Protein Kinase C Epsilon Overexpression Is Associated With Poor Patient Outcomes in AML and Promotes Daunorubicin Resistance Through p-Glycoprotein-Mediated Drug Efflux

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The protein kinase C (PKC) family of serine/threonine kinases are pleiotropic signaling regulators and are implicated in hematopoietic signaling and development. Only one isoform however, PKCε, has oncogenic properties in solid cancers where it is associated with poor outcomes. Here we show that PKCε protein is significantly overexpressed in acute myeloid leukemia (AML; 37% of patients). In addition, PKCε expression in AML was associated with a significant reduction in complete remission induction and disease-free survival. Examination of the functional consequences of PKCε overexpression in normal human hematopoiesis, showed that PKCε promotes myeloid differentiation, particularly of the monocytic lineage, and decreased colony formation, suggesting that PKCε does not act as an oncogene in hematopoietic cells. Rather, in AML cell lines, PKCε overexpression selectively conferred resistance to the chemotherapeutic agent, daunorubicin, by reducing intracellular concentrations of this agent. Mechanistic analysis showed that PKCε promoted the expression of the efflux pump, P-GP (ABCB1), and that drug efflux mediated by this transporter fully accounted for the daunorubicin resistance associated with PKCε overexpression. Analysis of AML patient samples also showed a link between PKCε and P-GP protein expression suggesting that PKCε expression drives treatment resistance in AML by upregulating P-GP expression.

Keywords: protein kinase C epsilon, acute myeloid leukemia, daunorubicin, drug resistance, P-glycoprotein (ABCB1 protein)
INTRODUCTION

Acute myeloid leukemia (AML) describes an aggressive group of hematological malignancies characterized by the accumulation of clonal, abnormally differentiated myeloid cells in the bone marrow (BM) and peripheral blood. For most patients, prognosis remains poor using conventional chemotherapeutic strategies, with an average survival of <6 months (1). Consequently, understanding the molecular mechanisms which underpin this malignancy with the aim of developing targeted therapeutic approaches to improve patient outcomes is a central focus in this field.

The PKC family of serine/threonine kinases consists of 11 isoforms which are classified according to their structure, cofactor activation and substrate specificity into classical (cPKC; α, βI/II, γ), novel (nPKC; δ, ε, η, θ) and atypical (aPKC; ϵ, λ/λ) isoforms (2). Of these, the nPKC isoform PKCe is unique within its family having shown transforming oncogenic properties and is frequently upregulated in solid cancers, where it is associated with aggressive disease phenotypes (3–6). In a hematological context, PKCe promotes erythrocyte and megakaryocyte lineage commitment and differentiation (7, 8). PKCe has also been implicated in malignant cell survival (9–12) and in AML cells PKCe has been reported to protect from TRAIL-induced apoptosis and to modulate reactive oxygen species (ROS) homeostasis (13, 14). Here we examine the frequency of PKCe dysregulation, as well as the pathophysiological associations and mechanistic contribution of PKCe misexpression in AML.

P-glycoprotein (P-GP) is an ATPase efflux pump from the ATP-binding cassette (ABC) transporter group. Substrates for ABC efflux pumps such as P-GP include amino acids, organic ions, peptides, chemotherapeutic agents, and xenobiotics (15). In AML, P-GP overexpression is associated with poor outcomes in newly diagnosed and relapsed disease (16). Particularly pertinent in AML is the ability of pumps such as P-GP to confer resistance to anthracyclines but has also been linked to increased invasiveness of AML cell lines (17). The mechanisms regulating efflux pump expression and activity are highly complex and have not been fully resolved. Despite this, some aspects of efflux pump regulation are outlined below, with a focus on P-GP, as this is the best characterized drug transporter. A wide range of transcription factors including AP-1 and NF-κB have been implicated in P-GP regulation (18, 19). The regulation of P-GP is post-translational as well as transcriptional. P-GP is exported from the endoplasmic reticulum as a 150-kDa protein which is subsequently glycosylated in the Golgi apparatus and yields the mature 170-kDa protein which mediates drug efflux (20). In addition to expression, the activity of efflux pumps is also central to drug transport. A role for PKC activity in activating P-GP has primarily been established using PKC agonists and inhibitors (21, 22). Three PKC phosphorylation residues (ser661, Ser667 and Ser 671) have been identified within the intracellular linker region of the P-GP peptide, suggesting potential roles for this family of kinases in P-GP regulation (23); however, directed mutagenesis of the PKC phosphorylation sites did not affect P-GP expression or activity in a yeast model (24). PMA treatment has been shown to promote P-GP expression and drug efflux (23, 25) while inhibiting PKC isoforms can overcome these phenotypes (26). However, the causality of these findings is complicated by the fact that some PKC inhibitors, including Chelerythrine and Enzastaurin can suppress P-GP-mediated drug resistance by directly binding P-GP and inhibiting its function (23, 27, 28).

