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ORIGINAL ARTICLE

PKCζ and PKMζ are overexpressed in TCF3-rearranged paediatric acute lymphoblastic leukaemia and are associated with increased thiopurine sensitivity

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Both tumour suppressor and oncogenic functions have been ascribed to the atypical zeta isoform of protein kinase C (PKCζ), whereas its constitutively active form PKMζ is almost exclusively expressed in the brain where it has a role in long-term memory. Using primers unique for either isoform, we found that both PKCζ and PKMζ were expressed in a subset of paediatric acute lymphoblastic leukaemia (ALL) cases carrying a TCF3 (E2A) chromosomal rearrangement. Combined PKCζ and PKMζ (PKC/Mζ) protein as well as phosphorylation levels were elevated in ALL cases, especially TCF3-rearranged precursor B-ALL cases, compared with normal bone marrow ($P < 0.01$). Furthermore, high PKC/Mζ expression in primary ALL cells was associated with increased sensitivity to 6-thioguanine and 6-mercaptopurine ($P < 0.01$), thiopurines used in ALL treatment. PKCζ is believed to stabilize mismatch-repair protein MSH2, facilitating thiopurine responsiveness in T-ALL. However, PKC/Mζ knockdown in a TCF3-rearranged cell line model decreased MSH2 expression but did not induce thiopurine resistance, indicative that the link between high PKC/Mζ levels and thiopurine sensitivity in paediatric precursor B-ALL is not directly causal. Collectively, our data indicate that thiopurine treatment may be effective, especially in paediatric TCF3-rearranged ALL and other patients with a high expression of PKC/Mζ.

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INTRODUCTION

The protein kinase C (PKC) family consists of at least 12 isozymes, divided into the classic or conventional PKCs, novel PKCs and atypical PKCs. All isozymes are characterized by an autoinhibitory regulatory N-terminal domain, connected through a flexible hinge region to a catalytic C-terminal domain. PKCζ (PKCzeta) and PKCι (PKCiota) are atypical PKCs, which function independently of Ca^{2+} and diacylglycerol. Instead, activation is established by the release of the regulatory region into an open conformation under influence of protein–protein interaction, and phosphorylation and autophosphorylation of two residues in the catalytic region: T410 and T560, respectively, in the case of PKCζ.[1]

PKCζ plays a critical role in mitogen-activated protein kinase-mediated nuclear factor kappa B activation[2–5] and stabilization of DNA mismatch-repair proteins,[6] and is involved in laeukemic cell differentiation,[7] cell migration and chemotaxis.[8,9] Several studies have addressed the role of PKCζ in cancer and describe diverse and seemingly contradicting functions. Overexpression of PKCζ induced apoptosis in ovarian carcinoma cells[10] and PKCζ deficiency increased cell proliferation and tumorigenesis in a mouse model for lung cancer.[11] In contrast, PKCζ-depleted cells displayed decreased tumorigenic activity in a colon cancer mouse model[12] and PKCζ inhibition impaired the migration of acute monocytic leukaemia and breast cancer cells *in vitro*.[8,9] PKCζ mediated drug sensitivity in a human leukaemia cell line, since PKCζ knockdown increased resistance to the thiopurines 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP),[13] compounds that are extensively used in the treatment of acute lymphoblastic leukaemia (ALL). Thus, PKCζ can have both tumour suppressor and oncogenic properties depending on the cellular context.

Several PKCζ isoforms have been described to date, among which a shorter form lacking the N-terminal regulatory domain of the protein, rendering it constitutively active (Figure 1a). This protein was first discovered in 1977 in bovine cerebellum[14] and named protein kinase M zeta (PKMζ). Some studies proposed that PKMζ could be formed by cleavage of full-length PKCζ protein.[14–16] Other studies, however, have gathered evidence that PKMζ expression in fact requires new protein synthesis,[17] and showed that transcription may be regulated by an alternative promoter within the PKCζ gene (*PRKCZ*),[18] resulting in a transcript identical to the 3′-end of *PRKCZ* but preceded by a unique 5′-end (Figure 1b).[18–21] Except for very low mRNA expression in rat kidney, PKMζ is considered brain-specific,[15,19,22] playing an important role in the maintenance of synaptic transmission, also known as long-term potentiation, and long-term memory.[23,24] Its expression is not detected in a range of other organ tissues.[19]

In this study, we describe the elevated expression of PKCζ and, more remarkably, PKMζ in paediatric TCF3-rearranged precursor B-ALL. This type of leukaemia occurs in approximately 5% of childhood ALL cases and is characterized by a chromosomal rearrangement involving the *TCF3* gene (also known as *E2A*). Most commonly, it concerns a t(1;19) translocation, resulting in a fusion between the transcription factors TCF3 and PBX1.[25] Here, we show that thiopurine treatment may be most effective in ALL patients with a high PKCζ and/or PKMζ expression.

