The protein kinase C agonist prostratin induces differentiation of human myeloid leukemia cells and enhances cellular differentiation by chemotherapeutic agents

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ABSTRACT

As acute myeloid leukemia (AML) cells are characterized by uncontrolled self-renewal and impaired cellular differentiation, induction of terminal differentiation of leukemia cells by differentiating agents has been proposed as an attractive therapeutic strategy to treat AML. Here, we demonstrated that prostratin, a potent protein kinase C (PKC) activator, inhibited the growth of myeloid leukemia cells by a predominant G1 arrest with variable induction of apoptosis. Conversely, prostratin induced significant differentiation of AML cell lines and primary AML blasts as evidenced by morphology and immunophenotyping. The effects of prostratin were PKC dependent, and activation of mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 by PKC was required for prostratin-induced cell differentiation. Consequently, prostratin reprogrammed transcriptional factor expression, and ectopic expression of c-Myc in HL-60 cells significantly eliminated prostratin-mediated cellular differentiation and cell cycle arrest, indicating an essential role for c-Myc suppression in the differentiation-inducing effects of prostratin. Finally, prostratin was able to potentiate cellular differentiation induced by chemotherapeutic agents such as Ara-C. Together, we proposed that prostratin alone or administered with other anticancer agents may be effective in differentiation therapy of AML.

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Introduction

Acute myeloid leukemia (AML) is the most common type of leukemia in adults and occurs in approximately one third of newly diagnosed patients. AML is characterized by the proliferation of clonal precursor myeloid cells with arrested differentiation [1]. Current therapy of AML relies on remission induction with cytosine arabinoside (Ara-C) and anthracyclines (eg, idarubicin, daunorubicin) with significant morbidity and mortality, particularly in elderly patients [2]. Although remission can be achieved in most de novo AML patients, relapse is common and long-term survival is poor for most cases. In contrast to the poor prognosis for the majority of patients with AML, the use of differentiation therapy with all-trans retinoic acid (ATRA) for acute promyelocytic leukemia (APL), a unique subtype of AML, introduced a paradigm for success of cell differentiation therapy for AML [3]. Unfortunately, the other AML subtypes demonstrate inherent resistance to ATRA-mediated differentiation. Furthermore, many APL patients treated with ATRA fail to respond or invariably relapse. Thus, it is imperative to develop novel agents for the treatment of AML, particularly the ones that exploit differentiation pathways.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases involved in the regulation of cell proliferation, differentiation, cell survival, apoptosis, and carcinogenesis [4]. There is emerging evidence that activation of PKC has important functional roles in the regulation of differentiation of hematopoietic cells [5]. Also, various studies have demonstrated that some activators of protein kinase C might be potential differenti-

Abbreviations: PKC, protein kinase C; AML, acute myeloid leukemia; PMA, phorbol-12-myristate-13-acetate; Ara-C, cytosine arabinoside; MAPK, mitogen-activated protein kinase.

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agents with potent antileukemic activity. For example, phorbol-12-myristate-13-acetate (PMA), a classic PKC activator, has been shown to induce terminal differentiation of myelomonocytic leukemia cells and have activity as a differentiating agent for the treatment of patients with myelocytic leukemia [6–8]. However, its potent tumor-promoting activity raises concerns about its therapeutic use. Another agonist of PKC, bryostatin 1, is undergoing several clinical studies against a few special types of hematological malignancies [9]. Unlike PMA, bryostatin 1 is not a complete tumor promoter and has been found to functionally antagonize phorbol ester responses that they themselves do not elicit [10–12]. However, the differentiation-inducing activity of bryostatin 1 remains inconclusive, since application of bryostatin 1 to different leukemia cell lineswelling, diarrhea, and yellow fever as a traditional remedy in treating a number of conditions, including back pain, abdominal swelling, diarrhea, and yellow fever [17]. This molecule has been shown to be a protein kinase C activator and a potential antagonist of HIV latency [17]. It has been shown to reactivate latent virus both in latently infected cell lines and primary cells from HIV-infected patients [18,19] and exhibit potent in vivo activity in promoting transcriptional activation of latent HIV proviruses in the SCID-hu mice model [20]. Notably, unlike many phorbol esters, prostratin is not tumor promoting and has been found to be an anti-tumor promoter [1721–23]. Preliminary data have also demonstrated that in HL-60 and THP-1 cells prostratin could drive their rapid adhesion to plastic and subsequent morphological changes indicative of differentiation [19]. However, the characterization of this drug induction of differentiation on extended myeloid leukemia cell lines and primary AML cells is still unknown. Therefore, in the present work we have sought to define the features that represent the differentiation–induction of prostratin and examined its potential role in interacting with other chemotherapeutic agents on leukemic cell differentiation. A series of studies dissecting the mechanism of prostratin induction of differentiation are also described here.

