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Article

Euphohelioscopin A Is a PKC Activator Capable of Inducing Macrophage Differentiation

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SUMMARY

To identify small molecules that selectively control hematopoietic stem cell differentiation, we performed an unbiased screen using primary human CD34$^+$ cells. We identified a plant-derived natural product, euphohelioscopin A, capable of selectively differentiating CD34$^+$ cells down the granulocyte/monocytic lineage. Euphohelioscopin A also inhibits proliferation and induces differentiation of the myeloid leukemia cell lines THP-1 and HL-60. Mechanistic studies revealed that euphohelioscopin A is an activator of protein kinase C (PKC), and that the promonocytic effects of this natural product are mediated by PKC activation. In addition to shedding insights into normal hematopoiesis, this work may ultimately facilitate the application of stem cell therapies to a host of myeloid dysfunctions.

INTRODUCTION

Among the best characterized adult stem cells are hematopoietic stem cells (HSCs), which can self-renew and differentiate into all blood lineages. At each step in this process, cells lose some of the differentiation and proliferation potential of their upstream progenitor. This phenomenon can be harnessed as a strategy against blood malignancies and leukemias that arise from the oncogenic transformation of a progenitor rather than a fully differentiated cell. In those cases, forcing the malignant progenitors to differentiate can put an end to their invasive proliferation (Leszczyniecka et al., 2001; Nowak et al., 2009, Petrie et al., 2009). In contrast, numerous conditions lead to the depletion of specific blood lineages, either as a direct result of bone marrow failure or as a consequence of leukemia, viral infection, or even aggressive treatments including radiation and chemotherapy. The ability to generate sufficient quantities of differentiated, functional cells to replenish the deficient compartment has long been a focus of regenerative medicine. To identify pharmacological agents that selectively differentiate hematopoietic progenitors toward a desired effector cell, we carried out an unbiased cell-based screen with primary human CD34$^+$ cells. The plant natural product euphohelioscopin A was identified that induces differentiation of HSCs and THP-1 and HL-60 cells to the granulocyte monocyte (GM) lineage by activation of protein kinase C (PKC).

RESULTS

Discovery of an HSC-Differentiating Natural Product

To identify molecules that modulate the differentiation of HSCs, a library of 704 pure microbial and plant natural products was screened in vitro using primary human CD34$^+$ cells isolated from mobilized peripheral blood. Cells were seeded into 384-well plates (2,500 cells per well) in medium optimized for their self-renewal, yet permissive to myeloerythroid differentiation (serum-free medium supplemented with thrombopoietin [TPO], stem cell factor [SCF], flt3 ligand [Flt-3L], and interleukin-6 [IL-6]) (Boitano et al., 2010; Murray et al., 1999). Compounds were added at a final concentration of 1 μM and the cells were incubated for 7 days, at which point the cultures, consisting of a mixture of CD34$^+$ and differentiated cells, were analyzed by flow cytometry. The number of cells of each lineage and the number of remaining progenitors in the culture were determined based on cell surface phenotype: CD34$^+$ (HSCs and progenitors), CD34$^-$ (lineage-committed cells) CD45ra$^+$ (GM lineage), and CD45ra$^-$ (megakaryocyte/erythrocyte lineage). Using this assay, we observed that euphohelioscopin A, a lathyrane diterpene natural product first isolated in 1983 (Shizuri et al., 1983) (Figure 1; see also Figure S1 available online), strongly induced the differentiation of CD34$^+$ cells toward the GM lineage (CD34$^-$/CD45ra$^+$).