MATERIAL AND METHODS

Primary Cell Material and Cell Culture

Diagnostic peripheral blood (PB) and BM samples were from patients who went on to receive intensive chemotherapy within the AML14 and AML15 NCRI trials (Supplementary Table 1). Cord blood was obtained from the University Hospital of Wales or NHS Blood and Transplant (NHSBT). All sample collections were in accordance with the 1964 Declaration of Helsinki. AML cell lines were purchased from ATCC and ECACC and were maintained as recommended. Cell line identity was confirmed using Cell Line Authentication Service (February 2021; Eurofins Scientific, Luxembourg). CD34+ hematopoietic stem/progenitor cells (HSPC) were isolated, cultured, and transduced with lentivirus as previously described (29). PKCe overexpression in HSPC and AML cell lines, employed a lentiviral expression construct, PKCe_GFP-Puro, expressing the PRKCE gene (NM0-05400.3) under the control of the EF1α promoter and EGFP and puroR genes linked by a T2A sequence driven by a CMV promoter. Control cells were transduced with vector lacking the PRKCE gene. Knockdown of PRKCE was achieved with shRNA lentiviral vectors (detailed in Supplemental Methods). Control cells were transduced with a non-targeted construct. All vectors were purchased from VectorBuilder Inc (Chicago, USA). Transduced AML cell lines were selected with puromycin at 1µg/ml.

Myeloid colony forming assays were performed by limiting dilution in U bottomed 96-well plates (0.3 cells/well) using transduced (GFP+) HSPC enriched for cells highly expressing CD34 (CD34+) by FACS on day 3 of culture. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Fisher Scientific, Esher, UK) supplemented with 5 ng/mL IL-3, SCF, G-CSF and GM-CSF and incubated at 37°C with 5% CO2. Following 7 days of growth, individual colonies (> 50 cells) and clusters (> 5, < 50 cells) were scored.

Western Blot Analysis

Western blot analysis was conducted as previously described (30) using the antibodies described in Supplementary Table 2. Prior to western blot and colony analysis of transduced HSPC (GFP+) cells were purified by FACS using AriAII (BD Biosciences). Band intensity on documented membranes were measured by densitometric analysis using ImageJ (Fiji; v. 2.0.0.71), unless otherwise stated. To do this, a region of interest (ROI) was constructed around a specific band. From this, a histogram of peak intensity was generated, and a baseline of background intensity was set from the area surrounding the band within the ROI. The area under the curve was then calculated to give an arbitrary intensity value. The band intensities of the protein for
each sample were normalized to the band intensity of the loading control (GAPDH expression). PKCe expression in AML patient samples were subsequently converted into fg/1000 cells by comparing the relative band intensity to a recombinant PKCe standard of a known concentration.

**Analysis of PKCe Expression**

PKCe (PRKCE) mRNA expression data from human (MicroArray; GSE42519 (31)), and murine (GSE14833 (32); and GSE6506 (33, 34)) HSPC were obtained from Bloodspot (35). For AML patient samples, PKCe mRNA expression was assessed using the TCGA, NEJM 2013 dataset (36) which was accessed using cBioPortal (37, 38). For survival analysis, only patients that received intensive cytarabine (Ara-C) and daunorubicin (DNR) treatment regimens were included. Patients were subsequently stratified according to PKCe expression using the upper (high PKCe) and lower (low PKCe) quartiles. PKCe (PRKCE) mRNA expression was also determined in AML14 and AML15 NCRI UK clinical trial samples by Affymetrix DNA microarray. The raw data from the Hu133A GeneChip® was normalized using MASS or Robust Multi Array Analysis. Two probe-sets were used (236459_at and 226101_at) as these had the highest correlation and the highest association with MASS present calls (20/22). The DEPMAP portal was used to analyze transcriptional correlations in 44 AML cell lines using the Expression 22Q1 Public RNASeq data set.