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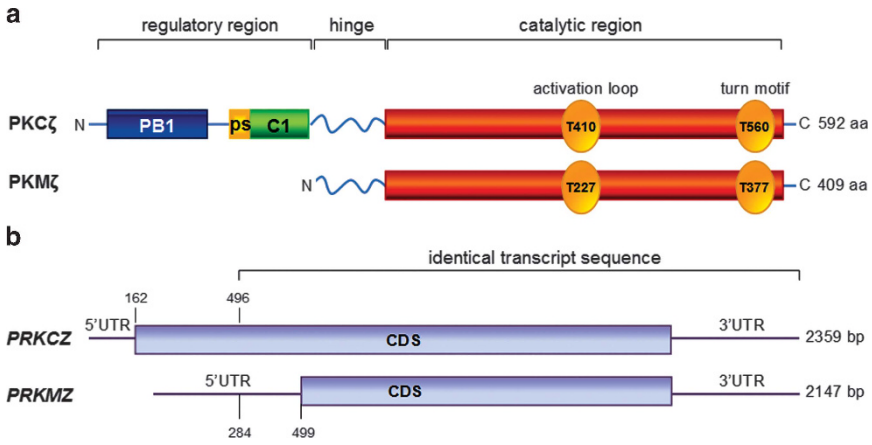

Figure 1. Schematic comparison of PKCζ and PKMζ protein and transcript domains. (**a**) The protein sequences of PKCζ and PKMζ contain an identical C-terminus including the complete catalytic domain. PKCζ activation requires phosphorylation at a threonine residue in the activation loop (T410) and subsequent autophosphorylation at the turn motif site (T560). A flexible hinge region connects the catalytic domain with the N-terminal regulatory region, which consists of a PB1 (Phox and Bem 1) domain not present in the conventional and novel PKCs and responsible for protein–protein interaction, an autoinhibitory pseudosubstrate domain (ps) and adjacent atypical C1 domain which both interact with the catalytic domain to keep PKCζ in a closed and inactive conformation.[26] PKMζ, however, lacks this N-terminal regulatory region and is therefore constitutively active. (**b**) *PRKCZ* and *PRKMZ* transcripts contain a unique 5′-end but share the final 1864 bp, including part of the coding sequence (CDS) and the complete 3′-untranslated region (UTR). The *PRKCZ* sequence was taken from the NCBI database (accession nr. NM_002744.4). The sequence of *PRKMZ* was derived from the NCBI database (*PRKCZ* isoform 2, accession nr. NM_001033581) and Hernandez *et al*.[19]

MATERIALS AND METHODS

Patient samples

Mononuclear cells were isolated from bone marrow or peripheral blood samples of children with newly diagnosed ALL or children without haematological disorder, as described previously.[27] Written informed consent was granted by parents or legal guardians to use leftover diagnostic material for research purposes, and studies were approved by the Erasmus Medical Center review board. ALL samples were enriched using immunomagnetic beads until morphological analysis of May–Grünwald–Giemsa-stained cytospins (Merck, Darmstadt, Germany) showed that samples contained at least 90% blasts. Patients were divided into T-ALL or precursor B-ALL by flow cytometry of cell surface markers, and precursor B-ALL was further divided as previously described[28] into the genetic subtypes MLL/11q23-rearranged, RUNX1-ETV6 (or TEL-AML1)/t(12;21)(p13;q22)-positive, TCF3 (or E2A)/19p13-rearranged and BCR-ABL1/t(9;22)(q34;q11)-positive by fluorescence *in situ* hybridization or reverse transcription-PCR, and hyperdiploid by karyogram (>50 chromosomes) and/or DNA index (⩾1.16). Precursor B-ALL patients were B-other when negative for above-mentioned features.

RNA was extracted from leukaemic cells using TRIzol reagent (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's protocol.

Cell culture

Leukaemic cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI+Glutamax and HEK293T cells in DMEM+Glutamax (Gibco BRL, Life Technologies), with 100 IU/ml penicillin, 100 µg/ml streptomycin (pen-strep; Gibco BRL), 0.125 µg/ml fungizone (Gibco BRL) and 10% or 20% fetal calf serum (Integro, Zaandam, The Netherlands). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. RNA was isolated with an RNeasy minikit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

Gene expression analysis

RNA was extracted from 859 ALL patients and 8 normal bone marrow (nBM) samples using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Affymetrix gene expression arrays (Santa Clara, CA, USA) were run and analyzed as described before.[28] Data normalization and correction for batch effects was performed as published earlier.[29] *PRKCZ* expression was determined for different subtypes of ALL and normal mononuclear bone marrow cells using the gene expression data of Affy probe ID 202178_at.

Reverse transcription-PCR

cDNA synthesis and PCR were performed as described before.[30] The following primer sets were ordered from Eurogentec (Maastricht, The Netherlands): *PRKCZ* foreward 5′-GGGGGACATCTTCATCA-3′, reverse 5′-CTC GGGAAAACATGAATG-3′; *PRKMZ* (encoding PKMζ) forward 5′-GGCCTTCCGT TAAATA-3′, reverse 5′-ATTCGGCTTTCTTCTCCT-3′; and Ribosomal Protein S20 (*RPS20*) forward 5′-AAGGGCTGAGGATTTTTG-3′, reverse 5′-CGTTGCGGC TTGTTAG-3′. *RPS20* expression was used as a control for cDNA input.

