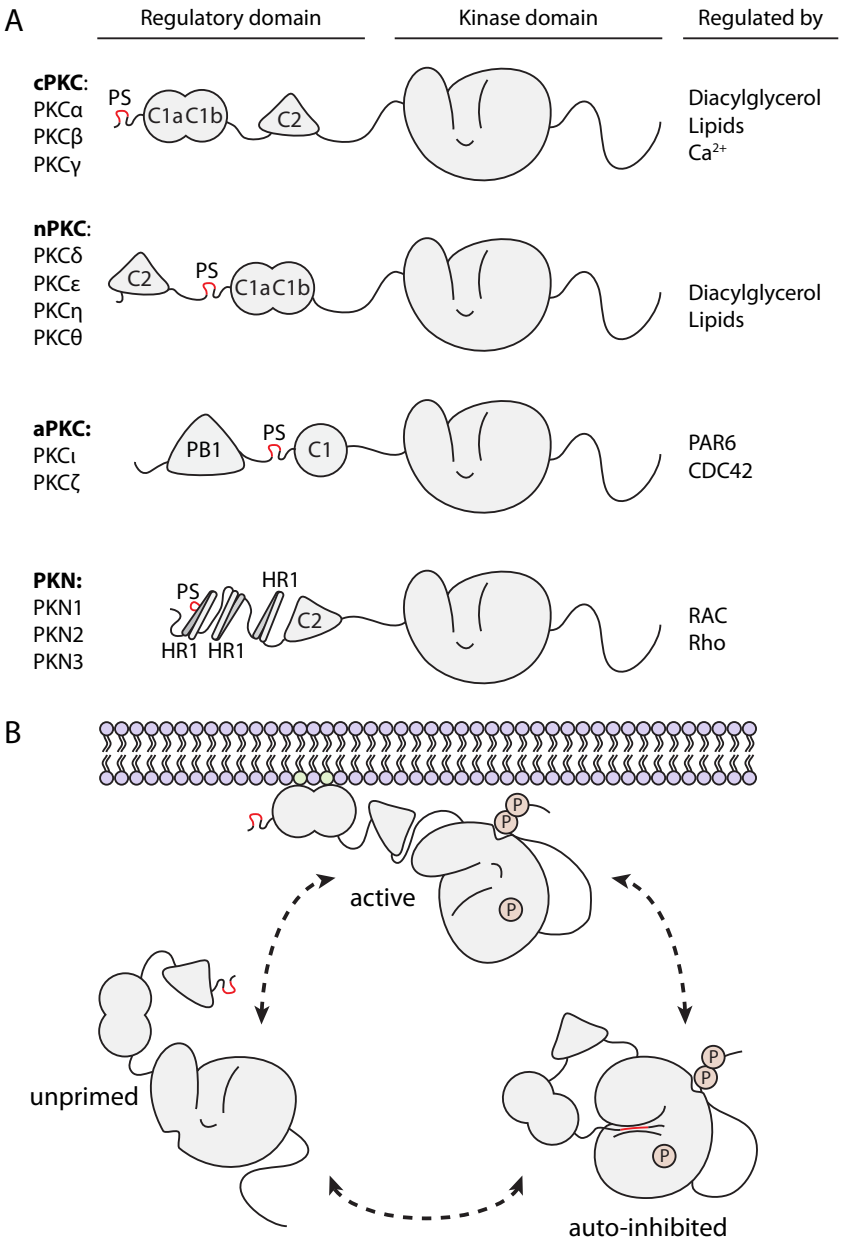

Fig. 1. Domain architecture and activation of PKC. (A) The four main subfamilies of PKCs (classical, novel, atypical and PKN) all share a conserved kinase domain, but differ in their modular regulatory domains. The different subfamilies are therefore activated by a variety of cofactors, including diacylglycerol, lipids, Ca^{2+} and others. All PKCs have a pseudo-substrate domain (PS), which is able to autoinhibit the kinase through binding in the kinase domain. (B) General activation states of PKC. Unprimed PKC is recruited to the plasma membrane, where lipid binding opens up the conformation. This allows other kinases to phosphorylate the priming sites on the activation loop and C-terminal tail of PKC. The phosphorylated tail folds back onto a hydrophobic pocket in the kinase domain, and the kinase is fully activated. Primed PKC is able to bind the pseudo-substrate motif in its kinase domain in an auto-inhibitory fashion.

GISTs are thought to be derived from the interstitial cells of Cajal that coordinate the peristaltic action of the gastrointestinal tract. The majority of GISTs have activating mutations in the gene for stem cell factor receptor-*KIT* or, less commonly, platelet derived growth factor receptor alpha *PDGFRA*, which are initiating or transforming events [43]. Introduction of the tyrosine kinase inhibitor (TKI), imatinib, has had a major impact on the disease, however most patients will develop resistance to tyrosine kinase inhibition and new treatment targets are required [44,45]. Strong overexpression of PKCθ is found in the majority of GISTs but not in other sarcoma subtypes, for example Blay et al. demonstrated that all 26 gastrointestinal stromal tumours in their study had strong cytoplasmic staining of the PKCθ antibody but no immunoreactivity was seen in 12 samples of other STS [46]. This specificity of PKCθ staining in GIST was confirmed in 27 FFPE tumour samples by Duensing et al., irrespective of *KIT* or *PDGFRA* mutational status [47]. Activation-loop phosphorylation of PKCθ, a marker of PKC-theta activity [48], was also detected at high levels in GIST lysates compared to Jurkat cell controls [47]. Not only is PKCθ strongly expressed and catalytically competent in GISTs, but a number of groups have demonstrated its utility as a diagnostic marker [49–51].