Materials and methods

Chemicals and reagents

Prostratin was purchased from LC Lab (Woburn, MA). SB203580 and SP600125 were purchased from Cayman (Cayman Chemical, MI). 5-(3,4-Dimethoxyphenyl)-4-(1H-indol-5-ylamino)-3-pyridinecarboxonitrile was purchased from Merck Millipore (Darmstadt, Germany). U0126 and antibodies against phospho-Raf, phospho-ERK, phospho-JNK, phospho-p38, P21, Cyclin D3, phospho-Rb on ser795 and ser809/11, CDK4, CDK6, Rb, C/EBPα, C/EBPβ, and C-Myc were purchased from Cell Signaling Technologies (Cambridge, MA). Raf, ERK, JNK, p38, and β-actin antibodies were purchased from Cayman (Cayman Chemical, MI). 5-(3,4-Dimethoxyphenyl)-4-(1H-indol-5-ylamino)-3-pyridinecarboxonitrile was purchased from Merck Millipore (Darmstadt, Germany). U0126 and antibodies against phospho-Raf, phospho-ERK, phospho-JNK, phospho-p38, P21, Cyclin D3, phospho-Rb on ser795 and ser809/11, CDK4, CDK6, Rb, C/EBPα, C/EBPβ, and C-Myc were purchased from Cell Signaling Technologies (Cambridge, MA). Raf, ERK, JNK, p38, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phycocyanin-labeled antibodies against CD11b and CD14 were purchased from BD Biosciences (San Jose, CA). Other chemicals and reagents were all from Sigma (St. Louis, MO).

Cell culture

The human myeloid leukemia cell lines HL-60 and U937 were obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China) and cultured under recommended conditions. NOD cells (kindly gifted by Dr. Bingwen Deng, Beijing Proteome Research Center, Beijing, China) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) at 37 °C, 5% CO2 humidified atmosphere. All cultural reagents were the product of GIBCO/BRL (Grand Island, NY). Fresh blasts from 3 cases of de novo AML patients were obtained with informed consent. Patients were diagnosed according to French-American-British classification (Appendix: Supplementary Table S1). Mononuclear cells were isolated by Ficoll density-gradient centrifugation and cultured in the presence of RPMI-1640 containing 10% FBS. Cells were then seeded at 5 × 10^5 cells/ml in the media described earlier and prepared for differentiation assay.

Lentiviral construction, production, and infection

Lentivector Expression System was purchased from System Biosciences. Packaging and production of lentivirus were performed according to the manufacturer’s protocol. Briefly, the coding sequence of full length human c-myc was inserted into the EcoRI and BamHI sites of the pCdh-MCS-T2A-CGGFP-MSCV cDNA expression vector. The new expression vector and Lentivirus Package plasmid mix (System Biosciences, SBI) were cotransfected into 293TN cells with Lipofectamine 2000 (Invitrogen). Forty-eight hours later, viral supernatant was obtained and mixed with polybrene (5 μg/ml), filtered through a 0.45 μM filter (Millipore), and added to HL-60 cell line. The infected HL-60 cells were subsequently expanded and analyzed by FACS for GFP-positive populations.

