Introduction

Improvements in drug therapy and in patient care have progressively improved survival among younger patients with acute myeloid leukemia (AML). However, the risk of AML increases with age and success rates in older patients remain very poor.1 As a result, the overall outcome remains very poor. In a study of 214 unselected AML patients in Sweden in whom the median age of patients was 69.5 years, median survival was 5.8 months and probable survival at 5 years was calculated as 9.3%.2 The clinical patients was 69.5 years, median survival was 5.8 months and unselected AML patients in Sweden in whom the median age of result, the overall outcome remains very poor. In a study of 214 years,3 and the impact of this statistic on AML and indeed other graphics in developed countries toward a more elderly population. The problem of AML is exacerbated by the shifting population demo-
vously improved survival among younger patients with acute myeloid leukemia (AML). However, the risk of AML increases in the treatment of chronic myelogenous leukemia (CML).4 However, a second and potentially invaluable approach to identifying therapeutically useful small molecules is to revisit remedies supported by this belief, with a biopsy-verified case study demonstrating remission of a basal cell carcinoma after home treatment with crude E peplus sap.5 The active agent in E peplus sap has now been identified as a hydrophobic diterpene ester, ingenol 3-angelate (PEP005), which has been shown to have topical antitumor activity against human cancer cell lines grown as subcutaneous tumors in mice.7

PEP005 is a potent activator of protein kinase C (PKC),8 a family of signaling isoenzymes that regulate many cell processes including proliferation, differentiation, and apoptosis.9,10 This signaling pathway has already been the target of several novel anticancer agents,11-13 and the potential for therapeutic activity of PEP005 beyond skin cancer therefore seemed likely.

Here we have investigated the in vitro antileukemic activity of PEP005 against both AML cell lines and primary AML blast cells and determined more precisely its mode of action. Our data show
that PEP005 was able to induce apoptosis in cell lines and primary AML blasts, whereas, in contrast, nonmalignant myeloid blasts were resistant to PEP005-induced apoptosis but were induced to partially differentiate. Importantly, not all myeloid cell lines were equally sensitive to PEP005, and we noted that the resistance displayed by KG1a cells was associated with the failure to express PKC-δ. However, transfection of KG1a cells with enhanced green fluorescent protein (EGFP)–PKC-δ restored not only PKC-δ expression but also an apoptotic response to PEP005. Analyses using selective PKC inhibitors and nuclear relocation further indicated that the antineoplastic action of PEP005 against AML is mediated via activation of PKC-δ. Together our findings provide a compelling case for the targeted activation of PKC-δ as a rational therapeutic intervention in acute myeloid leukemia and, by extension, other cancers in which tumor progression has not involved suppression of this PKC isoenzyme.

### Patients, materials, and methods

#### Cell cultures

The myeloid leukemia cell lines HL60, NB4, U937, K562, and KG1a were all grown in RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (Sera Laboratories International, Crawley, United Kingdom), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich, Poole, United Kingdom) at 37°C and 5% CO2. AML blasts were isolated from patient bone marrow aspirates and all grown in RPMI 1640 medium (Life Technologies, Paisley, United Kingdom). The myeloid leukemic cell lines HL60, NB4, U937, K562, and KG1a were also used to prepare cytopsins, which were then differentially stained using a commercial May-Grünwald-Giemsa stain (Diff-Kwik; Gamidor, Abingdon, United Kingdom) to identify cells with a blast or differentiated or apoptotic morphology. Slides were examined using a Zeiss III RS brightfield microscope equipped with a 40×0.75 water-immersion objective (Carl Zeiss, Jena, Germany). Images were captured with a SPOT2 camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Image-Pro 4.0 software (Media Cybernetics, Silver Spring, MD).

#### Protein kinase C activation and expression assays

PKC isoenzyme expression in leukemic cell lines was determined by Western blotting. Cells (0.5 × 106) were lysed in lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.4, containing 150 mM NaCl, 0.5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of aprotinin, leupeptin, and pepstatin A, and 1% Triton-X-100). The lysate was spun at 1000g for 10 minutes to isolate nuclei and was then combined with 1:1 sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 minutes. The extracts were then analyzed by Western blotting using antibodies to the major isoenzymes found in myeloid cells, namely PKC-α, PKC-β, PKC-δ, and PKC-ζ (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using an enhanced chemiluminescence (ECL) method (Amer sham Pharmacia, Buckinghamshire, United Kingdom).