Functional and biochemical studies have suggested an interesting relationship between PKCθ and c-KIT. Immunoprecipitation using PKCθ as bait in GIST882 cells confirms interaction with KIT and treatment with imatinib led to a reduction in phosphorylated KIT and a reduction in immunoprecipitated c-KIT [52]. In both imatinib sensitive (GIST882) and insensitive (GIST48 and GIST 430) cell lines, shRNA silencing of PKCθ decreased the total KIT protein expression although this decrease had a faster onset and was more pronounced in the

imatinib insensitive cells lines [53]. Interrogation of downstream signalling in the imatinib insensitive cells lines four days following PKCθ silencing demonstrated a reduction in AKT, p70S6K and STAT1 phosphorylation. Consistent with this signalling modulation, PKCθ silencing led to increased apoptosis and decreased cell viability in the imatinib insensitive GIST cell lines [53]. It is not known whether this PKCθ and KIT inter-play is due to a protein scaffold function or an involvement in transcription regulation. Both possibilities are plausible as the C2 domain of PKCθ is known to bind to phosphotyrosine residues [54] at similar motifs to those found in c-KIT and PKCθ has been shown to bind to chromatin and regulate transcription [55]. Unravelling the mechanisms of this interaction could be crucial in establishing whether PKCθ is a valuable target in imatinib resistant GIST.

In summary, PKCθ is involved in GIST cell survival, is a useful diagnostic marker and may have potential as a therapeutic target.

3.2. Osteosarcoma

Osteosarcoma (OS) is the most common primary high-grade sarcoma of bone with a peak incidence occurring in children aged 10–14 years and a second smaller peak in adults over 40 [56]. The cellular roles of the PKC family of proteins in OS have long been reported and include the cellular response to cisplatin [57], induction of differentiation [58], growth, survival and metastases [59].

Osteosarcomatous growth could be dependent on PKCδ which has been shown to be necessary for fibroblast growth factor (FGF) induced bone development by controlling the expression and post-translational modification of RUNX2 [60,61]. RUNX2 protein forms a transcription complex that regulates osteogenesis, particularly differentiation, survival and growth [62]. RUNX2 expression is elevated in OS [63], correlates with the stage of disease and may be predictive of treatment response [64]. Multiple signalling pathways converge to affect RUNX transcription including Hedgehog, canonical Wnt, mitogen activated protein kinase (MAPK), transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) cascades [65]. Kim et al. demonstrated that in the presence of FGF there was enhanced expression of RUNX2 mRNA and increased binding of RUNX2 protein to the osteocalcin gene promoter [60]. RUNX2 binding to DNA was critically dependent on its phosphorylation at Serine 247 by PKCδ [61]. The importance of PKCδ in this process was further indicated by dominant negative mutants of PKCδ or chemical inhibition of PKC using Calphostin C (broad specificity; [66]) or Rottlerin (questionable specificity; [67]) which caused decreased RUNX2 mRNA expression and decreased promoter binding [60].

During migratory phases of metastasis, PKC dynamically regulates ezrin [59,68], a protein that influences cellular shape, adhesion, motility, signal transduction and is highly expressed in OS [69,70]. PKC dependent phosphorylation of ezrin at threonine 567 leads to a conformation change of the latent form of ezrin, allowing its C-terminal domain to interact with the actin cytoskeleton and the N-terminal end to bind to membrane associated proteins [71]. In murine and human OS cells, ezrin and PKC co-immunoprecipitated and in lung metastases phospho-PKCα and phospho-ezrin co-localised at the invasive front of cells suggesting that they work together during migration [59]. In a wound-healing assay, knockdown of ezrin or PKC inhibition with Ro31-8220 (broad specificity) or Gö6976 (cPKC selective) caused a delay in migration. Normal migration could be rescued if the PKC inhibitor treated murine OS cells were transfected with a phosphomimetic ezrin protein (T567D), further supporting the requirement of ezrin phosphorylation by PKC in migrating osteosarcoma cells [59]. PKC is probably required for proliferation of human OS cell lines as treatment of these cell lines with PKC412, a multi-targeted kinase inhibitor including PKC, led to a decrease in PKCα phosphorylation (unclear how this is effected) and cell proliferation at a concentration of 1 μM [72]. Specific inhibitors of both cPKC and nPKC are being developed and could usefully be tested in osteosarcoma.

3.3. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a group of aggressive neoplasms of striated muscle and the most common soft tissue sarcomas in children and adolescents [73]. Rhabdomyosarcomas in children are broadly subdivided into two subgroups based on histopathology — alveolar RMS (ARMS) and embryonal RMS (ERMS). ARMS are characterised (75% of cases) by the reciprocal balanced translocations t(2;13)(q35;q14) or t(1;13)(p36;q14) that generate the fusion genes PAX3-FOXO1 and PAX7-FOXO1 respectively [74,75]. ERMS have loss of heterozygosity of Chr11p15.5 (the locus of the IGF2 gene), copy number gains at the ALK and GLI1 loci and gene mutations/deletions including RB1, TP53, CDKN2A/2B, RAS, NF1, PIK3CA and CTNNB1 [1].

Differential expression of PKC isoforms has long been described as correlating with tumourigenicity of rhabdomyosarcoma [76,77]. For example, in one study of rat rhabdomyosarcoma cell lines, low PKCα or PKCε and high PKCβ mRNA were associated with neoplastic growth in syngeneic rat models [76]. Immunohistochemistry of tumour microarrays containing 64 tumour samples of alveolar and embryonal rhabdomyosarcoma demonstrated increased PKC phosphorylation compared to the control tissue. In particular, phosphorylation levels of PKCα, PKCθ and PKCζ were elevated by 57%–69% in alveolar rhabdomyosarcoma and 43%–72% in embryonal rhabdomyosarcoma, suggesting that those isoforms may play a role in the development or progression of rhabdomyosarcomas [77], although as noted above, it is not evident whether this truly reflects a change in activity state.