Growth inhibition assay

For trypan blue dye exclusion assay, cells were plated at a cell density of 2 × 10^5 cells/well in 60-mm culture plates and were treated with varying concentrations of prostratin. At the end of desired treatment times (0–72 h), the total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted microscope. Each treatment and time point had three independent plates.

Analysis of apoptosis, cellular differentiation, and cell cycle distribution

Annexin V/PI (BD Pharmingen, San Diego, CA, USA) double staining analysis of apoptosis was carried out as per the manufacturer’s instructions using a Beckman Cytomics FC500 flow cytometer (Beckman Coulter, USA, USA). Differentiation induction with prostratin was confirmed by morphology and cell surface marker expression. For morphological assessment, cytospin preparations of treated cells stained with Giemsa were evaluated with light microscopy. Analysis of myeloid maturation with cell-surface markers was performed by fluorescence-activated cell sorting (FACS) with phycocyanin-labeled antibodies for CD11b and CD14. Live cells were gated based upon forward and side scatter patterns. For cell cycle analysis, cells were treated with varying concentrations of prostratin and harvested at the end of desired treatment time. Briefly, cells were washed twice with PBS and fixed with ice-cold 70% ethanol overnight at 4 °C. These fixed cells were washed with PBS, treated with 1% RNase A for at least 15 minutes at 37 °C, then stained with 50 μg/ml propidium iodide (PI), and subjected to flow cytometry. Finally the data were analyzed by the CELLQuest software.

Western blot analysis

At the end of the desired treatment, total cell lysate preparation and Western blot analysis were performed according to the procedure described before [24]. In brief, the cell lysates were clarified by centrifugation at 12,000 × g for 10 minutes at 4 °C. Equal amounts of protein (20 μg) were separated through 12.5% SDS-PAGE and transferred to NC membranes. The membranes were probed with the respective specific primary antibodies and followed by horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected with an enhanced chemiluminescence (ECL) system detection kit (Cell Signaling, Beverly, MA). Membranes were stripped and reprobed with an anti-β-actin antibody to check the equal protein loading. Signal intensity of proteins was normalized against β-actin using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA). Densitometry data presented below the bands are “fold change” as compared to control for prostratin or other treatments in each case.

Statistical analysis

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 0.05 was taken as a significantly different value.

Results

Prostratin inhibits AML cell growth with variable levels of increase in apoptosis

To assess the ability of prostratin to affect leukemic cell growth, its effects on proliferation of three AML cell lines (HL-60, NB4, and U937 cells) were investigated. As shown in Fig. 1A, prostratin exerted concentration- and time-dependent growth inhibition on all 3 cell lines to a similar degree. To elucidate the mechanism of prostratin-
induced growth suppression of AML cells, we next studied the role of apoptosis in prostratin-induced growth inhibition. It was shown that a mild increase in apoptosis was observed in HL-60 and NB4 cells after 72 hours of prostratin treatment (Fig. 1B). In contrast, prostratin triggered a dramatic induction of apoptosis in U937 cells under the same conditions (Fig. 1B). However, in all 3 tested cell lines, especially HL-60 and NB4 cells, the apoptosis-inducing capacity of prostratin was not comparable to its growth-inhibiting ability. Obviously, after 72 hours of treatment with prostratin (125–1000 nM) HL-60 cells showed 20%–70% growth inhibition, which was much stronger than the degree of apoptosis induction. These results thus indicated that the significant inhibition of AML cell growth induced by prostratin was not completely due to the induction of apoptosis.

Prostratin induces G1 arrest of AML cells with effects on key regulators of cell cycle progression

To further gain insights into the mechanism of the antiproliferative activity of prostratin, we next examined cell cycle progression using flow cytometric analysis. It was revealed that prostratin (125–500 nM) induced a G0/G1 phase accumulation of HL-60 cells in a concentration-dependent manner, accompanied by a reduction in the percentage of cells in the S and/or G2/M phase (Fig. 1C). High concentration of prostratin at 1000 nM led predominantly to accumulation of cells in the G2/M phase, with a lesser extent of increase in the percentage of cells in the G1 phase. Consistently, prostratin induced a concentration-dependent cell cycle arrest in the G1 phase in both NB4 cells and U937 cells (Fig. 1C).