PCR was run at the following temperatures: 2 min 50 °C, 10 min 95 °C, 40× (15 s 95 °C and 1 min 60 °C). PCR products with 0.1% Orange G (Merck) and 3% Ficoll (Pharmacia, Stockholm, Sweden) were run on a 1% agarose (Roche Applied Science, Almere, The Netherlands) gel containing 1:30 000 GelRed (Biotium, Hayward, CA, USA). Gels were scanned with a Gel Doc XR imager and Quantity One v4.6.3 software (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot

Cells were lysed in a cold buffer containing phosphatase inhibitors and the protein concentration was determined with a bicinchoninic acid assay (Pierce, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. In total, 20 µg of denatured protein in Laemmli's loading buffer was run on a 10% acrylamide gel and subsequently blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in cold methanol-containing blotting buffer. C-terminally binding PKC ζ (H-1) antibody was ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA; sc-17781), N-terminally binding (personal communication with the manufacturer) PKCζ Antibody from Cell Signaling Technology (Danvers, MA, USA; #9372), β-actin from Abcam (Cambridge, UK) (ab6276), and a fluorescently labeled secondary IRDye antibody from LI-COR Biosciences (Lincoln, NE, USA). Blots were scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Reverse-phase protein arrays

Paediatric ALL and nBM samples were lysed in Tissue Protein Extraction Reagent (T-PER; Pierce, Thermo Scientific), containing 300 mM NaCl, 1 mM orthovanadate and protease inhibitors. Reverse-phase protein arrays were then performed and analyzed as described previously,[30] in collaboration with E. Petricoin, George Mason University, Manassas, VA, USA. PKC/Mζ protein expression was analyzed in 277 ALL patient samples, divided over two separate arrays and normalized together by using the data for 20 samples that were included on both the arrays. PKCζ phosphorylati on was determined on one array containing 171 patient samples

(see Supplementary Table S1 for patient characteristics). Primary antibodies were PKC ζ (H-1) (Santa Cruz Biotechnology; sc-17781) and Phospho-PKC ζ/λ (Thr410/403) (Cell Signaling Technology; #9378).

MTS and MTT cytotoxicity assay

Drug sensitivity of leukaemic cells to PKCζ pseudosubstrate (Tocris Bioscience, Abingdon, UK), 6-TG and 6-MP (Sigma-Aldrich, Zwijndrecht, The Netherlands), was determined with an MTS or 3-(4,5-dimethylthiazol-2-yl)-. 2,5-diphenyltetrazolium bromide (MTT) assay as described before.[27,30] Cell survival was determined after 72 h (cell lines) or 96 h (primary patient cells) of the exposure and expressed as a percentage of untreated control cells after subtraction of background signal. Measurements were performed in duplicate.

Lentiviral transfection of short hairpin RNAs

Using FuGENE transfection reagent (Promega, Madison, WI, USA), HEK293T cells were transfected with lentiviral helper vectors psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259), and a pLKO.1 Mission vector (Sigma-Aldrich) containing a short hairpin RNA (shRNA) against *PRKCZ* (TRCN0000010114) or a non-targeting control hairpin (SHC002) in addition to a puromycin selection marker. Virus was collected and concentrated by ultracentrifugation as described before.[30]

A virus titration was performed on KASUMI-2 cells to determine the virus concentration necessary for an optimal infection efficiency. KASUMI-2 cells were spin-infected and subsequently selected on puromycin as described previously.[30] Cell counting was performed with a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) after staining with propidium iodide and gating on live, propidium iodide-negative cells. Three independent experiments were performed.

Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 20 (Armonk, NY, USA). Significance of differential protein expression was determined with the non-parametric Mann–Whitney *U* test. The Student's *t*-test was used to determine statistical significance of growth inhibition and thiopurine sensitivity after PKMζ knockdown in three independent experiments. Correlation between drug sensitivity and protein expression levels was calculated with the Spearman's rank (r_s) test. $P < 0.05$ was considered statistically significant.

RESULTS

PKCζ and PKMζ mRNA and protein are expressed in paediatric ALL cases

Using primer sets that distinguish between *PRKCZ* and *PRKMZ* (registered in the NCBI database as *PRKCZ isoform 2,* encoding only the C-terminal part of the PKCζ protein; see Supplementary Figure S1 for sequence alignment of *PRKCZ* and *PRKMZ*), we tested cDNA samples of 20 paediatric precursor B-ALL patients for expression of these two genes. Strikingly, *PRKMZ* was abundantly expressed in most TCF3-rearranged cases and two other precursor B-ALL cases, whereas *PRKCZ* was expressed more moderately (Figure 2a).

Western blot analysis of PKCζ and PKMζ protein expression using a C-terminus-binding PKCζ antibody revealed an 80-kDa band, representing full-length PKCζ, and smaller bands including one corresponding to a protein of about 55 kDa, the estimated size of PKMζ (Figure 2b, left panel). We repeated the western blot using an antibody that recognizes an N-terminal epitope of PKCζ. As expected, only the full-length protein was detected (Figure 2b, right panel), suggesting the smaller bands detected with the C-terminal antibody could indeed be PKCζ isoforms lacking the N-terminal part of the protein.

High PKC/Mζ protein expression is especially associated with TCF3-rearranged ALL cases

We then investigated PKC/Mζ protein expression with reverse-phase protein arrays using the C-terminal PKC/Mζ antibody in a larger series of primary ALL samples ($n = 277$) and nBM mononuclear cells ($n = 14$). Compared with nBM, PKC/Mζ protein levels were elevated in ALL patients ($P < 0.001$) regardless of genetic subtype. Interestingly, the median expression in primary precursor B-ALL cells carrying a TCF3 translocation was nearly 14-fold higher than in precursor B-ALL cases without the translocation ($P < 0.001$; Figure 2c). Gene expression array analysis confirmed that elevated expression in TCF-3-rearranged cases was present on the mRNA level as well ($P < 0.001$; Supplementary Figure S2).