Focusing on the role of PKCι as a possible therapeutic target in alveolar rhabdomyosarcoma, Kikuchi et al. demonstrated in human and orthotopic mouse tumour samples, an increase in PKCι mRNA which occurs in the absence of genomic amplification [78]. Concordantly, there was an increase in PKCι protein and enhanced PKCι phosphorylation compared to striated muscle controls. Perturbation of PKCι in alveolar rhabdomyosarcoma mouse primary cell cultures using either siRNA–PKCλ (the mouse homolog of PKCι) or a PKCλ/ι inhibitor (ATM) decreased non-adherent colony formation, a surrogate phenotype for tumourigenicity. In vivo, ATM had limited single agent activity in a rhabdomyosarcoma orthotopic mouse model, but led to a trend of enhanced anti-tumour activity when used in combination with vincristine, a microtubule inhibitor commonly used to treat rhabdomyosarcoma [78].

PKCι has previously been demonstrated in epithelial cells to interact with the polarity protein Par6 leading to normal mitotic spindle alignment, normal cell polarity [7] and Rac1 activation to support transformed growth [8]. In rhabdomyosarcoma primary cells, ATM inhibits PKCι–Par6 binding and Rac1 activation leading to an increase in cells in G2 phase of the cell cycle [78]. Aside from its interaction with polarity proteins, a further role for PKCι as an oncogenic kinase via cell intrinsic Hedgehog (HH) signalling has emerged [79]. This additional role was identified from a proteomic screen of basal cell carcinoma (BCC) cells where PKCι was revealed as an interacting partner to missing-in-metastasis (MIM), a scaffold protein necessary for Hedgehog signalling. PKCι was found to directly bind to and phosphorylate GLI1, the major transcription factor of the HH pathway, at residues within its zinc finger DNA binding region [79]. When these residues were mutated to non-phosphorylatable alanines (S243A and T304A) there was decreased DNA binding detected by chromatin immunoprecipitation (ChIP) and thus PKCι would appear to phosphorylate GLI1 to potentiate transcription of HH signalling components. As PKCι is itself a GLI1 target gene, this sets up a positive feedback system that can amplify HH signalling independent of upstream inputs (Smoothened (SMO) and Suppressor of Fused (SuFu) mutations) and lead to tumour growth (Fig. 2A) [79,80]. Of course this is a BCC model, but HH signalling may be activated by a number of genomic aberrations in rhabdomyosarcomas. In fact both BCCs and rhabdomyosarcoma are found in transgenic mice with germline mutations of the tumour suppressor gene Patched1 (*PTCH*) which leads to the activation of HH signalling [81]. In humans, these mutations of *PTCH*, a gene located on chromosome 9q22.3, causes Gorlin Syndrome, a condition that in

addition to BCC and RMS [82] predisposes to ovarian fibromas, meningiomas and medulloblastoma [83]. Furthermore in rhabdomyosarcoma, loss of heterozygosity mutations of the tumour suppressor gene *SuFu* [84], somatic loss of chromosome 9q22, [85] and amplification of chromosome 12q13–15 (that encompasses *GLI1*) are frequently detected and are proposed to activate HH signalling.

Given these data, the role of PKCι as a therapeutic target in alveolar rhabdomyosarcoma deserves further investigation. A number of Hedgehog pathway inhibitors are being developed and could be considered in combination with PKCι inhibition.

3.4. Ewing sarcoma

Ewing sarcoma (ES) is the second most frequent childhood bone tumour [86] and metastasises early in its clinical course. Although there have been significant improvements in the treatment of this disease using combined modality treatments and intensive chemotherapy with long term survivors [87], therapies for relapsed disease are disappointing and new treatments are desperately needed. Most ES harbour a reciprocal chromosomal translocation, t(11;22)(q24;q12), which links a strong transcriptional activation domain from the EWS to the ETS DNA-binding domain of FLI-1. This EWSR1-FLI1 fusion is required for ES oncogenesis [88]. A study by Surdez et al. provided insights into the relationship between this chromosomal translocation and PKCβ [89]. Compared to other tumours, ES was found to have at least 13 fold higher expression of *PRKCB* (the PKCβ gene) which corresponded to strong PKCβ protein overexpression in a tissue microarray. siRNA depletion of EWSR1-FLI1 led to fast and proportional reduction of PKCβ mRNA and protein levels, indicating that PKCβ expression in ES cells is EWSR1-FLI1 dependent. ChIP analysis in ES cells demonstrated