**Flow Cytometric Analysis of Drug Sensitivity, Immunophenotype, and Cell Cycle**

Flow cytometric data were acquired using an Accuri C6 cytometer (BD Biosciences, USA) and analyzed using FCS Express v6 (DeNovo, California, USA). For cell growth, viability, and drug sensitivity assays, cells were seeded at 2x10^5/mL. After 48 hours, harvested cells were stained with a TOPRO-3 staining solution (RPMI supplemented with 100 mM HEPES and 50nM TOPRO-3 (T3605; Invitrogen, California, USA). For flow cytometric analysis, debris acquired during sample acquisition, defined as TOPRO-3 negative events with an FSC of <5x10^6, were excluded and viability and viable cell counts were determined from within the viable cell population of TOPRO-3 negative events. Absolute counts/mL were determined from a 10 µL fixed volume acquisition. For the drug sensitivity assays, cell growth and viability were normalized to cells treated with the vehicle control. Drug sensitivity was compared by calculating the drug concentration which gave a half-maximal response (EC50). Master stocks of each agent were generated according to the manufacturer’s instructions and are outlined in Supplementary Table 3. Once generated, all stocks were stored at -20°C in 100 µL aliquots for long-term storage unless otherwise stated. DNR accumulation was determined by measuring the arithmetic mean fluorescence (585/40nm) for cells treated with 100nM DNR, compared to vehicle controls. Immunophenotypic analysis was performed as previously described (39). P-glycoprotein (P-GP) expression on transduced AML cell lines was determined using the CD243-APC antibody (UIC2; BioLegend; Supplementary Table 2), while expression in patient samples has been described previously (40). Full details of the all the antibodies used for immunophenotypic studies are outlined in Supplementary Table 2.

For cell cycle analysis, transduced AML cell lines were seeded at 2x10^5 cells/mL and expanded for 48 hours before being harvested to 1 mL flow tubes and washed with 1 mL PBS. The washed cells were subsequently resuspended in PBS and fixed for 30 min on ice by adding 700 µL absolute ethanol. After fixation, the tubes were stored at -20°C overnight. The next day, the cells were centrifuged at 270xg and washed with 1 mL PBS before being resuspended in 50 µL staining buffer (PBS+0.5% (w/v) BSA+0.02% (w/v) sodium azide). The cells were then stained with 25 µL staining solution containing 40 µg/ml propidium iodide, and 0.1 mg/ml RNase (Sigma-Aldrich) diluted in PBS, for 30 min at 37°C. Samples were acquired within 20 min of this incubation using the Accuri™ C6 Plus flow cytometer. For analysis, debris and doublets were excluded, before the cell cycle status was resolved using the fluorescence intensity of propidium iodide (Sigma-Aldrich). Cell cycle analysis was performed using the Multicycle AV DNA analysis tool plug-in for FCS Express (DeNovo, Pasadena, USA).

**Statistical Analysis**

Statistical significance was determined by the tests outlined in figure legends and were performed using GraphPad Prism v 9.0.

**RESULTS**

**PKCe Is Frequently Upregulated in AML and Is Associated With Poor Clinical Outcome**

PKCe has demonstrated oncogenic properties and in solid cancers misexpression is associated with poor clinical outcomes and aggressive disease phenotypes (3–5, 41). To elucidate the frequency and pathophysiological attributes associated with PKCe upregulation in AML, we assessed PKCe expression in two independent patient sample cohorts. Initial data showed PKCe protein to be broadly expressed in AML (Figure 1A). We next carried out quantitative assessment in 70 AML patient samples. Compared to normal CD34+ BM blasts, PKCe protein was significantly overexpressed in 37% (26/70) of samples analyzed (Figure 1B). Patients overexpressing PKCe protein had a significantly lower complete remission (CR) rate compared to those with low PKCe protein expression (65% vs 84%; Figure 1C) with no significant association between any other prognostically significant patient characteristic. A possible explanation for this is that PKCe overexpression is associated with chemotherapy resistance. To validate these findings, we used a clinically annotated mRNA dataset. We first established a significant positive correlation between PKCe mRNA and protein expression in AML patient samples (Figure 1D) which indicated that mRNA is a reasonable predictor of PKCe protein expression in this context but found no relationship to FAB subtype or LSC phenotype (data not shown). We subsequently
evaluated the impact of PKCe mRNA expression in AML patients using the TCGA dataset (36). High PKCe mRNA expression was not significantly associated with any AML subset or known prognostic factors (Supplementary Figure 1). While the impact on overall survival (OS) was not significant (Figure 1E; p=0.052), high PKCe expression was significantly associated with reduced disease-free survival (DFS; Figure 1F). Although CR status was not available, reduced DFS is consistent with a treatment resistant phenotype. This data therefore supports the protein analysis in suggesting that high PKCe expression is associated with poor outcomes in AML. Together these data show that PKCe is frequently upregulated in AML and indicate that high PKCe expression is associated with poor patient outcomes, particularly in terms of response to initial chemotherapy.