Euphohelioscopin A Induces Differentiation to the GM Lineage

Euphohelioscopin A decreased the percentage of CD34$^+$ cells after 7 days from 29% in the vehicle-treated cultures (DMSO 0.04%) to less than 13%, while the total number of viable nucleated cells (TNCs) doubled compared to vehicle-treated cultures (Figures 2A and 2B). Concurrently, CD45ra$^+$ cells increased from about 50% of the vehicle-treated cultures to 79% of the total population when treated with euphohelioscopin A, suggesting that the compound promotes selective differentiation of

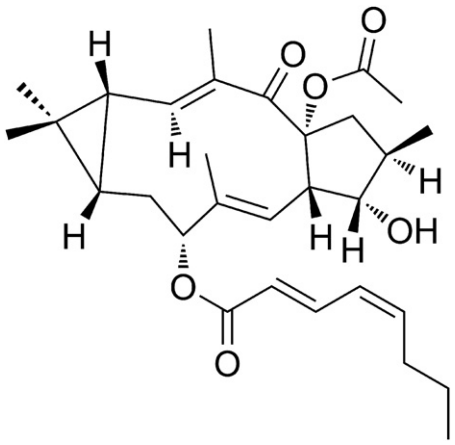

Figure 1. Structure of Euphohelioscopin A
See also Figure S1.

CD34$^+$ cells toward the myeloid lineage (EC_{50} = 600 nM). This was further supported by the observation that CD34$^-$/CD45ra$^+$ cells make up to 71% of the final cell population after treatment with compound for 7 days. The change in phenotype and TNC resulted in no net change in the number of CD34$^+$ cells compared to vehicle, while the number of CD34$^-$/CD45ra$^+$ increased 4-fold. Finally, CD34$^+$ cells treated with euphohelioscopin A for 7 days generated 10-fold more cells positive for CD11b, an integrin characteristic of monocytes and macrophages, than the vehicle. Together these results suggest that euphohelioscopin A selectively promotes terminal differentiation of hematopoietic stem and progenitor cells toward the myeloid lineage.

A key characteristic of stem and progenitor cells is the ability to form colonies when plated in semisolid media. The composition of the colonies reflects the lineage potential of the expanded cells. The different colonies include macrophage (M), erythroid (E), mixed GM, and mixed GM, E, and megakaryocyte (GEMM) and represent a stage of hematopoietic differentiation between HSCs and more terminally differentiated cells. The number of colonies obtained is linearly proportional to the colony-forming cell content of the input cell suspension where colonies containing cells of two or more lineages (mixed colonies) arise from an earlier progenitor than those containing cells of a single lineage. To confirm the differentiating effect of euphohelioscopin A, we examined the colony forming potential of CD34$^+$ cells expanded with vehicle or euphohelioscopin A (2.5 μM) for 7 days. Euphohelioscopin A-treated cells showed a 3-fold decrease in total colony-forming unit (CFU) potential compared to control cultures (Figure 2C), suggesting that the cells had differentiated and the cultures had decreased numbers of progenitors. Interestingly, GM colonies were the only colonies that did not experience a significant decrease in numbers; all other colonies decreased by more than 4-fold. These results are consistent with the differentiated phenotype of the 7 day cultured cells and support the hypothesis that euphohelioscopin A treatment of CD34$^+$ cells selectively leads to an increase in nonproliferating, lineage-restricted myeloid cells by driving the differentiation of CD34$^+$ progenitor cells rather than inducing lineage-restricted CD34$^-$/CD45ra$^+$ cells to proliferate.