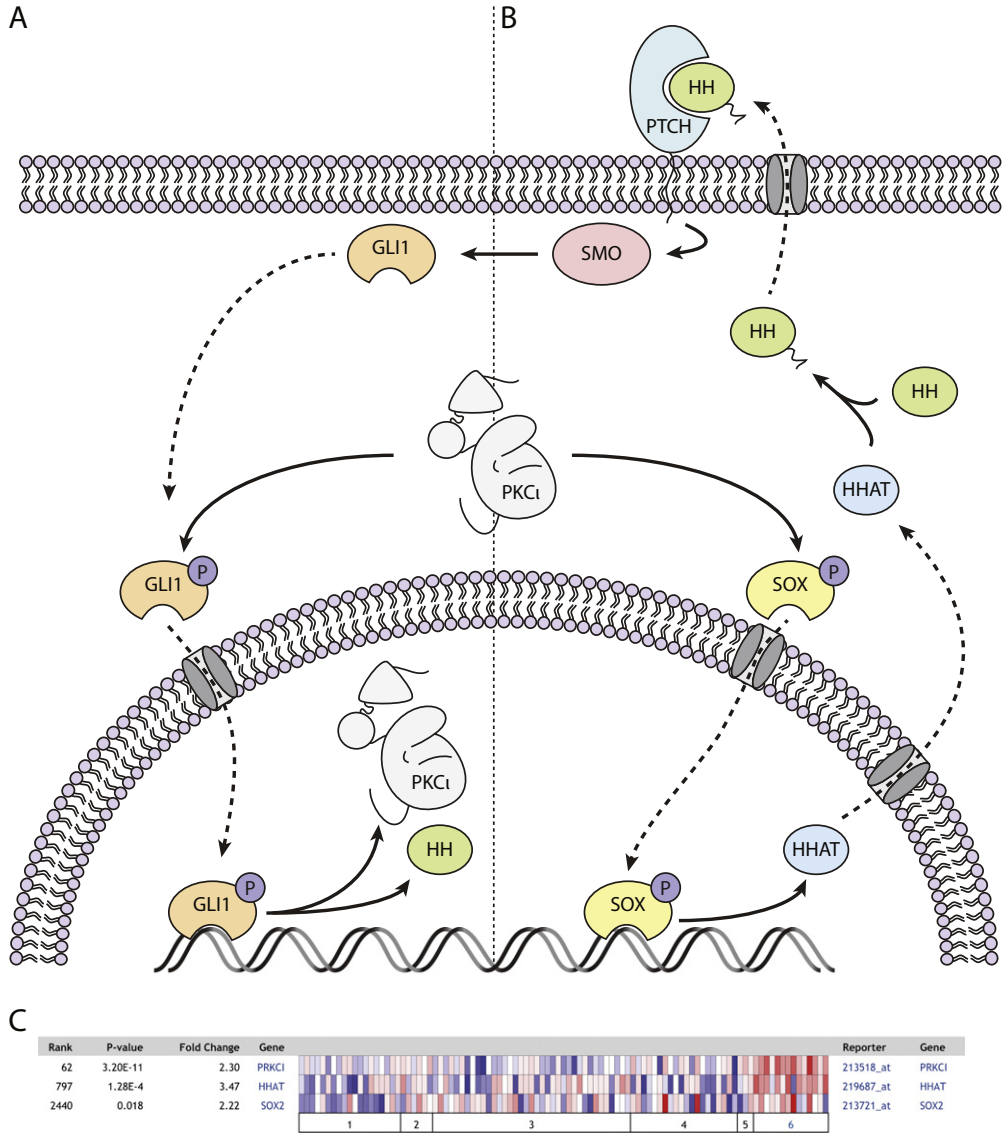

Fig. 2. Possible cooperative PKCι and Hedgehog (HH) signalling in sarcoma. (A) Model of cell-intrinsic signalling in basal cell carcinoma and rhabdomyosarcoma. PKCι phosphorylates and activates GLI1 increasing gene expression of GLI1 targets. (B) Model of autocrine/paracrine signalling in squamous cell carcinoma of the lung and synovial sarcoma. PKCι phosphorylates and activates SOX2 increasing gene expression of SOX2 targets including HHAT. HHAT leads to a lipid modification of HH which is then secreted by the cell and binds to Patched 1 receptors (PTCH) in an autocrine or paracrine mechanism leading to HH signalling via smoothened (SMO) and transcription of GLI1 dependent genes. (C) Representative expression array of *PRKCI*, *HHAT* and *SOX2* in sarcoma. 1, Fibrosarcoma (19); 2, leiomysosarcoma (6); 3, liposarcoma (37); 4, malignant fibrous histocytoma (20) samples; 5, malignant peripheral nerve sheath tumour (3); and 6, synovial sarcoma (14). Colours are z-score normalised to depict relative values within rows: red, most expressed; blue, least expressed; and grey, not measured. For each gene, an expression profile is shown across sarcoma samples. The statistical significance of over or under expression of that gene between synovial sarcoma and other sarcomas is reported. Oncomine use a Student's t-test for two class differential expression analyses. P-values are corrected for multiple hypothesis testing using the false discovery rate method, as described by Storey and Tibshirani [167] therefore a P-value of <0.01 is usually significant (www.oncomine.com) [168]. These results are consistently seen in sarcoma expression array datasets [169–171].

occupancy of the *PRKCB* promoter by EWSR1-FLI1 and accordingly *PRKCB* promoter activity, in a luciferase reporter assay, was significantly decreased upon doxycycline induced shRNA knockdown of EWSR1-FLI1. To test the phenotypic significance of PKCβ in cell culture, 3 separate ES cell lines were infected with lentiviral shRNA–PKCβ resulting in decreased proliferation and increased apoptosis. The specificity of the shRNA was confirmed by partially rescuing the proliferation upon exogenous expression of a shRNA-resistant version of PKCβ cDNA. To corroborate this finding, the cell lines were treated with a PKCβ inhibitor, enzastaurin, which was cytotoxic with an IC50 of 5 μM (note that at this fairly high dose of this PKCβ selective inhibitor, PKCα will also be inhibited). In SCID mice inoculated in the flanks with human ES cell lines, doxycycline induction of shRNA–PKCβ led to the prevention of tumour engraftment and reduced tumour growth for 5 days followed by tumour shrinkage thereafter. The treatment of inoculated mice with enzastaurin also led to reduced tumour growth. The signalling downstream of PKCβ remains unclear but one possibility is that it modulates Histone methylation. In prostate cancer, PKCβ was shown to phosphorylate Threonine 6 of Histone H3 (H3T6ph) which leads to stabilisation of tri-methylation of Lysine 4 of Histone H3 (H3K4me3) potentiating transcription [90]. While siRNA against PKCα or PKCβ individually did not affect histone H3 phosphorylation in ES cells, the combination treatment led to diminished H3T6ph and H3K4me3. These findings suggest functional redundancy between the classical PKC isoforms and that the demethylation effect is not specific to EWSR1-FLI1 promoters, instead it represents a more global role in maintaining H3T6ph steady-state [89]. The profound effects of genetic and chemical inhibition of PKCβ in Ewing sarcoma make it a promising therapeutic target although further mechanistic studies are required.