The phosphorylation levels of PKCζ/λ (T410/403) displayed a similar pattern as PKC/Mζ protein levels, with median phosphorylation levels being 2.6-fold higher in ALL cases ($N = 171$) than nBM ($N = 10$) ($P < 0.001$) and levels in TCF3-rearranged cases being 1.16-fold higher than in other precursor B-ALL cases ($P < 0.01$; Figure 2d).

In vitro cytotoxicity of PKCζ pseudosubstrate is not specific for high PKC/Mζ-expressing cell lines

Expression analysis in a cell line panel showed that *PRKMZ* mRNA was expressed in two out of three TCF3-rearranged precursor B-ALL cell lines: KASUMI-2 and 697, but not MHH-CALL-3 (Figure 3a). *PRKCZ* expression, on the other hand, was detected in KASUMI-2 but was more pronounced in the acute myeloid leukaemia cell line THP-1 and the human embryonic kidney cell line HEK293T. Elevated expression of PKC/Mζ was confirmed in KASUMI-2 and HEK293T on the protein level (Figure 3b). KASUMI-2, 697 and THP-1, and REH as a cell line negative for both PKCζ and PKMζ, were cultured in the presence of different concentrations of the PKCζ-inhibiting PKCζ pseudosubstrate. Cytotoxicity of the compound only occurred at concentrations above 2 μM, with no specificity for the cell lines with the highest PKC/Mζ expression, that is, KASUMI-2 and THP-1 (Figure 3c).

Primary TCF3-rearranged cells and ALL cells with a high PKC/Mζ expression are more sensitive to thiopurines

PKCζ stabilizes the DNA mismatch-repair protein MSH2. Diouf *et al.*[13] showed that loss of PKCζ leads to degradation of MSH2 and increased resistance of the paediatric T-ALL cell line CCRF-CEM to the thiopurines 6-TG and 6-MP. Based on this finding, we hypothesized that primary patients' leukaemic cells with high PKC/Mζ protein levels, that is, primarily TCF3-rearranged ALL cases, may be more sensitive to thiopurines than patients with a lower level of PKC/Mζ. To test this, we cultured primary ALL samples with high and low PKC/Mζ protein expression (with high expression defined as PKC/Mζ expression levels above the 90th percentile when ranking 277 patient samples) in the presence of 1.6 μg/ml 6-TG or 15.6 μg/ml 6-MP, and determined the effect on cell viability after 96 h. Primary leukaemic cells with a high expression, including three TCF3-rearranged cases and one non-TCF3-rearranged case, were more sensitive to both thiopurines than those with a low expression ($P < 0.01$; Figure 4a). There was a significant negative correlation between PKC/Mζ expression levels and leukaemic cell survival upon exposure to 6-TG ($r_s = -0.78$ and $P < 0.01$) and 6-MP ($r_s = -0.85$ and $P < 0.001$; Figure 4b). Examination of *PRKCZ* and *PRKMZ* mRNA expression revealed that both genes were highly expressed especially in the thiopurine-sensitive group (Figure 4c).

Primary cells of different genetic subtypes of precursor B-ALL, including TCF3-rearranged, hyperdiploid, ETV6-RUNX1 and B-other cases, were then exposed to a dilution range of 6-TG ($n = 135$) and 6-MP ($n = 130$). Median IC50 values of TCF3-rearranged cases were approximately fivefold lower for 6-TG ($P = 0.033$) and ninefold lower for 6-MP ($P = 0.021$), than for other precursor B-ALL cases (Supplementary Figure S3).