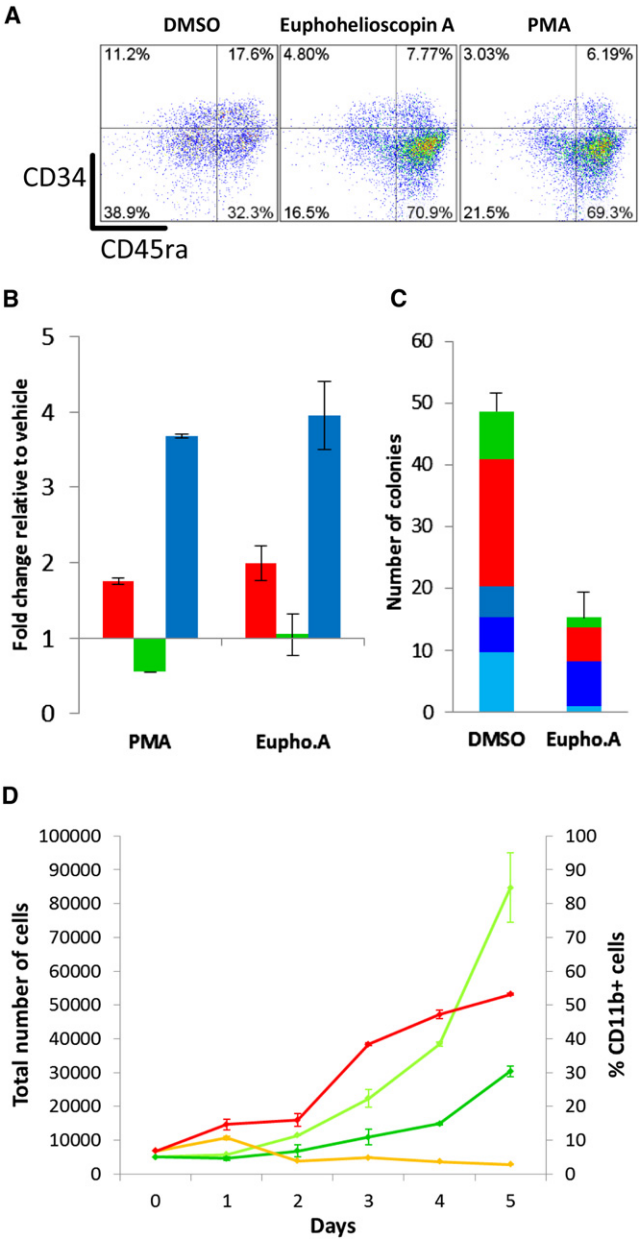

Figure 2. Euphohelioscopin A Induces the Differentiation of CD34$^+$ and THP-1 Cells
(A)–(C) characterize the population of cells generated by primary human mobilized peripheral blood-derived CD34$^+$ cells cultivated for 7 days, with vehicle (DMSO 0.04%), euphohelioscopin A (600 nM), or PMA (0.6 nM). (A) CD34 and CD45ra expression, analyzed by flow cytometry. (B) Fold change in TNC (red), number of CD34$^+$ cells (dark green), and number of CD34$^-$/CD45ra$^+$ cells (medium blue), relative to vehicle. (C) Colony forming potential of 80 CD34$^+$ cells following 7 days of culture with vehicle or euphohelioscopin A: GEMM, dark green; E, red; G, medium blue; GM, dark blue; M, light blue.
(D) THP-1 cells were seeded in a 96-well plate (5,000 cells per well) in RPMI +10% FBS, and treated with vehicle (0.01% DMSO) or euphohelioscopin A (1 μM). TNC (DMSO, light green; eupho.A, dark green) and CD11b expression (DMSO, orange; eupho.A, red) were quantified by flow cytometry after the indicated number of days in culture. Data are represented as mean ± SD.
See also Figure S2.