PKC translates from the soluble to the particulate fraction of cells when activated, and this method was used to assess activation of PKC-δ by PEP005 and PMA. Briefly, cells were incubated for 10 minutes in medium alone or 20 nM PEP005 or 20 nM PMA, washed twice in phosphate-buffered saline (PBS), and then lysed by homogenization in hypotonic lysis buffer in the absence of detergent. Lysis was checked using trypan blue uptake. Nuclei were isolated by centrifugation at 1000g for 10 minutes. Centrifugation at 100 000g for 45 minutes at 4°C was used to isolate the cytosol (supernatant) and cell membrane (pellet) fractions. All were taken up in SDS sample buffer and analyzed for PKC-δ by Western blotting.

#### Caspase-3 phosphorylation

To determine if PEP005 induced caspase-3 phosphorylation, HL60 cells were incubated with 1.5 × 107 Bq/mL 32PO4 (Amersham Pharmacia) in phosphate-free medium for 3 hours prior to addition of 20 nM PEP005 or PEP005 and 5 μM rottlerin (Calbiochem, Nottingham, United Kingdom). Cells were then lysed and caspase-3 was immunoprecipitated with an anti–caspase-3 antibody (BD Pharmingen, Oxford, United Kingdom) or an irrelevant isotype-matched control antibody (DAKO), and the immunoprecipitate was isolated using an anti–mouse immunoglobulin G (IgG) antibody (DAKO) and protein G–coated magnetic beads (microbeads; Miltenyi Biotec). The isolate was taken up in SDS sample buffer and run on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel and the labeled proteins were visualized using a fluorophorimager.

#### PKC transfection studies

KG1a cells were transiently transfected with EGFP-tagged mouse PKC-δ subcloned into pEGFP-N1 plasmid (kindly provided by P. Blumberg, National Cancer Institute [NCI], Bethesda, MD) using an Amaxa nucleofection apparatus (Amaxa, Koeln, Germany). Transfection efficiency was approximately 35% as judged by fluorescence-activated cell sorter (FACS) analysis, and cells were treated with PEP005 (0.2 μM-20 μM) 24 hours after transfection. Cell viability in EGFP-positive cells was assessed by analysis of cell morphology (forward scatter and side scatter profile) by FACS and loss of viability confirmed in the total cell culture by MTT assay after 3 days. Briefly, 24 hours after transfection, 2 × 105 cells were plated in 5 wells in 96-well plates and exposed to 0, 0.2, 2, and 20 μM PEP005. At 72 hours, 20 μL MTT substrate at 5 mg/mL was added and plates were incubated at 37°C. After 3 hours, 150 μL media was removed and replaced...
with 200 μL dimethyl sulfoxide (DMSO). Absorbance at an optical density (OD) of 550 nm was read on a Biotech plate reader (Amersham Pharmacia) and corrected for absorbance obtained from blank media controls.

Immunofluorescence imaging of PKC-δ-GFP activation

KG1a cells were transfected with PKC-δ-EGFP or pEGFP-N1 (vector control). At 24 hours after transfection, cells were treated with 0, 0.2, 2, and 20 μM PEP005 and cytospins prepared after 15 minutes. Slides were fixed with 2% paraformaldehyde in PBS for 20 minutes, rinsed briefly in PBS, and mounted in Vectashield containing DAPI (4,6 diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). Slides were examined using a Leica Fluorescence microscope (Leica, Heidelberg, Germany) fitted with a ×60 oil immersion objective. Images were captured using a Hamamatsu C4742-95 camera (Grafters Imaging, Austin, TX) and analyzed using OpenLab 3.1 software (Improvision, Coventry, United Kingdom).

Statistics

Data presented here represent a minimum of 3 experiments and, where appropriate, data are expressed as means plus or minus SD. Statistical significance was assessed by Student t test, and a P value less than .05 was taken as a significantly different value.