We next explored whether prostratin modulated levels of the cell-cycle-related molecules in HL-60 cells by Western blot analysis. Rb phosphorylation is a critical step in the G1 to S phase transition [25]. We found that prostratin suppressed the pRb phosphorylation on ser795 as well as ser807/811 in HL-60 cells in a concentration-dependent manner (Fig. 1D). Interestingly, a marked decrease in protein levels of Rb was evident in HL-60 cells treated with 1000 nM prostratin. Phosphorylation of Rb is regulated primarily by complexes of cyclin D associated with CDK4/6 and later by cyclin E associated with CDK2 [26]. Prostratin treatment resulted in a concentration-dependent decrease in the protein levels of CDK4 and CDK6 in HL-60 cells (Fig. 1D). And in case of cyclin D3, obvious reduction was observed only in the higher concentrations of prostratin-treated HL-60 cells (Fig. 1D). The CDK inhibitor p21 plays an important role in the regulation of G1-S transition by binding to and inhibiting kinase activity of CDK/cyclin complexes [27]. We showed that the protein level of p21 was dramatically increased in HL-60 cells treated by prostratin in a concentration-dependent manner (Fig. 1D).

Prostratin induces differentiation in AML cell lines and primary blasts from AML patients

Similar with other PKC activators, prostratin may have potential differentiation-inducing activity in myeloid leukemia cells. In order to assess this assumption, we first evaluated the role of prostratin in inducing differentiation of the AML cell lines including HL-60, NB4, and U937 cells. When treated at 1000 nM prostratin for 24–72 hours, cells in suspension became adherent to the tissue
culture plate. Furthermore, all 3 cell lines treated with prostratin showed monocytic morphological features such as larger cell size and decreased nucleus–cytoplasm ratio (Fig. 2A). We next examined the cell-surface markers associated with myeloid maturation, CD11b and CD14. As shown in Fig. 2B, HL-60 cells, upon prostratin, showed a concentration- and time-dependent increase in the expression of CD11b. Similar results were observed in U937 and NB4 cells treated with prostratin (Fig. 2C). Furthermore, all three AML cell lines showed a significant increase in expression of CD14 (Fig. 2D, E), which is generally associated with a more monocytic phenotype. Finally, we determined the potential differentiation-inducing effects of prostratin on primary blast cells from 3 cases of AML patients, according to the percentage of CD11b-positive cells. Prostratin induced evidence of differentiation in the evaluable AML patient samples (Fig. 2F). Together, these results indicated the significant activity of prostratin in driving AML cells into differentiation.