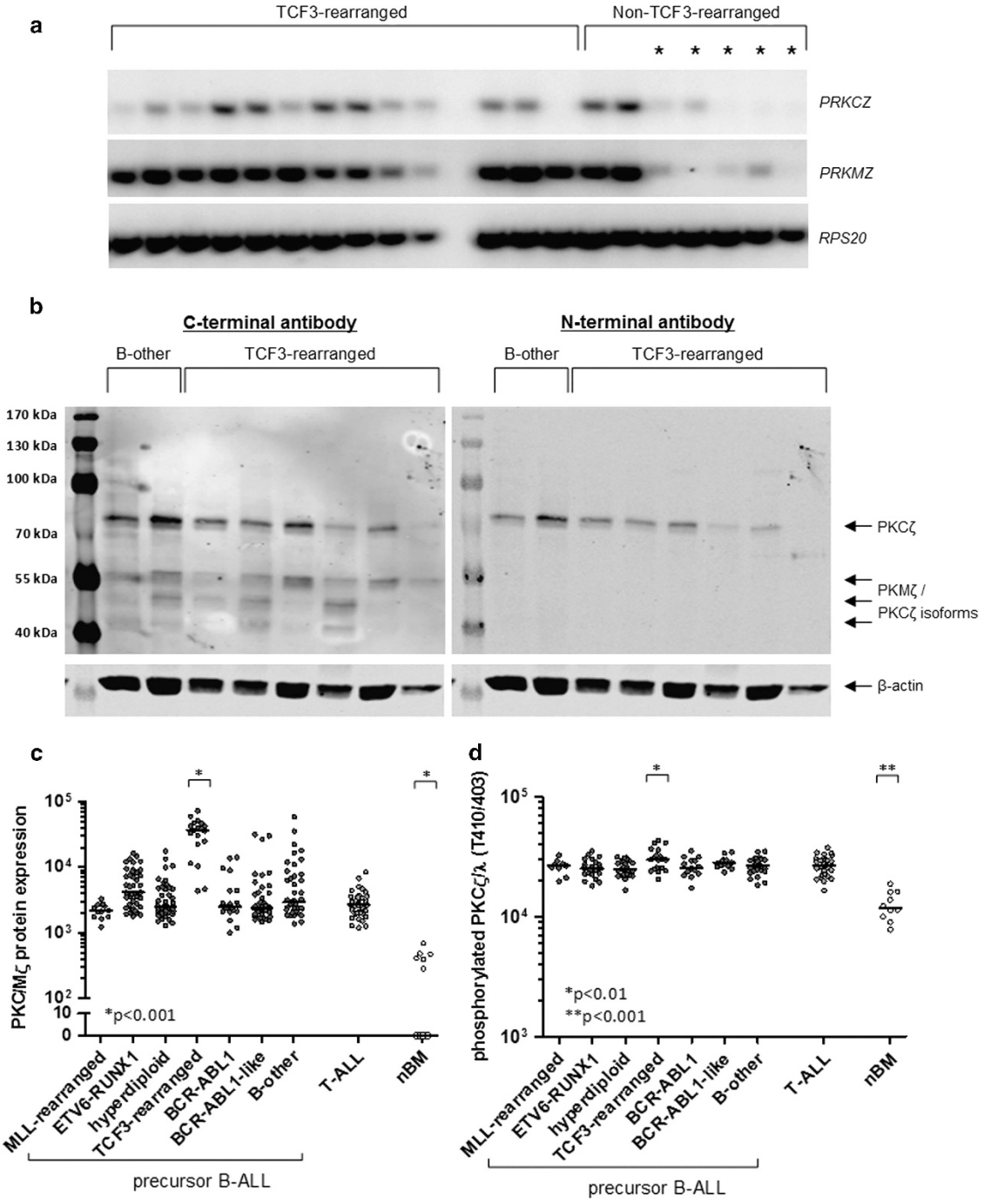

Figure 2. PKCζ and PKMζ expression and phosphorylation levels in ALL patients. (**a**) *PRKCZ* and *PRKMZ* mRNA expression in paediatric ALL patient samples ($n = 20$). *RPS20* was used as a loading control. Asterisks indicate samples with PKC/Mζ protein levels below the median as determined in Figure 2d. (**b**) Western blot analysis of PKCζ and PKMζ expression using a C-terminus- (left panel) or *N*-terminus (right panel)-binding antibody detected with a red or green fluorescent secondary antibody, respectively, enabling simultaneous hybridization. β-actin was used as a protein loading control. Reverse-phase protein arrays (RPPA) were used to determine PKC/Mζ protein levels (**c**) and T410/403 phosphorylation levels of PKCζ/λ (**d**) in genetic subtypes of precursor B-ALL ($n = 237$), T-ALL ($n = 40$) and normal bone marrow (nBM; $n = 14$). *Y* axes present protein expression in units of fluorescence, corrected for background and total protein signals. Statistical significance is indicated for nBM vs ALL, T-ALL vs precursor B-ALL, and for each genetic subtype of precursor B-ALL vs all other precursor B-ALL subtypes.

PKMζ knockdown in a cell line model does not convey resistance to thiopurines

Next, we investigated whether PKC/Mζ is directly responsible for increased sensitivity of TCF3-rearranged cases to thiopurines. The KASUMI-2 cell line, expressing high levels of PKMζ, was chosen as a model representative for TCF3-rearranged precursor B-ALL. An shRNA designed to bind *PRKCZ* was used to establish knockdown of *PRKMZ*, since its target sequence is located in the identical region of both mRNAs. Expression analysis showed that this method effectively achieved PKMζ knockdown on the mRNA (Figure 5a) and protein (Figure 5b) level. In addition, a modest reduction in MSH2 protein expression was observed (Figure 5b).