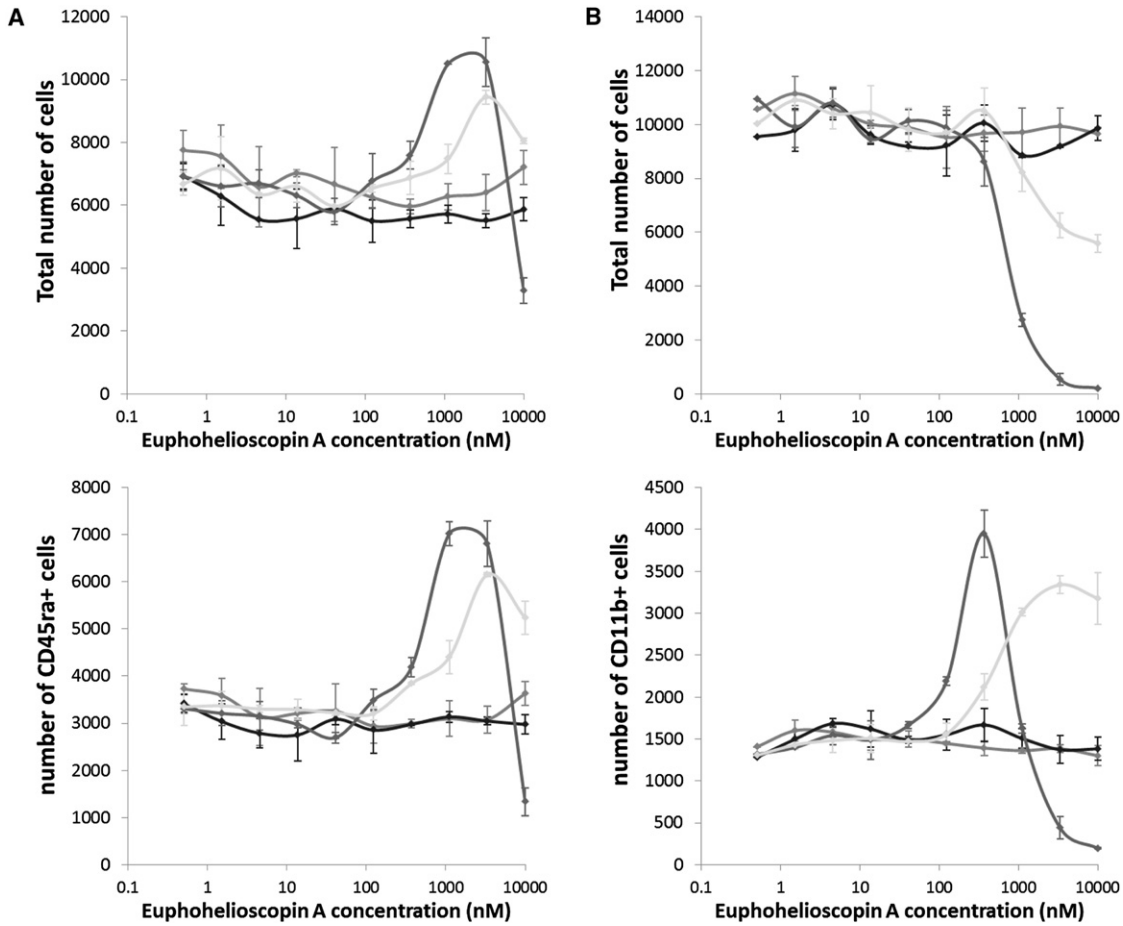

Figure 3. The Differentiating Activity of Euphohelioscopin A Is Mediated by PKC

(A) Human mobilized peripheral blood CD34$^+$ cells were seeded in 96-well plates (5,000 cells per well) and treated with vehicle (DMSO) or euphohelioscopin A at the indicated doses, with or without the PKC inhibitor Gö6983 (500 nM). Cell number and CD45ra expression were measured by flow cytometry after 7 days of culture.

(B) THP-1 cells were seeded in 96-well plates (5,000 cells per well) and treated with vehicle (DMSO) or euphohelioscopin A at the indicated doses, with or without the PKC inhibitor Gö6983 (500 nM). Cell number and CD11b expression were measured by flow cytometry after 4 days of culture. Data are represented as mean ± SD. DMSO, medium gray; DMSO + Gö6983, black; euphohelioscopin A, dark gray; euphohelioscopin A + Gö6983, light gray.

See also Figure S3.

To determine whether euphohelioscopin-A can induce the differentiation of leukemic cells with myeloid potential, we tested its activity on two myeloid leukemia cell lines, HL-60 and THP-1. After 4 days of culture with euphohelioscopin A (1 μM), 50% of THP-1 cells were CD11b$^+$ compared to less than 5% of vehicle-treated cells, and the total number of cells was 2.6-fold lower than in the control, indicating that the inhibition of proliferation was concurrent with differentiation to mature macrophages (Figure 2D). A similar effect was observed with HL-60 cells: after 4 days of treatment, 26% of cells were CD11b$^+$ (compared to less than 5% of control cells), and the total cell number was decreased 8-fold (Figure S2).