Recently, PKCι was shown to be necessary for non-canonical Wnt signalling in an Ewing sarcoma cell line, TC-32 [91]. Wnt and its downstream effectors regulate various processes important for cancer progression [92], and neurite outgrowth in TC-32 cells, has been successfully used as a cancer model to interrogate Wnt signalling pathways [93]. Greer et al. demonstrated in TC-32 cells that Wnt3a ligand treatment leads to neurite extension which can be abrogated by siRNA or chemical inhibition of either PKCι or casein kinase 1 delta (CK1δ) [91,94]. Interrogation of this pathway demonstrated that Wnt3a leads to casein kinase 1 delta (CK1δ) dependent phosphorylation of Dishevelled 2 (Dvl2) and in this phosphorylated form, Dvl2 forms an immunocomplex with PKCι extracted from cells [91]. This Wnt3a > CK1δ > Dvl2 > PKCι axis is further supported by the finding that Wnt3a leads to pericentrosomal distribution of PKCι and co-localisation with CK1δ. Whether this is a general phenomenon or idiosyncratic wiring in this tumour derived cell line remains to be determined.

3.5. Synovial sarcoma

Synovial sarcoma is one of the most aggressive forms of STS that can occur at any site in the body, often in young people, and frequently metastasises to the lungs (>50%) and lymph nodes (20%). The majority of cases carry a t(X;18) balanced translocation and the resulting SS18-SSX oncoprotein disrupts the BAF/BAF47/SS18 complex dependent repression of the SOX2 transcription factor [95]. This leads to increased expression of SOX2 and in turn SOX2 dependent proteins that are required for transcription, cell cycle regulation and proliferation [96]. SOX2 has recently been implicated in a model of lung cancer as a PKCι substrate which cooperates with Hedgehog signalling pathway (Fig. 2B) [97]. In vitro kinase assays and mass spectrometry have demonstrated that PKCι phosphorylates the SOX2 protein at Threonine 118. Chromatin immunoprecipitation studies in organotypic lung cell cultures have shown that phosphorylation of SOX2 leads to increased occupancy of the Hedgehog acyl transferase (HHAT) promoter. Knockdown of SOX2 decreased HHAT mRNA and protein providing supporting evidence for the PKCι–SOX2–HHAT signalling axis. HHAT contributes to the activation of Hedgehog (HH) by adding a palmitoyl moiety to the amino-terminus of the ligand after autoproteolytic cleavage. The cleaved and lipid modified HH is secreted by the cell and binds to the Patched1 receptor (PTCH) in an autocrine or paracrine fashion. This releases the PTCH inhibition of Smoothened (SMO) resulting in translocation of GLI (a transcription factor originally isolated in Glioblastoma) to the nucleus and transcription of GLI dependent genes, many of which are oncogenic. In synovial sarcoma, similar to lung cancer, publically available expression array datasets suggest that *PRKCI* (the *PKCι* gene) and *HHAT* are co-expressed. Unlike lung cancer however, in synovial sarcoma *SOX2* is not consistently overexpressed (Fig. 2C). This co-expression data would support a hypothesis that PKCι–SOX2–HHAT axis may be conserved between lung cancer and synovial sarcoma with SOX2 protein being activated in synovial sarcoma by loss of BAF47 induced repression rather than *SOX2* gene amplification.

4. PKC therapeutic strategies in clinical trials

The compelling cancer linkage data for PKCs (reviewed in [10,11]) has focused attention on how to therapeutically target these molecules. PKCs share close homology of their kinase domain sequence and thus many early inhibitors have lacked isotype specificity. Crystallographic data, which is available for the kinase domains of PKCθ, PKCι and PKCβII [98–100] has identified potential liabilities of the tertiary structure enabling development of isoform specific inhibitors. Also, increased mechanistic understanding about the activation of PKC, proximal binding partners and distal effectors of PKCs has opened up a range of targeting strategies which are discussed below [27,32,101–103]. A multitude of PKC inhibitors are being used as tool compounds but here we limit our discussion to those drugs that are being tested in, or are poised to enter, clinical trials (Table 1).

4.1. Inhibition of PKC activation

Priming phosphorylations of PKC lead to catalytic competence and are regulated by the upstream kinases PDK1 and TORC2. A number of rationally designed PDK1 inhibitors have been developed (see [130] for review) but have not yet entered clinical trials. Retrospectively defined PDK1 inhibitors such as UCN-01, a multi-targeted inhibitor of PDK1, Chk1 and PKC, have been tested in trials. For example, in a phase II study of 25 patients with triple-negative breast cancer, UCN-01 was shown to inhibit phosphorylation of p70S6 kinase, one of the direct PDK1 substrates, in peripheral blood mononuclear cells at 24 h. One patient had a partial response to treatment while 8 patients maintained stable disease suggesting minor activity of this agent in triple negative breast cancer [104].

Mammalian target of Rapamycin (mTOR) inhibitors have demonstrated benefit in the treatment of a number of malignancies, including Temsirolimus for renal cell carcinoma [131], Everolimus in HER2 negative breast cancer [132] and ridaforolimus in soft tissue sarcoma [133]. However, PKC isoforms are phosphorylated by TORC2 rather than TORC1 and TORC2 itself contributes to a transformed phenotype in cancer model systems [80]. Dual mTORC1/2 inhibitors are currently recruiting for clinical studies including a phase II comparison between the novel TORC1/2 inhibitor AZD2014 and the TORC1 inhibitor Everolimus in metastatic renal cancer (NCT01793636).

4.2. Nucleotide pocket/substrate binding inhibitors

The majority of PKC inhibitors are structural analogues of ATP and compete for interaction with the nucleotide binding pocket.

4.2.1. Midostaurin

The first PKC inhibitor to be evaluated in clinical trials was midostaurin (PKC412). Midostaurin (PKC412), N-benzoyl-staurosporine, potently inhibits PKCα, VEGFR2, c-KIT, PDGFR and FLT3 kinases.

Table 1
Summary of PKC inhibitors in clinical trials.