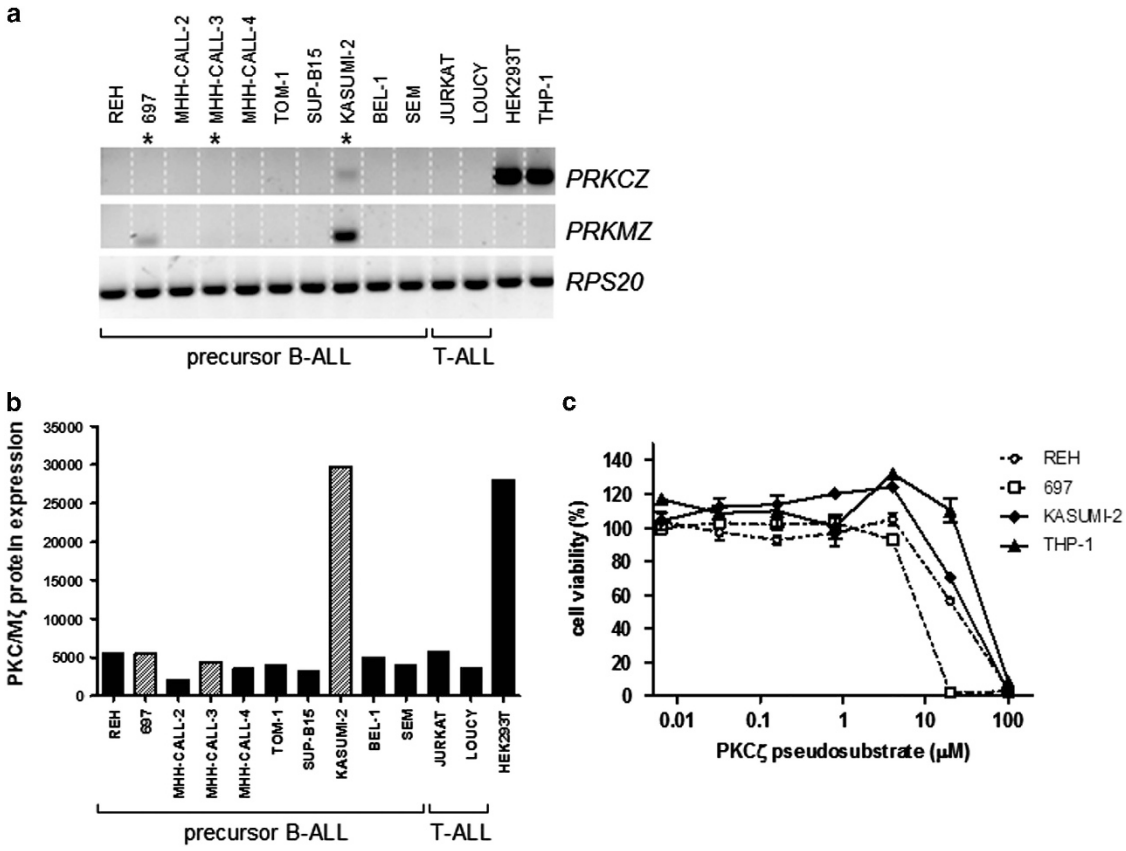

Figure 3. PKCζ and PKMζ expression and PKCζ pseudosubstrate efficacy in ALL cell lines. (**a**) *PRKMZ* and *PRKCZ* mRNA expression in 12 ALL cell lines. One embryonic kidney cell line (HEK293T) and one acute myeloid leukaemia cell line (THP-1) were included as positive controls. *RPS20* was used as a loading control. Asterisks denote precursor B-ALL cell lines carrying a TCF3 translocation. (**b**) PKC/Mζ protein expression in ALL cell lines and HEK293T as determined by Reverse-phase protein arrays (RPPA). TCF3-rearranged cell lines are represented by hatched bars. Protein levels are expressed in units of fluorescence, corrected for background and total protein signals. (**c**) Survival curve of a PKMζ-high (KASUMI-2), PKMζ-low (697), PKCζ-high (THP-1) and PKC/Mζ-negative (REH) leukaemic cell line after 72 h of exposure to PKCζ pseudosubstrate. Cell viability was determined with an MTS assay and expressed as a percentage of control cells exposed for 72 h to solvent only.

PKMζ knockdown inhibited normal cell proliferation, as determined by comparing cells transfected with the targeting shRNA or a control vector (Figure 5c). These cells were then exposed to 6-TG or 6-MP and IC50 values were determined. Repression of PKMζ and reduction of MSH2 expression did not increase the level of resistance to thiopurines. On the contrary, even a slight sensitizing effect (significant for 6-TG) was seen upon PKMζ knockdown (Figure 5d).

DISCUSSION

The current multi-drug therapy regimen for children with ALL achieves an event-free survival rate of approximately 80%,[25] but is intensive and has a large impact on the quality of life during and after treatment. The identification of patients that could benefit from a new, more specific and potent drug or from dose reduction of a currently used drug would enable customization of protocols to be more effective and less toxic. In this study, we described a group of paediatric ALL patients whose leukaemic cells highly express PKC/Mζ and appear to be relatively sensitive to the thiopurines 6-TG and 6-MP.

Before this study, the only non-neuronal tissues believed to be expressing PKMζ were rat kidney, although in very low amounts and undetectable on the protein level.[19] Human leukaemic cells, however, had never been investigated. We have shown that predominantly TCF3-rearranged paediatric ALL cells express PKMζ and/or PKCζ, both at the mRNA and protein level. Moreover, also the phosphorylation levels of these proteins, indicative for activation, were higher in these cases. The chimeric transcription factor arising from the TCF3-PBX1 translocation may enhance the expression of *PRKCZ* and/or *PRKMZ*. However, several findings argue with this theory. First, the TCF3-PBX1 fusion protein binds the genomic sequence ATCAATCAA.[31,32] Screening of 10 000 nucleotides upstream of the *PRKCZ/PRKMZ* genomic sequence did not result in the identification of a putative TCF3-PBX1-binding site (data not shown). This is supported by a recent study by Diakos *et al.*,[33] showing that *PRKCZ* was neither among the top-most downregulated genes upon TCF3-PBX1 silencing, nor identified as a direct binding target of the fusion protein in chromatin immunoprecipitation-on-chip experiments. Second, high PKC/Mζ levels were also detected in non-TCF3-rearranged precursor B-ALL patients, which suggests an alternative mechanism for transcriptional activation may exist in ALL. Since PKMζ protein is identical to the active form of PKCζ protein, there is no reason to believe that it performs a different function in ALL cells than PKCζ. Whether there is a regulatory mechanism for transcription of either isoform, and if so, what the function may be of PKMζ expression in ALL, are questions remaining to be answered.