**Cell differentiation induced by prostratin is dependent on the activation of PKC**

Previous studies have established that prostratin is an agonist of protein kinase C. PKC is a multi-isoenzyme family with differential tissue expression and involvement in cell proliferation, differentiation, and apoptosis. We therefore examined the potential contribution of PKC isozymes to AML cell differentiation in response to prostratin stimulation. HL-60 cells were pretreated with chemical inhibitors for 1 hour and then challenged with prostratin. Pretreatment of cells with GF109203X (GFX) and Gö6983, which are blockers of both cPKCs and nPKCs [28], completely blocked prostratin-mediated monocytic differentiation of HL-60 cells, as revealed by morphological alterations and CD11b expression (Fig. 3A, B). Furthermore, pretreatment with the PKC inhibitor GFX antagonized prostratin-induced p21 expression (Fig. 4D) and G1 cell cycle
Fig. 3. Prostratin induces PKC-dependent differentiation. (A, B) After pretreatment with or without 4 μM GFX or 4 μM Gö6983 for 1 h, HL-60 cells were treated with 1 μM prostratin for 72 h. Giemsa’s staining morphology (A) and the percentage of CD11b+ cells (B) were measured. Bars, 50 μm. Data represent mean ± SD of three independent experiments. (C, D) After pretreatment with or without 4 μM GFX or 10 μM U0126 for 1 h, HL-60 cells were treated with 1 μM prostratin for 24 h (C) or 72 h (D) and DNA content (C) and annexin V/PI binding (D) was assessed by flow cytometric analysis. The results are representative of three independent experiments.
Fig. 4. The PKC/ERK axis in prostratin-induced differentiation. (A) HL-60 cells were treated with indicated concentrations of prostratin for 15 min. After treatments, total cell lysates were prepared and examined for p-Raf (Ser338), p-MEK (Ser217/Tyr221), p-ERK (Thr202/Tyr204), p-JNK (Thr183/Tyr185), and p-p38 (Thr180/Tyr182), and total Raf, MEK, ERK, JNK, and p38 protein levels by Western blot analysis using the respective specific antibodies. The results are representative of three independent experiments. (B, C) After pretreatment with or without 10 μM MEK inhibitor (U0126), 10 μM p38 inhibitor (SB203580), or 10 μM JNK inhibitor (SP600125) for 1 h, HL-60 cells were treated with 1 μM prostratin for 72 h. Giemsa’s staining morphology (B) and the percentage of CD11b+ cells (C) were measured. Bars, 50 μm. Data represent mean ± SD of three independent experiments. (D) HL-60 cells were pretreated with or without 4 μM GFX or 10 μM U0126 for 1 h followed by cotreatment with or without 1 μM prostratin in the presence of the inhibitor for 30 min at 37°C. After treatments, total cell lysates were prepared and analyzed by Western blotting. The results are representative of three independent experiments.
PKC-dependent activation of the MEK/ERK/MAP kinase signaling pathway is required for differentiation induced by prostratin

It has been well documented that the MEK/ERK/MAP kinase signaling pathway plays an important role in myeloid differentiation [29,30]. We next examined whether the MEK/ERK/MAP kinase pathway could be activated and involved in the prostratin-mediated AML cell differentiation. As shown in Fig. 4A, prostratin induced a concentration-dependent increase in the phosphorylation levels of Raf, MEK, and ERK, suggesting the activation of the MEK/ERK/MAPK pathway upon prostratin treatment. Similarly, the expression of phosphorylated p38 and JNK was also increased by prostratin (Fig. 4A). In contrast, prostratin treatment did not affect the total levels of Raf, MEK, ERK, p38, and JNK. To evaluate whether these kinase pathways are involved in the regulation of cell differentiation induced by prostratin, specific inhibitors of MEK, p38, and JNK were used in our study. Notably, analysis of differentiation by using morphological evaluation and CD11b staining revealed a block in monocyctic differentiation when HL-60 cells were pretreated with U0126 (Fig. 4B, C). These results strongly suggested that MEK/ERK activation is required for prostratin-induced differentiation. However, no involvement of p38 and JNK in HL-60 cell differentiation was found, since inhibition of the p38 and JNK pathways by specific pharmacological inhibitors, SB203580 and SP600125, respectively, was ineffective in preventing HL-60 cell differentiation (Fig. 4B, C). To determine that the activation of the MEK/ERK/MAPK pathway resulted from activation of PKC, the consequences of inhibiting PKC for prostratin-induced effects on MAPKs were determined. GFX pretreatment gave a nearly complete inhibition of the effects of prostratin on Raf-1, MEK, and ERK phosphorylation (Fig. 4D), suggesting that prostratin affects the MAPK via activation of PKC. Consistent with these results, pretreatment with the MEK inhibitor U0126 blocked prostratin-induced ERK phosphorylation and p21 expression (Fig. 4D) and G1 cell cycle arrest (Fig. 3C), as well as prostratin-mediated apoptosis (Fig. 3D). Taken together, these results demonstrate that the MEK/ERK/MAP kinase signaling pathway is required for prostratin-induced myeloid differentiation.