Mechanism of action	Drug	Study	Results	Reference
Inhibition of PKC activation	UCN-01	Phase II trial in combination with irinotecan in recurrent triple-negative breast cancer	n = 25 1 PR 8 SD	[104]
Nucleotide pocket/substrate binding inhibitors	Midostaurin (PKC412)	Phase I/II trial in acute myeloid leukaemia and myelodysplastic syndrome	Greater than 50% reductions in blast counts	[105]
		Phase I/II trial in combination with imatinib in imatinib-resistant GIST	Reduced imatinib blood levels. Trial discontinued.	[106]
		Phase I trial in solid tumours	n = 32 1 PR and 1 SD in cholangiocarcinoma	[107,108]
		Phase IIA trial in metastatic melanoma	n = 17 No responses.	[107,108].
	Enzastaurin (LY317615)	Phase I trial in solid tumours	n = 47 21 SD	[109]
		Phase II trial in diffuse large B-cell lymphoma	n = 55 3 CR	[110]
		Phase II in colorectal cancer	n = 28 12 SD	[111]
		Phase II in ovarian/peritoneal cancer	n = 27 2 PR	[112]
		Phase II trial in multiple myeloma	n = 14 1 minimal response	[113]
		Phase II trial in NSCLC	n = 55 19 SD	[114]
		Phase II trial in combination with paclitaxel and carboplatin in ovarian cancer	n = 142 3.7 m longer median PFS compared with standard treatment not statistically significant	[115]
		Phase II trial in combination with pemetrexed and carboplatin in NSCLC	Trend to worse OS with standard chemotherapy plus enzastaurin	[116]
		Phase II trial in combination with radiotherapy in GBM	n = 60 24-week PFS 53.6%	[117]
	Sotrastaurin (AEB071)	Phase I trial in melanoma	Ongoing	NCT01430416
		Phase I trial in diffuse large B-cell lymphoma	Ongoing	NCT01402440
		Phase IB/II in combination with MEK162 in uveal melanoma	Ongoing	NCT01801358
		Phase IB/II in combination with Everolimus in diffuse large B-cell lymphoma	Ongoing	NCT01854606
Protein–protein interactions inhibitors	Bryostatin 1	Phase II trial in combination with vincristine in B-cell lymphoma	n = 14 2 CR 2 PR 6 SD	[74]
		Phase I in combination with temsirolimus in renal cell carcinoma	n = 17 3 PR	[118]
		Phase II trial sequentially with paclitaxel in gastric or gastro-oesophageal junction adenocarcinoma	Trial discontinued due to severe myalgia in more than 50% of patients	[119]
	ATM (aurothiomalate)	Phase I trial in NSCLC, ovarian cancer and pancreatic cancer	n = 15 Good tolerance	[120]
Translation inhibitors	ISIS 3521	Phase I trial in advanced cancer (21-day infusion)	n = 21 3 PR (ovarian cancer) 2 CR (lymphoma) 1 SD (NSCLC)	[121]
		Phase I trial in advanced cancer (24-hour infusion)	Not well tolerated	[122]
		Phase II trial in low-grade non-Hodgkin's lymphoma	n = 26 3 PR 10 SD	[123]
		Phase II trial in colorectal cancer	n = 16 No clinical activity	[124,125]
		Phase II trial in high-grade astrocytomas	n = 21 No clinical activity	[124,125]
		Phase II trial in ovarian cancer	n = 36 0 CR/PR 1 CA125 response	[126]
		Phase I/II trial in combination with carboplatin and paclitaxel in NSCLC	n = 53 Response rate 48%	[127]
		Phase III trial in combination with gemcitabine in NSCLC	n = 670 No clinical activity	[128]
	Atu027	Phase I trial in advanced cancer	n = 34 8 SD 2PR (neuroendocrine and breast cancer)	[129]

CR, complete response; GBM, glioblastoma multiforme; m, months; NSCLC, non-small-cell lung cancer; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease.

Midostaurin is orally bioavailable and is well tolerated with activity observed in phase I/II trials of acute myeloid leukaemia and myelodysplastic syndrome with greater than 50% reductions in blast counts. This blast response was significantly greater in patients with mutant FLT-3 indicating that the mode of activity may be due to inhibition of FLT-3 rather than PKC [105].

Pre-clinical data suggests that midostaurin has activity in GIST or BaF3 cells that carry certain primary or secondary imatinib resistant c-KIT mutations with IC50s in the nM range [134]. A phase I/II trial was therefore initiated in patients with imatinib resistant GIST evaluating the combination of imatinib and midostaurin, however the combination led to reduced imatinib blood levels and the trial was abandoned [106]. Furthermore, midostaurin monotherapy has failed to demonstrate activity in trials in a number of solid tumours [107,108].

4.2.2. Enzastaurin

Enzastaurin is an orally bioavailable ATP inhibitor of PKCβ, and less potently against PKCα and AKT, which exhibited proapoptotic and anti-proliferative activities in various cancer cell lines [135] and animal models [136,137]. In phase I, [109] and phase II studies of monotherapy in glioblastoma multiforme (GBM) [138], and diffuse large B-cell lymphoma (DLBCL) [110], enzastaurin was well tolerated and had better response rates than historical controls including complete responses. However, there was no prolongation of progression free survival and in studies in colorectal cancer, [111] ovarian/primary peritoneal cancer, [112] myeloma, [113] and non-small cell lung cancer, [114] there was minimal activity. In view of the lack of activity as a monotherapy, combination strategies were pursued. In 142 ovarian cancer patients enrolled into a randomised phase II trial of carboplatin and paclitaxel with or without enzastaurin (concomitant and then maintenance therapy) there was a trend to a 3.7 month improvement in PFS although in this small study this did not reach statistical significance (HR = 0.8, p = 0.37) [115]. A number of studies have shown no benefit of enzastaurin in combination with chemotherapy, for example a randomised phase II study in 218 patients with NSCLC actually showed a trend to worse overall survival when standard chemotherapy was given with enzastaurin [116]. Such differences are likely to be disease specific and highlight the necessity to identify the molecular subgroups that are most likely to benefit. One such predictive biomarker has been described in a phase II study of enzastaurin in GBM. In particular, 60 patients with GBM that lacked hypermethylation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) promoter (a subgroup typically resistant to treatment) were treated with enzastaurin before, during and after radiotherapy which led to a 24 week progression free survival rate (PFR24) of 53.6% [117]. This was substantially better than historical data of radiotherapy alone (PFR24 = 35.2%) or chemoradiotherapy (PFR24 = 40%) [139]. This promising data needs to be followed up with a randomised controlled trial and reinforces the necessity for biomarker integration into clinical trials in order to robustly evaluate the potential benefit of novel therapies.