PKCe Overexpression Promotes Monocytic Differentiation

Having determined that PKCe is frequently upregulated in AML, the mechanistic contribution of PKCe misexpression was evaluated. Initially we focused on whether overexpression contributes to the pathogenesis of AML by disrupting myeloid cell growth and differentiation. We first examined PKCe mRNA expression throughout normal myeloid development. In human
and murine myeloid progenitors, PKCε was highly expressed in HSC and MPP, however expression was observed throughout hematopoiesis (Supplementary Figure 2), indicating that PKCε may influence myeloid lineage commitment and differentiation. To support this, western blot analysis of human HSPC confirmed PKCε expression in all myeloid progenitor subsets (Figure 2A). The migratory differences observed are attributable to different phosphorylation states of PKCε (42). Further, when HSPC were cultured in conditions primarily supporting monocytic and granulocytic differentiation, PKCε expression increased with maturation (Figure 2B). Together these data indicate a potential developmental role for PKCε in myelopoiesis; however, we were unable to show any developmental consequences arising from PKCε knockdown in this context (Supplementary Figures 3-6) though we did observe a significant reduction in the growth of granulocytic cells (Supplementary Figure 4).

We next examined the functional consequences of PKCε overexpression. CD34+ HSPC stably overexpressing PKCε (Supplementary Figures 7B-C) were analyzed for changes in growth and differentiation by flow cytometry. Briefly, the myeloid lineages were resolved using the lineage discrimination markers CD13 and CD36, into monocytic (CD13highCD36high) and granulocytic (CD13lowCD36low) progenitors.

**FIGURE 2** | PKCε is expressed throughout hematopoiesis and promotes monocyte differentiation. (A) Western blot showing PKCε (MW-84kDa) protein expression in human umbilical cord blood (CD34+) derived monocyte (mono; CD14high), erythrocyte (ery; CD14negCD36high) and granulocyte (gran; CD14lowCD36low) progenitors. (B) Western blot analysis showing PKCε protein (MW-84kDa) expression in CD34+ HSPC over 13 days of culture where PKCε expression is shown alongside GAPDH (MW-36kDa) expression which was used as a loading control. For densitometry analysis of this western blot see Supplementary Figure 3A. PKCε expression was detected using the Cell Signaling Technologies antibody (22B10; Supplementary Table 2) while GAPDH was detected using the ThermoFisher Scientific antibody (GA1R; Supplementary Table 2). (C) Cumulative fold expansion of CD34+ HSPC total culture and (D) of monocytic progenitors (CD13highCD36high) transduced with the control or PKCε overexpression constructs. Immunophenotypic analysis of (E) CD11b and (F) CD14 expression (MFI) on transduced monocytic progenitors (CD13highCD36high) over 10 days of culture. Antibodies used for this analysis are outlined in Supplementary Table 2; n=4, *p < 0.05, **p < 0.01, ***p < 0.001 using two-way ANOVA with Bonferroni post-test comparison between the control and PKCε overexpression cells at day 10 of culture.
granulocytic (CD13<sup>low</sup>CD36<sup>low</sup>) progenitor populations (Supplementary Figure 8). Overall PKCe overexpression was associated with a 2.6-fold reduction in HSPC fold expansion over 10 days of culture (Figure 2C) and had a similar impact on colony formation (Supplementary Figure 9). Subset analysis revealed that PKCe overexpression significantly reduced the growth of monocytic progenitors (1.6-fold), compared to the control cultures (Figure 2D). This was associated with significant upregulation of the maturation markers CD11b (Figure 2E) and CD14 (Figure 2F). In contrast, we observed no significant impact on granulocytic proliferation (Supplementary Figure 10A) or differentiation (Supplementary Figures 10B, C). Together, these data indicate that increased PKCe expression promotes monocyte differentiation and does not support an oncogenic role for PKCe upregulation in the pathogenesis of AML, which is characterized by a block in terminal differentiation.