Results

PEP005 has antileukemic effects against cell lines and primary AML blasts

PEP005 is a small molecule activator of the 8 classical and novel PKC isoenzymes. PEP005 has already been shown to have antineoplastic potential against skin cancers, and an initial screen of the cytotoxic effects of PEP005 against other cancer cell types revealed potent effects on leukemic cell lines (data not shown). The antileukemic potential of PEP005 was therefore investigated further.

There were 5 myeloid leukemia cell lines treated with PEP005, and differentiation and apoptosis were determined. Differentiation was initially assessed by gain of CD11b expression (Figure 1A). Apoptosis was measured by FACS analysis of sub-G1 DNA (Figure 1C) and caspase-3 activation (data not shown). There were 3 cell lines (HL60, NB4, and U937) induced to express CD11b (Figure 1A) and enter apoptosis (Figure 1C) and caspase-3 activation (data not shown). There were 5 myeloid leukemia cell lines treated with PEP005, and differentiation and apoptosis were determined. Differentiation was initially assessed by gain of CD11b expression (Figure 1A). Apoptosis was measured by FACS analysis of sub-G1 DNA (Figure 1C) and caspase-3 activation (data not shown). There were 3 cell lines (HL60, NB4, and U937) induced to express CD11b (Figure 1A) and enter apoptosis (Figure 1C) and caspase-3 activation (data not shown). There were 5 myeloid leukemia cell lines treated with PEP005, and differentiation and apoptosis were determined. Differentiation was initially assessed by gain of CD11b expression (Figure 1A). Apoptosis was measured by FACS analysis of sub-G1 DNA (Figure 1C) and caspase-3 activation (data not shown). There were 3 cell lines (HL60, NB4, and U937) induced to express CD11b (Figure 1A) and enter apoptosis (Figure 1C) and caspase-3 activation (data not shown).
therapeutic window for PEP005 that spans at least 2-log concentrations.

**PEP005 responsiveness correlates with PKC-δ expression**

Recent studies have already established that PEP005 is an activator of PKC signaling pathways.⁸ PKC is a multi-isoenzyme family with differential tissue expression and involvement in cell proliferation, differentiation, and apoptosis.⁹,¹⁷ We therefore determined if the varied responsiveness of leukemic cell lines to PEP005 was related to differential expression of PKC isoenzymes. We measured expression of the 4 isoenzymes that predominate in myeloid cells, namely PKC-α, β, -δ, and -ζ. There was no consistent association between PEP005 responsiveness and expression of PKC-α, PKC-β, or PKC-ζ (Figure 4A). However, expression of PKC-δ did correlate with PEP005 sensitivity, with good levels of this isoenzyme expressed in HL60, U937, and NB4 and no significant expression detected in KG1a (Figure 4B). In addition, PEP005 induced activation of PKC-δ in HL60 cells, detected by translocation of the enzyme from the cytosol to the membrane fraction of cells (Figure 4C). Interestingly, initial translocation of PKC-δ was to the nuclear and cell membranes following treatment with PEP005, whereas PMA induced translocation only to the cell membrane (Figure 4C). This differential effect of PEP005 and PMA on PKC-δ subcellular location was also reported in COS cells transfected with PKC-δ.⁸ PKC-δ is known to undergo proteolysis by caspase-3 to release a proapoptotic 40-kDa catalytic fragment, and we therefore determined if PEP005 treatment resulted in the production of the 40-kDa fragment of PKC-δ. Figure 4D shows that the 40-kDa fragment was produced rapidly in HL60 cells. The likelihood that the proapoptotic activity of PEP005 was mediated through activation of PKC-δ was further supported by the observation that PEP005-induced apoptosis of HL60 cells was inhibited by the broad range
PKC inhibitor bisindolylmaleimide 1 (Bis-1, Figure 4E), but not by Go6976, which is an inhibi
tor of the classical PKC isoenzymes PKC-α and -β.18 Interestingly, the differentiating actions of ATRA on leukemic cells have been shown to be in part mediated via activation of PKC-δ and phosphorylation of retinoid acid receptor α (RARα).19 This may explain why PEP005-induced partial differenti
ation was seen only in the ATRA-responsive cell lines. To investigate the possible proapoptotic mode of action of PKC-δ, we determined the effect of PEP005 treatment on caspase-3 phosphorylation. A very recent publication reported that PKC-δ was able to
phosphorylate and activate caspase-3 in monocytes.20 Figure 4F shows that PEP005 was able to induce caspase-3 phosphorylation and that this was ablated by the PKC-δ inhibitor rottlerin.