4.2.3. AEB071

AEB071 (sotrastaurin or STN) is an orally potent drug with nM activity against classical and novel isoforms of Protein kinase C (PKC) [140]. In pre-clinical studies AEB071 has been demonstrated to have at least two separate actions — suppression of autoimmunity and anti-cancer phenotypes. Studies of cancer have focused on uveal melanoma, a rare cancer that commonly has activating mutations of Gα which lead to the activation of PKC/Erk and NF-κB signal transduction, growth and survival [141]. Single agent AEB071 causes melanoma cells to undergo G1 arrest and apoptosis [141] and has synergistic effects with ionising radiation [142], with an inhibitor of PI3K α (BYL719) [143] and the MEK inhibitors PD0325901 and MEK162 [144]. A number of phase II studies in patients requiring immune modulation (post-organ transplant, plaque psoriasis, uveitis and ulcerative colitis) have established that AEB071 doses of 200–300 mg twice daily are well tolerated but with sufficient followup there was a lack of significant activity as an immunosuppressant [145–147]. The role of AEB071 as an anti-cancer agent is currently being actively pursued with ongoing recruitment to phase I studies in melanoma and diffuse large B-cell lymphoma either as monotherapy (NCT01430416 and NCT01402440) or in phase IB/II studies of AEB071 in combination with a MEK inhibitor (phase IB/II NCT01801358) or mTOR inhibitor (NCT01854606).

4.2.4. CRT0066854

CRT0066854 is an ATP-competitive thieno[3,2-d]pyrimidine based PKCι inhibitor which also displaces Phe543, a critical contact within the ATP binding cleft, presumably inducing an inactive kinase state (Fig. 3) [148]. The inhibitor was identified from a high through-put immobilised metal ion-affinity fluorescence polarisation (IMAP) screen and then the potency and selectivity was refined using structure based design [148]. In particular, the potency was improved by changing components of the molecule (scaffold hop) to access a pocket adjacent to the gatekeeper residue [149]. PKCι inhibition with CRT0066854 was able to partially reverse the aberrant cellular polarity (a cancer phenotype) induced by oncogenic Ras, [150] — a mutation found in 6–35% of human sarcomas [151–153]. Derivatives of these aPKC inhibitors suitable for clinical studies are eagerly awaited.

4.3. Protein–protein interaction inhibitors

Functional inhibitors of C1A/B binding domains have been developed such as Bryostatin 1 which is a macrolide lactone compound isolated from the marine bryozoan *Bugula neritina* [154]. Acute treatment with Byrostatin 1 acts as an activator of DAG/phorbol responsive PKCs with translocation from the cytosol to the active membrane fraction [155] however chronic treatment leads to membrane dissociation and proteosomal degradation of PKC [81]. Initial studies of infusional (chronic) Bryostatin 1 in combination with chemotherapy [156] or mTOR1 inhibition [118] suggested promising activity (see Table 1). However, treatment was poorly tolerated in some chemotherapy pretreated patients [118] leading to the discontinuation of at least one trial [119]. There are currently no active trials using this agent but further efforts using novel medicinal chemistry to refine the biological activity of bryostatins are ongoing.

As the C2 region of PKCs have a divergent structure between the classical and novel isoforms (Fig. 1), this domain has been explored for potential inhibitor specificity. The C2 domain is required for calcium dependent binding to phospholipids in the cPKCs and for novel mode of phosphotyrosine binding in certain nPKCs. The C2 domain has also been shown to be involved in protein–protein interactions such as the binding to receptor for activated C-kinase (RACKs) thus contributing to the subcellular localization of PKCs. Short peptides with sequence homology to the predicted interaction sites have been shown to act as inhibitors

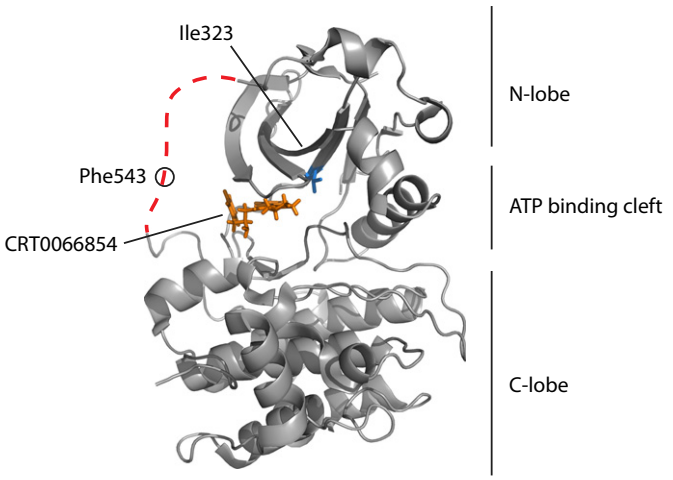

Fig. 3. PKCι bound to the specific inhibitor CRT0066854. The inhibitor binds in the ATP-binding pocket, adjacent to the gatekeeper residue Ile323. CRT0066854 displaces Phe543 from the ATP binding cleft and stabilises an inactive conformation of the kinase. PDB ID: 3ZH8 [148].