Myc antagonizes the differentiation induced by prostratin in AML cells

Transcription factors, e.g., CCAAT/enhancer-binding proteins (C/EBPs) and c-Myc, are pivotal for normal myeloid differentiation and are dysregulated in leukemia [31,32]. Here, we observed that upon prostratin treatment, HL-60 cells showed a concentration-dependent increase in protein levels of C/EBPα and C/EBPβ (Fig. 5A). Accordingly, prostratin treatment resulted in a strong concentration-dependent loss of c-Myc protein level in HL-60 cells (Fig. 5A). Furthermore, pretreatment of HL-60 cells with GFX or U0126 reversed the prostratin-induced upregulation of C/EBPα and C/EBPβ expression and downregulation of c-Myc expression, suggesting that alterations in the expression of these transcription factors are downstream effects of PKC-activated ERK proteins. (Fig. 4D). The oncogene c-Myc blocks myeloid differentiation, and its down-regulation is critical for myeloid cell differentiation [32]. To evaluate whether suppression of Myc confers the differentiation-inducing effects of prostratin, we generated HL-60 cell cultures in which the Myc cDNA

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** The role of c-Myc in the prostratin-induced differentiation of HL-60 cells. (A) HL-60 cells were treated with indicated concentrations of prostratin for 24 h. After treatments, total cell lysates were prepared and examined for C/EBPα, C/EBPβ, and c-Myc protein levels by Western blot analysis using the respective specific antibodies. The results are representative of three independent experiments. (B) Western blotting of whole-cell lysates prepared from HL-60 cells transduced with empty vector or a Myc-cDNA-containing MSCV retrovirus. Cells were treated for 48 h with DMSO or 1000 nM prostratin. The results are representative of three independent experiments. (C, D) Quantification of the percentage of CD11b+ cells (C) and DNA content (D) in HL-60 cells transduced with empty control vector or the Myc cDNA. Cells were treated with prostratin for 72 h (C) or 24 h (D) at the indicated concentrations. Data represent mean ± SD of three independent experiments.
was ectopically expressed from a retroviral promoter, which resulted in Myc expression levels that were only slightly elevated but evidently resistant to prostratin-induced suppression (Fig. 5B). Furthermore, ectopic Myc expression conferred nearly complete resistance to myeloid differentiation and G1 cell cycle arrest induced by prostratin (Fig. 5C, D). Consistently, it was also shown that induction of Myc antagonized the upregulation of p21 by prostratin in HL-60 cells. Together, the results demonstrate that Myc impairs the myeloid differentiation mediated by prostratin in HL-60 cells.

Combination of prostratin and Ara-C improves myeloid leukemia cell differentiation

Ara-C is one of the most important antileukemic agents for the treatment of AML and is known to induce the differentiation of some myeloid leukemia cells [33,34]. Thus, we further examined the effects of the combination treatment of Ara-C and prostratin on differentiation in leukemia cells. As shown in Fig. 6A, combined treatment using Ara-C with a low concentration of prostratin resulted in the appearance of differentiated cells with the characteristics of mono-