**Expression of PKC-δ in KG1a cells confers responsiveness to PEP005**

To confirm the key role played by PKC-δ in mediating the effects of PEP005, KG1a cells were transfected with EGFP-tagged PKC-δ and 24 hours later were treated with PEP005 for 3 days. Transfection was confirmed by FACS analysis and transfection efficiency was approximately 35%. Expression of PKC-δ at the protein level was confirmed by Western blotting 24 hours after transfection (Figure 5A). Importantly, exposure of EGFP-positive PKC-δ–expressing KG1a cells to PEP005 resulted in apoptosis, assessed by increased forward scatter and decreased side scatter by FACS, at doses commensurate with those inducing apoptosis in HL60, U937, and NB4 cells. In contrast, EGFP-positive cells transfected with control vector were resistant to PEP005-induced apoptosis even at 20 μM PEP005 (Figure 5B). Loss of viability was confirmed in the total cell population by MTT assay (data not shown).

Finally, fluorescence microscopy confirmed that PEP005-induced apoptosis of EGFP–PKC-δ–expressing KG1a cells was preceded by the activation of PKC-δ. Figure 5C shows that prior to exposure to PEP005, EGFP–PKC-δ was located predominantly in the cytosol of KG1a cells but that PEP005 treatment induced its translocation to the nucleus and perinuclear region. Furthermore, we have shown previously that translocation of PKC-δ to the nucleus is associated with induction of apoptosis in human neutrophils and T cells21 and that key substrates of this isoenzyme involved in the induction of apoptosis include nuclear lamin B.22

We conclude therefore that PEP005 mediates selective and potent antineoplastic actions against AML cells that are mediated via the activation of PKCδ and its translocation to the nucleus.

**Discussion**

The key role of the PKC family of isoenzymes in the regulation of cell proliferation, differentiation, and apoptosis has identified them as important targets for drug design.23 In particular, 2 isoenzymes, PKC-α and PKC-δ, appear to play specific roles in tumor promotion and suppression. PKC-α promotes EGF-transforming activity24 and is generally antiapoptotic,25 whereas PKC-δ is antiproliferative,26 promotes retinoid-induced differentiation,19 and mediates proapoptotic signals.27-29

The screening of small molecule libraries is a topical and rational approach to the discovery of novel drugs that target these important signaling pathways, but our data illustrate that traditional medicines represent another powerful and relatively untapped source of therapeutic small molecular compounds that are not readily synthesized chemically.

Although PKC-δ selective activators30 and inhibitors31 have been isolated from natural sources, they have not been used clinically to date, possibly due to their acute toxicity or the lack of suitability for large-scale production. Consequently, targeting of PKC isoenzymes therapeutically has, to date, been predominantly via broad specificity agents. Bryostatin 1, a functional PKC antagonist, has been in clinical trial as an anticancer agent,22 as has the PKC-activating phorbol ester PMA.13 Ingenol derivatives, which are structurally closely related to phorbols, have received much less attention. Ingenol 3-angelate (PEP005) extracted from *Euphorbia peplus* has recently been shown to have activity against human skin tumors grafted on to mice2 and is also a potent activator of PKC.8 There are, however, key differences between the effects of PMA and PEP005 on promyeloid leukemic cells: most notably, PEP005 does not induce complete differentiation and apoptosis is the predominant effect, whereas PMA induced full differentiation,32 with no effect on cell viability.

The differential effects of PMA and PEP005 may derive from their differential activation of PKC isoenzymes in whole cells or the site of activation within the cell.33 PKC is a family of 11 isoenzymes, 8 of which are responsive to PEP005 and PMA in vitro by their binding to the C1 domain in the classical (α, β, β′, γ) and novel (δ, ε, η, θ) PKCs. While PEP005 shows little selectivity in its activation of PKC isoenzymes in vitro, in vivo it induces a distinct pattern of translocation to that seen with PMA. Most notably, 10 nM PEP005 induced a rapid translocation of PKC-δ from the cytosol to the nuclear membrane and perinuclear region in COS cells transfected with GFP-tagged PKCs.8 In contrast, 10 nM PMA had little effect and 100 nM PMA was required to give translocation of PKC-δ, and this was initially to the plasma membrane and not the nucleus or perinuclear region.8 In the studies reported here, we also showed that PEP005 induced a rapid nuclear translocation of PKC-δ in HL60 cells, whereas PMA induced translocation to the cell membrane. In KG1a cells transfected with...
PKC-δ, PEP005 also induced translocation to the nucleus. The direction of translocation of PKC-δ is important, as it will dictate where the enzyme is active within the cell and hence its substrate availability.