of protein–protein interactions [157]. In animal models of myocardial infarction, the tat-derived (cell membrane permeable) peptide inhibitor of PKCδ–RACK binding, Delcasertib (KAI-9803) led to reduced infarct size, enhanced recovery of myocardial metabolic activity and improved microvascular flow [158]. However, in a large phase IIB clinical trial of 1176 patients with acute anterior ST elevation MIs, Delcasertib administered intravenously prior to and during primary percutaneous coronary intervention failed to reduce the size of infarct or improve clinical outcomes. Subgroup analysis revealed a trend towards reduced infarct size (~14%) in patients with angiographically determined good prognostic disease who received high dose drug [159]. This less than emphatic result has tempered the enthusiasm for a similar approach in cancer studies.

The divergent C2 related PB1 domain of the aPKCs is a heterodimerisation module whose binding partners include Par6, a molecule critical for transformed growth. Aurothiomalate (ATM) was identified in a fluorescence resonance energy transfer (FRET) assay as an inhibitor of PKCι–Par6 interactions and ATM has been shown to reduce colony formation in soft agar and tumourogenicity in vivo [160]. ATM has been widely used in the treatment of rheumatoid arthritis and it is thought that it works by the formation of gold-cysteine adducts with cellular targets (see [161] for review). Molecular modelling based on the crystal structure of PKCι–Par6 predicts adduct formation between ATM and Cys69, a cysteine unique to the PKCι PB1 domain. In keeping with this prediction, the mutation of Cys69 to either an isoleucine or valine retains Par6 binding but renders it ATM insensitive [162]. In a lung cancer cell line panel, sensitivity to ATM correlated with PKCι mRNA and protein expression [163]. A subsequent phase I study of ATM in non-small cell lung cancer, ovarian cancer and pancreatic cancer was recently completed. This study showed that ATM was well tolerated at a dose of 50 mg daily and had a minor activity in a patient group that was not tested for tumour PKCι expression [120]. Further trials are ongoing with these gold compounds.

4.4. Translation inhibitors

ISIS 3521 is a phosphorothioate anti-sense oligonucleotide targeted to the 3′-untranslated region of PKC alpha mRNA. This agent led to a specific reduction in PKCα protein expression in cancer cell lines and human xenograft models [164,165]. In the phase I study, 21 day infusional ISIS 3521 every 4 weeks was well tolerated and responses were seen in 3 patients with ovarian cancer, 2 complete remissions in lymphoma and a prolonged stable disease in NSCLC [121]. A further phase I study of weekly 24 h infusion was also tested but this was not as well tolerated as the 21-day infusion [122]. In phase II studies of ISIS 3521 monotherapy there were 3 partial responses in NHL [123], one CA125 response in refractory ovarian cancer [126], but no activity in other tumour types [124,125]. Phase I and II studies of ISIS 3521 in combination with carboplatin and paclitaxel in patients with NSCLC ($n = 53$) looked extremely promising with an objective response rate of 48% [127]. However, two subsequent phase III trials failed to demonstrate any benefit of ISIS 3521 in combination with standard chemotherapy in NSCLC and one of these studies remains unpublished [128]. Consequently, clinical development of ISIS 3521 has been discontinued.

Depletion of PKN3 by siRNA contained in a liposomal particle, Atu027, has been shown to restrict tumour growth, invasion and distant metastasis in mouse xenograft models [166]. Data on the phase I trial of Atu027 was presented at the ASCO 2013 meeting and reported excellent tolerability and disease stabilisation in 8/34 patients including 2 patients with prolonged stable disease (9 and 22 months) [129].

5. Conclusions

PKC forms a nexus between a large number of sarcoma associated pathways and behaviours from the master growth regulators of PI3kinase and mTOR through to the specifics of cell polarity, apoptosis and migration. While mutations have their place in isolated cases, overexpression and mislocalisation appear to represent a recurrent theme for PKC in sarcoma. Consideration of expression, localisation, dynamics, allostery and substrate recruitment are all key when we try to understand the malign roles of PKC in sarcoma, and cancer in general.

The regulatory diversity of the PKC family have afforded a range of therapeutic strategies including isoform specific RNAi technologies, regulatory domain directed interventions, substrate mimetics and allosteric modulators. The mainstay however remains ATP competitive inhibitors targeted to the kinase domain nucleotide pocket. Use of structure based design has enabled the development of highly isoform specific inhibitors although, given the isoform redundancy it is still not clear whether specific inhibitors are superior to multi-targeted kinase inhibitors. Although many clinical trials involving PKC inhibitors have been clinically disappointing, there are signs that with the use of carefully developed biomarkers and combination treatment strategies, PKC interventions will be of significant value. Aberrant PKC signalling is prominent in many different forms of sarcoma and there are limited conventional treatment options for this disease. As such, sarcoma is ideally placed to act as a platform to test PKC targeted therapies early in the disease course with a view to improving treatments and patient outcomes.

Author contributions

J.M.-L. and M.L. authored the signalling section, A.C. wrote the structural section, J.C. designed the figures and M.L. wrote the therapeutic section. M.L. and A.C. wrote the introduction and conclusions. P.J.P., I.R.J. and all authors were involved in editing the manuscript.

Acknowledgements

J.M.-L, M.L. and I.J. were funded by the National Institute for Health Research/Royal Marsden Hospital Biomedical Research Center (BRC). M.L. is also funded by the NIHR University College London Hospital/UCL BRC. J.C., A.C. and P.J.P. are funded by Cancer Research UK. A.C. is also funded by a HEFCE award to the Barts Cancer Institute. We would like to thank Richard Mitter for the biostatistical support, Julien de Naurois for carrying out the initial literature search and to the members of the Parker Lab for the critical review.

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