**PKCe Overexpression Promotes Selective DNR Resistance in AML Cell Lines**

Given that the data above are not supportive of an oncogenic role for PKCe overexpression in AML, we next examined the capacity of PKCe to promote chemoresistance in AML cells as this is a known consequence of PKCe overexpression in solid cancers (10, 41). To model this, we overexpressed PKCe in AML cell lines (U937 and HEL) which exhibited low and undetectable levels of endogenous PKCe protein, respectively. Ectopic PKCe expression was validated by western blot...
analysis (Figure 3A) and the resulting impact on cell proliferation and viability were assessed by flow cytometry. PKCε overexpression resulted in a 1.5-fold (U937) and 2.8-fold (HEL) reduction in growth compared to the control lines (Figure 3B). This was accompanied by a modest increase in the proportion of cells in the G2 phase of the cell cycle (Supplementary Figure 11), suggesting that the reduced proliferation rate could be due to perturbed cell cycle progression. Although PKCε overexpression also resulted in a 10% reduction in cell viability (Figure 3C), the small effect size suggests this is unlikely to be biologically significant and may have arisen from the relative accumulation of non-viable cells due to the reduced proliferation rate of the PKCε cultures.

We next established the effect of PKCε overexpression on Ara-C and DNR chemosensitivity; two of the central agents used in AML treatment. Despite the reduced growth of these cell lines, PKCε overexpression significantly sensitized U937 and HEL cells to Ara-C (2.9-fold and 1.9-fold respectively; Figure 4A), therefore inconsistent with the hypothesis that PKCε overexpression confers chemoresistance to this nucleotide analogue. In contrast however, PKCε overexpression increased the EC50 of DNR from 60nM to >100nM in both cell lines investigated (Figure 4B), demonstrating that PKCε overexpression can selectively confer resistance to DNR.

**PKCε-Mediated DNR Resistance Arises From P-GP-Mediated Drug Efflux**

As PKCε overexpression in AML cell lines promoted opposing phenotypes to Ara-C and DNR, it was initially hypothesized that the mechanism of PKCε-mediated chemoresistance was specific to the mode of action of DNR. The cytotoxicity of DNR is, in part, mediated by oxidative stress (43). Since PKCε has been reported to promote antioxidant capacity in AML (14) we assessed whether the PKCε overexpression lines showed resistance to other agents which induce oxidative stress (glucose oxidase, antimycin A, and arsenic trioxide). We found no evidence of resistance to any of these agents (rather sensitization was observed; Supplementary Figure 12) indicating that enhanced antioxidant capacity is unlikely to explain DNR resistance.

We next investigated whether PKCε influenced intracellular DNR concentrations. DNR has intrinsic fluorescence enabling direct measurement of intracellular drug accumulation by flow cytometry. Following 2 hours of DNR treatment, the PKCε overexpression cell lines showed a 1.8-fold reduction in

**FIGURE 4** | PKCε overexpression selectively promotes resistance to DNR. Line graphs showing the effect of increasing (A) Ara-C and (B) DNR concentrations on the viability of U937 (left) and HEL (right) cells transduced with the control or PKCε overexpression constructs, following 48 hours of treatment. Viability was determined by flow cytometry using TOPRO-3 staining (Supplemental Methods) and was normalized to the viability of cells treated with the vehicle control (PBS), at the time of harvesting; n=3; data represents mean ±1SD; *p < 0.05, **p < 0.01, ***p < 0.001 comparing test and control cultures at the dose indicated using two-way ANOVA with Bonferroni post-test comparison.
intracellular DNR compared to the control lines (Figure 5A) with similar data observed at the timepoint used for cytotoxicity assessment (2 days of treatment; Supplementary Figure 13A). Reduced DNR accumulation can arise from decreased drug uptake or increased expulsion via efflux pumps. DNR enters the cell by passive diffusion and so is unlikely to explain the observed reduction in intracellular DNR. Instead, it was hypothesized that the reduced DNR accumulation was due to increased drug efflux. P-GP (aka ABCB1/MDR1/CD243) is the best characterized efflux pump and is associated with reduced OS and CR induction in AML (44, 45). P-GP has several substrates including DNR (46), but does not transport Ara-C (47), potentially explaining the differential phenotypes exhibited in response to these chemotherapeutic agents.