Euphohelioscopin A Is a PKC Activator

The only biological activity reported to date for euphohelioscopin A is inhibition of the drug transporter P-glycoprotein, as measured in a mitoxantrone efflux assay (Barile et al., 2008). However, this compound was significantly less effective than related diterpenes (IC_{50}: 14 μM, with maximal inhibition at 100 μM), suggesting that this mechanism does not account for the molecule's differentiation potential. A known pathway for selective macrophage differentiation is through the activation of PKC (Blumberg, 1988). Indeed, when tested in our cell culture assays, PKC inhibitors were able to modulate euphohelioscopin A-induced differentiation: addition of the PKC inhibitor Gö6983 (500 nM; higher concentrations resulted in significant cytotoxicity) shifted the EC_{50} of euphohelioscopin A in the CD34$^+$ differentiation assay (from 590 nM to 2 μM, Figure 3A), in the THP-1 differentiation assay (from 200 to 850 nM, Figures 3B and S3A), and in the HL-60 differentiation assay (from 1.7 to 14.5 μM, Figure S3B). Euphohelioscopin A was also able to induce the phosphorylation of known downstream targets of PKC including ERK1/2 and MARCKS (Figure 4A). These results suggest that the promonocytic effects of euphohelioscopin A are mediated through PKC activation.

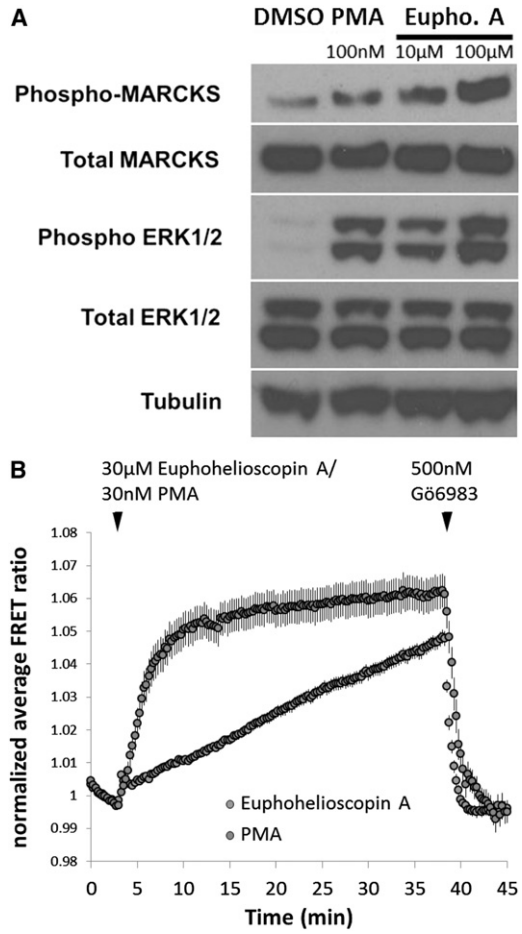

Figure 4. Euphohelioscopin A Is a PKC Activator

(A) HEK293T cells were treated for 10 min with vehicle, euphohelioscopin A or PMA at the indicated concentrations, and western blots were performed for phosphorylation of downstream targets of PKC. Data are representative of three independent experiments.

(B) HeLa cells transiently transfected with a PKC-specific FRET probe (CKAR) and rat PKC βII-RFP were imaged to measure PKC activation in real time upon stimulation with euphohelioscopin A or PMA. Base-line images were acquired for 3 min before addition of euphohelioscopin A or PMA. The PKC inhibitor Gö6983 was added 35 min after the addition of euphohelioscopin A or PMA. FRET ratios were measured by epifluorescence microscopy at 15 s intervals and normalized to the baseline, the average FRET ratio measured before compound addition. The normalized average FRET ratio is the average of these corrected values ± SE.