Highly expressed proteins could be suitable targets for cancer therapy when they are crucial for proliferation, survival or chemotaxis of the cancer cell. PKCζ inhibitors have been shown to have the potential to inhibit lung[34] and breast[35] cancer metastases. In this study, we investigated the direct cytotoxic effect of PKCζ pseudosubstrate *in vitro*. Cytotoxicity was observed in acute leukaemia cell lines in high concentrations, but within the

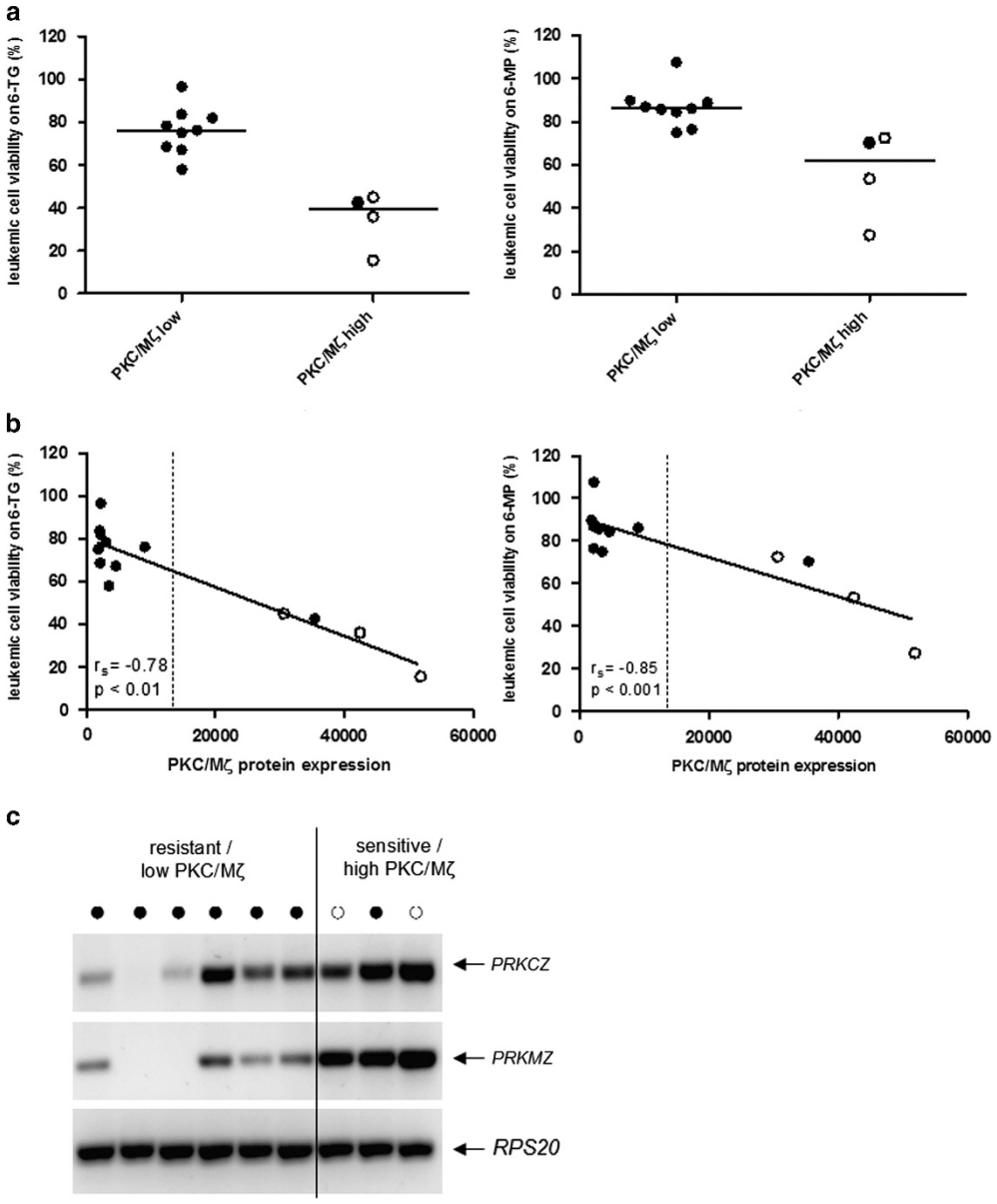

Figure 4. High PKC/Mζ protein expression is associated with increased sensitivity to thiopurines. Primary leukaemic cells of precursor B-ALL patients ($n = 13$) were exposed to 1.6 μg/ml 6-TG or 15.6 μg/ml 6-MP. Leukaemic cell viability was determined with an MTS assay after 96 h and expressed as a percentage of cells treated with solvent only. Cases were categorized into those with low and high PKC/Mζ protein levels with a cut-off of 13 513 fluorescence units (that is, the 90th percentile of 277 ranked patient samples). Open circles represent TCF3-rearranged cases. (**a**) Comparison of cell viability after exposure of low-PKC/Mζ and high-PKC/Mζ cases to 6-TG (left) and 6-MP (right). Horizontal lines indicate group medians. (**b**) Correlation plots of PKC/Mζ protein expression and leukaemic cell viability after exposure to 6-TG (left) or 6-MP (right). The vertical dotted line indicates the cut-off for low–high expression. Graphs present the best fit and Spearman correlation coefficient (r_s). (**c**) *PRKCZ* and *PRKMZ* mRNA expression in thiopurine-resistant low-PKC/Mζ and sensitive high-PKC/Mζ cases.

functional midmicromolar range that has been reported in literature.[34–36] Cell lines with a high PKCζ or PKMζ expression were not more sensitive to the inhibitor than cell lines with a lower or no expression, which would suggest that PKC/Mζ inhibition does not directly decrease leukaemic cell viability. However, PKMζ knockdown in the KASUMI-2 cell line did lead to decreased cell proliferation. It is therefore more likely that the general cytotoxicity of the PKCζ pseudosubstrate is due to off-target effects of the compound.