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**FIGURE 5** | PKCε overexpression promotes P-GP upregulation in AML cell lines. (A) Bar charts showing the DNR fluorescence [fold change relative to cells treated with the vehicle control, PBS] of U937 (left) and HEL cells (right) transduced with the control and PKCε overexpression constructs, following 2 hours of treatment with 100nM DNR (n=4); data represents mean±1SD; * p<0.05 using paired t-test. (B, C) Representative histograms showing P-GP expression (MFI) in U937 and HEL cells transduced with the control and PKCε overexpression constructs. P-GP expression was detected by flow cytometry using the CD243-APC (UIC2; Supplementary Table 2) antibody and was compared to an isotype control (IgG2k-APC). (D) Corresponding bar charts showing ratiometric P-GP expression. P-GP expression was determined by flow cytometry using the CD243-APC (UIC2; Supplementary Table 2) antibody and was compared to an isotype control (IgG2k-APC; Supplementary Table 2); U937 (left) and HEL (right) (n=4), data represents mean±1SD; *p < 0.05, **p < 0.01 using paired t-test.
To determine whether DNR resistance in the PKCε overexpression cell lines could be explained by P-GP-mediated drug efflux, we first evaluated P-GP expression by flow cytometry (Figures 5B, C). PKCε overexpression led to a >3.0-fold increase in P-GP expression compared to the control cell lines (Figure 5D), suggesting that PKCε-mediated DNR resistance is associated with P-GP upregulation. To establish a causal relationship between PKCε and P-GP, inhibition assays were conducted using the selective P-GP inhibitor Zosuquidar hydrochloride [ZSQ (48)]. In the PKCε overexpression cell lines, ZSQ increased DNR accumulation to levels equivalent to the control cell lines (Figure 6A), whilst there was no significant effect on DNR accumulation in the control lines. Furthermore, ZSQ treatment overcame PKCε-mediated resistance, restoring DNR sensitivity to that of the control lines (Figure 6B). Importantly, ZSQ treatment alone did not significantly impact the growth, viability, or autofluorescence of any of the cell lines investigated (Supplementary Figures 13B, C). Together these data demonstrate that P-GP drug efflux fully accounts for the PKCε-mediated DNR resistance observed.

Overexpression systems tend to give rise to much higher levels of protein expression than is observed in physiological or diseased states. Therefore, the relationship between high PKCε expression and P-GP expression was also assessed in the TCGA
Neither of these factors with PRKCE expression in AML (data not shown).

Having determined that PKCc is frequently upregulated in AML, functional connotations were investigated. Analysis of PKCc mRNA and protein in hematopoietic progenitor cells showed an expression profile indicative of a role in myeloid cell differentiation. Functionally, knockdown of PKCc in HSPC had no significant impact apart from growth inhibition of granulocyte progenitors which suggests that its role may be redundant in this context. Conversely, PKCc overexpression in human HSPC impaired growth and promoted monocyte differentiation. This latter phenotype is concordant with the literature regarding other PKC isoforms (51, 52, 57) but is inconsistent with a role for PKCc in leukaemogenesis which is characterized by arrested myeloid development.

We next modelled the impact of PKC overexpression in the context of AML. PKCc overexpression significantly reduced the growth of U937 and HEL cells, potentially because of perturbed cell cycle progression. Although PKCc overexpression has been associated with pro-proliferative phenotypes (3, 58), PKC isoforms have also been associated with G2-phase delay and ERK-dependent upregulation of p21; a negative regulator of cell cycle progression (59, 60). We also found that PKCc overexpression conferred selective resistance to DNR. Given that an antioxidant role for PKCc has previously been described (14, 61), we first investigated whether PKCc-mediated resistance could be explained by the capacity of DNR to promote oxidative stress (43). However, we were unable to demonstrate enhanced antioxidant capacity in cells overexpressing PKCc. Instead, further work showed PKCc-mediated DNR resistance was accompanied by reduced intracellular DNR accumulation. DNR is a substrate for several efflux pumps, including P-GP (62) which is associated with reduced CR induction in AML (45) and reduces intracellular DNR (46). Furthermore, associations between PKCc and P-GP expression have previously been implicated in prostate and breast cancer cells (63, 64). In the present study, both PKCc overexpression AML lines showed P-GP upregulation by flow cytometry. Functional P-GP inhibition, with ZSQ, restored DNR accumulation and ablated DNR resistance in the PKCc overexpression cell lines. This demonstrates that P-GP drug efflux fully explains PKCc-mediated DNR resistance.