To further corroborate this hypothesis, we performed a real-time fluorescence resonance energy transfer (FRET)-based PKC activity assay using C kinase activity reporter (CKAR), a reporter for PKC-mediated phosphorylation developed by Violin et al. (2003). CKAR is a genetically encoded, fluorescent PKC-specific substrate that undergoes a conformational change when phosphorylated, which can be monitored by intramolecular FRET. Euphohelioscopin A (30 μM) was able to induce an immediate, robust increase in PKC activity in HeLa cells cotransfected with CKAR and rat PKC-βII-RFP; the observed activation was reversed by the PKC inhibitor Gö6983 (Figure 4B). Euphohelioscopin A had no effect on CKAR T/A, a reporter lacking the phosphoacceptor site. These results confirm that euphohelioscopin A acts as a PKC activator.

A comparison of the differentiation activities of euphohelioscopin A with known PKC activators revealed that phorbol esters such as phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and mezerein afforded a very similar phenotype in the $CD34^+$ cell differentiation assay (Figure S2B) as well as in their ability to differentiate and halt the proliferation of myeloid leukemia cell lines HL-60 (Figure S2C) and THP-1 (Figure S3A). Importantly, PMA, a broad and ultrapotent PKC activator, showed a very similar activity profile and response to PKC inhibitors as euphohelioscopin A in all cell culture assays (Figures 2A, 2B, S2, and S3A). Ingenol appeared 10-fold less potent than euphohelioscopin A (Hasler et al., 1992), while bryostatin 1 showed a very different activity profile (Kraft et al., 1986); it showed a multiphasic dose-response curve in all differentiation assays except the HL-60 differentiation assay, where it failed to induce any increase in $CD11b^+$ cells or any decrease in total cell numbers comparable to euphohelioscopin A. ADMB was not active in any of the cell culture assays. These findings suggest that the properties of euphohelioscopin A as a PKC activator may be more closely related to those of phorbol esters than to other families of natural and synthetic PKC activators.

DISCUSSION

In summary, we have identified the natural product euphohelioscopin A as a PKC activator and inducer of myeloid differentiation. Due to its pivotal role in pathways governing cell proliferation and differentiation, PKC has been a focus of interest as a pharmacological target for cancer therapy for the last 3 decades. In particular, several PKC-activating compounds have been investigated for their ability to induce differentiation of malignant myeloid progenitors (Hampson et al., 2005; Han et al., 1998b) or to alleviate chemotherapy-induced leukopenia (Han et al., 1998a). Though phorbol esters were originally the most promising candidates due to their potency, the need to balance their benefits and their tumor-promoting effects severely limited the scope of potential therapeutic applications. This led to the development of alternative PKC activating natural products such as bryostatins and ingenols, which differed in their isozyme specificity, their kinetics of PKC activation and their induction of PKC translocation to various subcellular compartments (Clamp and Jayson, 2002; Hampson et al., 2005, 2010; Kedei et al., 2004).

As more PKC ligands came to be known, it became clear that, whether in vitro or in vivo, the spectrum of PKC-mediated responses was very broad. This revived the interest in finding molecules able to activate PKC with distinct activities and specificities. More recently, as structural details emerged on the mechanism of PKC activation, several attempts to rationally design PKC activating or inhibiting ligands have been described (Baba et al., 2004; Boije af Gennas et al., 2009; Keck et al., 2010; Kiriazis et al., 2011). In this report, we introduce another scaffold to explore in this context: a lathyrane diterpene (Figure S1). Although the full spectrum of activity of this novel class of activators on different PKC isozymes and leukemic cells remains to be investigated, our results with euphohelioscopin A hint that this

natural product could have properties distinguishing it from currently known PKC activators.