Diouf *et al.*[13] recently showed that shRNA-mediated knockdown of *PRKCZ* (and most probably also of *PRKMZ* since the used shRNAs hybridize to both isoforms) resulted in increased resistance of the paediatric T-ALL cell line CCRF-CEM to 6-TG and especially 6-MP by failure to prevent degradation of mismatch-repair protein MSH2. We were not able to establish this relation in our cell line model for TCF3-rearranged precursor B-ALL, as PKMζ knockdown in KASUMI-2 did not lead to resistance to thiopurines but even to a slightly increased 6-TG sensitivity. Either the reduction in MSH2 resulting from PKMζ repression was not sufficient to prevent thiopurine cytotoxicity, or KASUMI-2, with only limited PKCζ expression, is not an ideal representative of primary cells, which generally displayed both PKCζ and PKMζ expression. This cell line model therefore shows that increased thiopurine sensitivity is not linked to high PKMζ through MSH2, but this cannot be ruled out for PKCζ. Our data in primary cells did show that high combined expression levels of PKC/Mζ in ALL are associated with increased

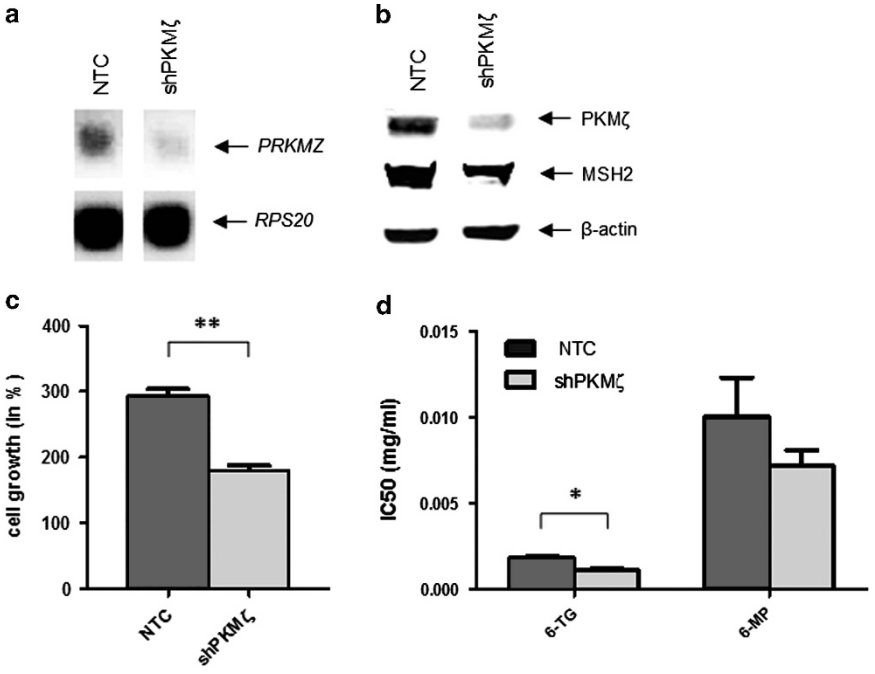

Figure 5. PKMζ knockdown in a TCF3-rearranged cell line. KASUMI-2 cells were transfected with a short hairpin against PKMζ (shPKMζ) or a non-targeting control hairpin (NTC), and effects were analyzed after 120 h. (**a**) *PRKMZ* mRNA knockdown was assessed by reverse-transcription-PCR (RT-PCR). *RPS20* was used as a reference gene. Results are representative of three independent experiments. (**b**) PKMζ and MSH2 protein expression was assessed by western blot. β-actin expression is shown as a loading control. Results are representative of three independent experiments. (**c**) The effect of PKMζ knockdown on cell growth. (**d**) 6-TG and 6-MP IC50 values for shPKMζ- or NTC-transfected cells. IC50 values were determined with an MTS assay by exposure of cells to a thiopurine dilution range for 72 h. $^{*}P < 0.01$, $^{**}P < 0.001$.

sensitivity to 6-TG and 6-MP. It is therefore likely that factors other than MSH2 contribute to thiopurine sensitivity in paediatric ALL, as is supported by data of Krynetskaia *et al.*,[37] showing that thiopurine sensitivity is only partly mediated by MSH2. It is a possibility that a common factor is responsible for both increased PKC/Mζ expression and thiopurine sensitivity in a subset of paediatric ALL cases.

A study by Frost *et al.*[38] previously showed that leukaemic cells from TCF3-PBX1-positive patients were relatively sensitive to 6-TG as compared with non-TCF3-PBX1 patients. Besides confirming these findings, our study additionally shows an increased sensitivity to 6-MP. This finding is relevant, because most study groups intensively use 6-MP during the different phases of their current treatment protocols.

In conclusion, our study suggests that both 6-TG and 6-MP may be effective especially in paediatric TCF3-rearranged ALL patients and possibly in other patients with a high expression of PKC/Mζ. Although further studies are required to establish the mechanistic relation, pre-treatment screening of patients for high PKC/Mζ expression, for which TCF3-rearrangement is an indication, will enable the selection of those patients that may benefit most from the use of thiopurines. This could be a step toward personalized medicine by treating individual patients in the most effective way.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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