We also attempted to determine the effect of PKCc knockdown on drug sensitivity in AML cell lines and in primary AML patient-derived lines, however none of the cells in these panels demonstrated overexpression of PKCc making the experiments difficult to interpret. Specifically, in our panel of 10 AML lines only 3 showed detectable PKCc expression (Mv-4;11, U937 and OCI-AML5; Supplementary Figure 16) and none demonstrated overexpression, unsurprisingly while we achieved knockdown in U937 and Mv-4;11 (Supplementary Figure 17) we observed no significant impact on cell growth, viability or chemosensitivity (Supplementary Figures 18, 19). A similar outcome was obtained for our panel of 8 primary AML patient-derived lines (Supplementary Table 4) in the 2 lines which had detectable (though again modest) expression of both PKCc and P-GP (Supplementary Figure 20) knockdown also had no significant

DISCUSSION

Despite evidence that PKC signaling plays a role in hematopoietic cell differentiation and survival (8, 50–54), little is known about the role of PKCc in hematological malignancies. To address this, we determined PKCc expression in two independent AML patient cohorts and showed that PKCc is frequently overexpressed. PKCc was associated with poor OS and DFS (mRNA) and CR-induction (protein), suggesting that PKCc could be an indicator of poor treatment response; findings which are supported by observations in solid cancer (4) and a study describing a risk prediction model which incorporated multiple AML molecular datasets (RNAseq, methylation, GEP and SNP) (55). We have no data that indicates what is controlling the regulation of PRKCE transcription in AML. While we did identify a study in breast cancer cell lines that highlighted a role for STAT-1 and Sip-1 in regulating PKCc transcription (56), we found no correlation with the expression of
effect on drug sensitivity (Supplementary Figure 21); additionally, as ZSQ also had no impact on DNR sensitivity, these data suggest that P-GP was inactive in these cells (with the caveat that there was insufficient material to validate the extent of knockdown in these cells). Given the prevalence of PKC overexpression in vivo, these findings suggest that overexpression of PKCc is not sustained or is incompatible with in vitro culture of AML cells making the consequences of knockdown difficult to model. We also investigated the potential role of PKCB which was co-expressed with PKCc in AML cell lines; however combined knockdown of both isoforms in OCI-AML5 cells also failed to impact drug sensitivity (data not shown).

P-GP expression is regulated at an mRNA and protein level (49, 65). No evidence for transcriptional regulation of P-GP by PKCc was observed in AML patient samples from the TCGA 2013 dataset; however, at a protein level, high PKCc was associated with high P-GP expression in AML cell lines and patient samples, indicating a post-translational mechanism of regulation. Promoting endogenous PKC activity using the cPKC and nPKC agonist, PMA, suggested that activation of endogenous P-GP activity alone was insufficient to explain DNR resistance and that increased expression of P-GP mediated by PKCc represents the main driver of this phenotype though we cannot rule out that activation of P-GP may also be a contributing factor.

The fact that Ara-C is not transported by P-GP is consistent with the observed selective effect of PKCc overexpression on increasing resistance to DNR. In patients, these agents are administered in combination, with Ara-C being infused continuously and DNR administered in pulses, which raises the question of the overall effect of PKCc overexpression under these circumstances. These pharmacokinetic conditions are difficult to model in vitro where drug levels remain largely static, we are therefore currently attempting to model the impact of PKCc overexpression on such a treatment regimen in vivo.

Overall, this study demonstrates that PKCc upregulation occurs frequently in AML, is a poor prognostic indicator associated with reduced patient responses to induction therapy and could contribute to poor outcomes by decreasing leukemia cell DNR chemosensitivity through promoting P-GP expression and drug efflux. Whilst clinically applicable inhibitors have yet to be generated, these data suggest that selective inhibition of PKCc may be effective in improving drug responsiveness in AML patients.

AUTHORS CONTRIBUTIONS

RN designed and carried out experiments, analyzed all data and co-wrote the manuscript. AM, AA, and AI helped to collect and process human cord blood. SD provided training and technical support. CS provided the P-GP protein expression data in AML14/15 patient samples as well as support with the analysis. AG provided patient data and materials. SK provided clinical related advice and edited the manuscript. RD and AT secured funding, contributed to experimental design, data analysis and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by South East Wales Local Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this study can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.840046/full#supplementary-material

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