PKC-dependent induction of myeloid differentiation is mediated by PKC-α and -δ but not PKC-βII (Mischak et al., 1993). The observation that euphohelioscopin A is capable of inducing differentiation suggests that the compound might activate PKC-α and -δ in addition to PKC-βII, and might therefore be an activator of both conventional and novel PKCs. However, the effects observed of euphohelioscopin A on $CD34^+$ and myeloid leukemia cells are very different from the activity profiles that have been reported for two potent activators of conventional and novel PKCs: bryostatin 1 and ingenol-3-angelate (I3A). Bryostatin 1 is not able to differentiate HL-60 cells (Figure S2; Kraft et al., 1986) and shows a very different dose-response profile than euphohelioscopin A in our HSC and THP1 differentiation assays (Figure S2). These characteristics have been attributed to the unique interaction between bryostatin 1 and PKC-δ (Lorenzo et al., 1997, 1999), a PKC linked to apoptosis in malignant cells. While phorbol esters translocate this isoform to the plasma membrane and cause its rapid downregulation, bryostatin 1 translocates it to the nuclear membrane (Wang et al., 1999), and protects it from downregulation in a biphasic manner (Szallasi et al., 1994). I3A is reported to have no antiproliferative effects against $CD34^+$ cells and to induce apoptosis rather than differentiation in HL-60 cells (Hampson et al., 2005), unlike the activity reported with PMA and unlike what we observed with euphohelioscopin A. This particularity of I3A is also thought to originate from its pattern of PKC-δ translocation, primarily to the nuclear and intracellular membranes (Hampson et al., 2005; Kedei et al., 2004). In contrast, we found that phorbol esters such as PMA closely recapitulated the phenotype of euphohelioscopin A in the three cell types we tested. Since the differences in activity between bryostatin 1, I3A and phorbol esters are mediated by their differences in PKC-δ activation and subcellular translocation, this suggests that euphohelioscopin A may behave similarly to phorbol esters in relation to this isozyme.

Importantly, euphohelioscopin A also bears significant structural differences relative to PMA (Figure S1), including a less constrained structure than daphnane, ingenane, and tigliane diterpenes (which comprise most known PKC-activating natural products isolated from *Euphorbiaceae*). Second, euphohelioscopin A lacks several functional groups that are thought to be important for the PKC binding and translocation induced by phorbol esters, such as an ester in position 13, the hydroxyl groups in positions 4, 9, and 20, and the carbonyl in position 3 (Blumberg, 1988; Nacro et al., 2000). Finally, the hydrophobic side chain is shorter and unsaturated, a characteristic that has been associated with modified intracellular distribution and lower tumor promotion in phorbol esters (Fürstenberger et al., 1981; Wang et al., 1999, 2000). It will be interesting to determine to what extent these differences in side chain hydrophobicity and ring system strain each contribute to the kinetic differences in PKC activation we observed between PMA and euphohelioscopin A: in addition to a 1,000-fold lower potency, the latter showed a much slower rate of PKC activation (Figure 4B). This is particularly significant since lower rates of activation have been associated with differential phenotypic outcomes, including decreased PKC inactivation and subsequent degradation, a more sustained signal, and lower tumor promotion (Hampson et al., 2010).

These particularities highlight euphohelioscopin A and related lathyrane diterpenes as a family of natural products worthy of further investigation in the efforts toward safe and efficient pharmacological induction of myeloid differentiation.

SIGNIFICANCE

Through an unbiased cell-based screen with primary human $CD34^+$ cells, we unveiled a biological activity of the plant natural product euphohelioscopin A. Euphohelioscopin A is able to induce myeloid differentiation in human hematopoietic stem and progenitor cells and in two myeloid leukemia cell lines. These findings suggest a potential utility of this scaffold for pharmacological intervention to alleviate a shortage or dysregulation of myeloid cells in the clinical setting. We were also able to determine the mechanism of this bioactivity by showing that euphohelioscopin A is an activator of protein kinase C; to our knowledge, this is the first evidence of a lathyrane diterpene activating PKC, and may shed new insights into the way ligands bind and activate this family of proteins.

EXPERIMENTAL PROCEDURES

$CD34^+$ Cell Culture

All experiments were performed in HSC expansion media (StemSpan SFEM, StemCell Technologies) supplemented with 1 × antibiotics and the following recombinant human cytokines: thrombopoietin, IL-6, Flt3 ligand, and stem cell factor (100 ng/ml, R&D Systems) unless otherwise indicated. Human mPB $CD34^+$ cells were purified from fresh human leukophoresed mobilized peripheral blood (AllCells) using direct CD34 progenitor cell isolation kits (Miltenyi Biotec) following manufacturer's protocols. $CD34^+$ cells were resuspended in HSC expansion medium (5×10^4 cells/ml) before being aliquoted in 384-well plates (Greiner Bio-One). Compounds were added immediately after plating. Cells were cultured at 37°C in 5% CO_2.