A role for PKC-δ in the nuclear events of apoptosis has been known for several years. Kufe’s group (Ghayur et al27 and Emoto et al28) showed that PKC-δ was cleaved and activated by caspase-3 during apoptosis in cell lines, and overexpression of the catalytic fragment of PKC-δ was sufficient to induce apoptosis. Previous studies in a leukemic cell line TUR have also shown that defects in the PKC-δ signaling pathway were responsible for resistance to apoptosis in these cells.24 Our own studies have shown that PKC-δ translocates to the nucleus and is required for apoptosis in normal T cells and neutrophils.35,36 PKC-δ is also involved in producing several of the characteristics of apoptosis, including activation of scramblase to produce flipping out of phosphatidylserine on the cell surface,37 inhibition of DNA repair enzymes,38 and dissolution of the nuclear lamina.22 The translocation of PKC-δ to the nucleus by PEP005 is therefore consistent with the proapoptotic effect of the compound on leukemic cells. More recently, PKC-δ has been shown to be able to phosphorylate and activate caspase-3, identifying another proapoptotic substrate for this PKC isoform.30 We show here that PEP005 treatment also results in PKC-δ-dependent phosphorylation of caspase-3.

Growth inhibition and differentiation have been reported previously when PKC-δ was overexpressed,39 but the mechanism for induction of the differentiated phenotype was not defined. In our studies, we noted that responsiveness to PEP005 also corresponded with the ability to differentiate in response to ATRA. HL60, U937, and NB4, each of which are ATRA responsive, were all PEP005 responsive, whereas KG1a, which is unresponsive to ATRA, was not PEP005 sensitive. ATRA is a potent inducer of cell differentiation in malignant cells and its use in acute promyelocytic leukemia (APL) has greatly improved the prognosis for these patients.40 Retinoids mediate their biologic effects by binding to nuclear receptors of the RAR or retinoid X receptor (RXR) family. ATRA binds to RARs and induces formation of RAR:RXR dimers, which bind to retinoid acid response elements (RAREs) in DNA and initiate the transcription of antiproliferative and prodifferentiating genes. Retinoids also regulate the activation of various signaling pathways including mitogen-activated protein (MAP) kinase and PKC, and these are also involved in mediating the effects of retinoids.9,41 Platanius’ group (Kambhampati et al19) has shown that ATRA treatment can induce association of PKC-δ with RARs in leukemic cell lines NB4 and HL60, leading to activation of RAR transcriptional activity and induction of the differentiation gene STAT1. Importantly, ATRA therapy has reaped benefit only in APL and has had little impact in other forms of AML. This relates to the fact that APL cells bear RARα fusion protein mutations not found in other AMLs. However, we observed additive differentiation effects of ATRA and PEP005 in HL60 and U937 cells, which are not APL cells, but not in NB4 cells, which are APL cells and carry characteristic RARα fusion proteins. These data may indicate that PEP005 and ATRA may be useful in combination differentiation therapy of non-APL AML. Moreover, preliminary toxicity data show that PEP005 can be given systemically at doses that were effective against the AML isolates.

In summary, PEP005 represents a novel small molecule PKC activator isolated from a plant source used in traditional medicine for treatment of skin cancer. Although it can activate a broad range of PKC isoenzymes in vitro, its ability to induce nuclear translocation of PKC-δ endows it with potent antileukemic effects. The fact that cells not expressing PKC-δ were resistant to PEP005 is also useful clinically as it provides a simple test for potential responsiveness to treatment with PEP005.

References


PEP005, a selective small-molecule activator of protein kinase C, has potent antileukemic activity mediated via the delta isoform of PKC

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