Fig. 6. Synergistic induction of differentiation by combination treatment of prostratin and Ara-C in AML cells. (A) HL-60 cells were treated with Ara-C (100 nM) or prostratin (250 nM) alone or in combination for 72 h. Cytospins for the treated cells were stained with Giemsa. Images were acquired with a Olympus BX-53 microscope (Olympus, Tokyo, Japan), 400× magnification, and SPOT software. Bars, 50 μm. Representative photos of three independent experiments are shown. (B, C) HL-60 cells were treated for 72 h with the indicated concentrations of Ara-C in the presence or absence of 250 nM prostratin in (B) or with the indicated concentrations of prostratin in the presence or absence of 100 nM Ara-C in (C). The percentage of CD11b+ cells was determined by FACS analysis. Data represent mean ± SD of three independent experiments. ### P < 0.001 between combination and prostratin treated cells. ** P < 0.01; *** P < 0.001 between combination and Ara-C treated cells. (D) NB4 or U937 cells were treated for 48 h with Ara-C (50 and 25 nM, respectively) or prostratin (250 nM) alone or in combination, after which the percentage of CD11b+ cells was determined by FACS analysis. Data represent mean ± SD of three independent experiments. ## P < 0.01 between combination and prostratin treated cells. ** P < 0.01 between combination and Ara-C treated cells. (E) HL60 cells were treated with either prostratin (250 nM) and/or Ara-C (100 or 200 nM) for 24 h. After treatments, total cell lysates were prepared and examined for C/EBPα, C/EBPβ, c-Myc, and P21 protein levels by Western blot analysis using the respective specific antibodies. The results are representative of three independent experiments.
Prostratin has been shown in previous studies to be a protein kinase C activator [17] with potential as an inductive adjuvant therapy for highly active antiretroviral therapy (HAART) due to its potential as an inductive adjuvant.

The above observations indicate that the prostratin-induced differentiation of AML cells is associated with some key transcription factors in myelopoiesis. Consistent with this, the expressions of C/EBPβ and c-Myc were both affected by the combination treatment of prostratin and Ara-C, whereas the expression of C/EBPα showed no alteration under the same conditions (Fig. 6E). In addition, the cell cycle analysis showed that Ara-C could induce slight G1 arrest in HL-60 cells (Appendix: Supplementary Fig. S1). Notably, this arrest was further enhanced by the co-treatment with prostratin with evident increased p21 expression (Appendix: Supplementary Fig. S1 and Fig. 6E). However, combination treatment with the two agents for 48–72 hours did not significantly increase apoptosis in AML cells (Appendix: Supplementary Fig. S2), suggesting that their synergistic activity only contributed to the induction of differentiation.

**Involvement of PKC/ERK signaling pathway in cellular differentiation induced by the combination of prostratin and Ara-C**

As described above, prostratin has been shown to activate the PKC–ERK pathway which is essential for myeloid differentiation induced by prostratin itself. We next examined the role of the PKC–ERK signaling pathway in the cellular differentiation by the combination of prostratin with Ara-C. Analysis of differentiation using morphological assessment of Giemsa-stained cytospin preparations revealed an inhibition of monocytic differentiation when HL-60 cells were pretreated with GFX or U0126, respectively (Fig. 7A). Flow cytometric analyses of CD11b differentiation markers also confirmed the marked inhibition of monocytic differentiation by GFX or U0126 (Fig. 7B). These results suggested that the activation of PKC and ERK signaling pathways may participate in HL-60 cell differentiation induced by prostratin with Ara-C. Additionally, we further examined whether prostratin could enhance the induction of differentiation by other differentiation-inducing agents. It was shown that a significant increase of cellular differentiation was also observed when the cells were cotreated with prostratin and decitabin or VD3, respectively (Fig. 7C). Furthermore, pretreatment of cells with GFX or U0126 also blocked the differentiation caused by combined prostratin with decitabin or VD3 (Fig. 7B). Thus, these results suggested that the activation of PKC/ERK signaling pathways may play an essential role in these synergies.

**Discussion**

In the present study, we demonstrated differentiation-inducing activity of prostratin in acute myeloid leukemia cell lines and primary AML cells, and examined its mechanism of action. We demonstrated further the synergistic activity of prostratin with other anticancer drugs in inducing differentiation of AML cells.