Flow Cytometry

Cells were stained in staining medium (HBSS supplemented with FBS [2%] and EDTA [2 mM]) at 4°C for 1 hr with PerCP anti-human CD34 (BD Bioscience), PECy7 anti-CD45ra (eBiosciences), and PE anti-CD11b (BD Bioscience), then washed with staining medium and analyzed. Multicolor analysis for cell phenotyping was performed on a LSR II flow cytometer (Becton Dickinson).

Colony Forming Assays

The progeny of 80 $CD34^+$ cells after 7 days in culture were seeded in serum-free methylcellulose containing SCF, IL-6, erythropoietin, IL-3, granulocyte and granulocyte-macrophage colony stimulating factor (MethoCult SFH4436, StemCell Technologies), supplemented with 1× antibiotics, thrombopoietin, and Flt3 ligand. Colonies were scored on day 14 with an inverted microscope at 40× magnification. Numbers reported represent the average of the number of colonies scored from three dishes.

Western Blots

HEK293T cells (2×10^6) were serum starved overnight, then treated with euphohelioscopin A, PMA or vehicle. After 10 min of compound treatment, cells were washed with cold PBS, then lysed with 100 μl of RIPA buffer supplemented with a protease inhibitor cocktail (Sigma). Following incubation on ice for 20 min, the lysed cells were passed through a $26^{1/2}$ syringe needle and centrifuged (15,000 × *g*, 20 min at 4°C). Cell lysates were denatured by boiling in SDS sample buffer (Invitrogen) containing 5% β-mercaptoethanol. Proteins were electrophoresed, transferred onto a polyvinylidene fluoride

membrane and probed with anti-ERK1/2, antiphospho-ERK1/2, anti-MARCKS, and antiphospho-MARCKS (all from Cell Signaling Technology), as well as antitubulin (Sigma) antibodies at suggested concentrations in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk. Blots were then incubated with HRP conjugated secondary antibodies and detected with a chemiluminescent substrate (Thermo Scientific).

Materials

Phorbol 12-myristate 13-acetate (PMA) and Gö 6983 were purchased from Calbiochem. Euphohelioscopin A was obtained from the Shanghai Institute of Materia Medica, Shanghai, China.

Cell Culture

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum (GIBCO), at 37°C, in 5% CO_2. THP-1 and HL-60 cells were maintained in RPMI1640 (Cellgro) containing 10% fetal bovine serum, at 37°C, in 5% CO_2.

Plasmid Constructs

The construction of CKAR and of C-terminally tagged rat PKC βII-RFP has been previously described (Gould et al., 2009; Violin et al., 2003).

Cell Transfection

Cells were plated onto Lab-Tek chambered #1.0 borosilicate cover glass (Nunc) prior to transfection. Transient transfection of CKAR and rat PKC βII-RFP DNA was carried out using jetPRIME (Polyplus transfection). Cells were imaged approximately 24 hr following transfection.

FRET Imaging and Analysis

HeLa cells were rinsed with and imaged at room temperature in Hanks' balanced salt solution (Cellgro) supplemented with 1 mM $CaCl_2$. CFP, YFP, FRET, and RFP images were acquired and analyzed as described previously (Gallegos et al., 2006). RFP emission was monitored during the imaging experiments as a control for PKC expression levels. Baseline images were acquired for 3 or more minutes before ligand addition. PKC inhibitor Gö6983 was added 35 min after ligand addition. Individual data traces were normalized to 1 by dividing by the average base-line FRET ratio, and data from at least three different imaging dishes were referenced around the ligand addition time point. The normalized average FRET ratio is the average of these corrected values ± SE.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.06.010.

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