In this study, prostratin was first shown to induce a concentration- and time-dependent decrease in cell number of three human myeloid leukemia cell lines (HL-60, NB4 and U937) with an increase of apoptosis to different degrees. However, it was evident that the percentage of apoptotic cells does not account for that of growth arrest cells in the prostratin-treated AML cell lines. In this context, we further examined the effect of prostratin on the cell cycle progression and found induction of predominant G1 cell cycle arrest, which is essential for the terminal differentiation of AML cells [35]. We assume that prostratin has potent differentiation-inducing activity, and, as expected, prostratin induces differentiation based on morphological assessment, immunophenotype changes, and differentiation-promoting transcription factor expression. Indeed, terminal differentiation is usually coupled with permanent exit from the cell cycle, and this process may require the coordinated activities of the retinoblastoma (RB) family of proteins and cyclin–dependent kinase (CDK) inhibitors [35]. Consistently, the cell cycle arrest associated with prostratin-induced differentiation was shown to involve a marked increase in p21 level and down-regulation of CDK4, CDK6, and cyclin D3 with resultant dephosphorylation of RB at Ser795 and Ser807/811.

Prostratin has been shown in previous studies to be a protein kinase C activator [17] with potential as an inductive adjuvant therapy for highly active antiretroviral therapy (HAART) due to its
ability to up-regulate viral expression from latent virus [18–20]. It has been established that protein kinase C (PKC) signaling pathway plays important role in the HIV-1 antiviral activity of prostratin [36,37]. In this study, we have shown that the activation of PKC appears necessary for prostratin-induced differentiation, cell cycle arrest, and apoptosis, since the pan PKC inhibitors Gö6983 and GF120918 both block the prostratin response in HL-60 cells. However, pre-treatment with the cPKCs inhibitor Gö6976 and nPKC inhibitors 5-(3,4-Dimethoxyphenyl)-4-(1H-indol-5-ylamino)-3- pyridinecarboxitrile [38] and rottlerin [39] had no effect (Appendix: Supplementary Fig. 53), suggesting that prostratin signaling involved multiple PKC isoforms rather than a single isoform irrespective of specific types. Thus, the specific role of each PKC isoforms in the prostratin-induced myeloid differentiation of AML cells still remains obscure. Further careful analysis of activation of specific PKC isoforms by prostratin using various other techniques such as cells expressing dominant negative mutants of PKCs, specific inhibitory peptides, analyses of PKC knock-out cells or short interfering RNA techniques, is essential.

Previous studies have clearly demonstrated an important role for MEK/ERK activation in myeloid differentiation [29,30]. In agreement with these reports, treatment of HL-60 cells with prostratin resulted in the modulation of multiple signaling pathways, including Raf/MEK/ERK, p38, and JNK. Our data also suggest that only PKC-mediated activation of the MEK/ERK/MAP kinase pathway is required for induction of myeloid differentiation by prostratin. As we have described above, prostratin treatment was found to be able to act on some key transcription factors in myelopoiesis and led to the upregulation of C/EBPα and C/EBPβ and the downregulation of c-Myc. We showed that prostratin-induced changes in the expression of such transcription factors were all dependent on PKC-mediated ERK activation. Importantly, we demonstrated an essential role of c-Myc in cellular differentiation by prostratin. Our results are in accordance with the described inhibition by Myc of AML cell differentiation in different cellular models [40,41]. It is well known that cell cycle progression by c-Myc activation is generally incompatible with terminal differentiation [42]. c-Myc enhances cell cycle progression by several mechanisms, including the repression of the cyclin-dependent kinase inhibitors p21 and p27Kip1 [43]. In this regard, we observed that c-Myc abrogated the prostratin-induced G1 cell cycle arrest and p21 upregulation concomitantly with the differentiation inhibition. Collectively, these data demonstrate the importance of the PKC–ERK–c-Myc pathway for prostratin-mediated differentiation of AML cells.

Ara-C is one of the most commonly used chemotherapeutic agents for AML. Treatment with low concentrations of Ara-C can induce the differentiation of human myeloid leukemia cells in vitro [44,45]. Under certain circumstances, the mechanism of Ara-C at low-dose was suggested to be mediated through leukemic cell differentiation in the treatment of AML patients [34]. In this study, we demonstrated that combined treatment with Ara-C and prostratin resulted in a synergistic differentiation effect in AML cells, which may lead to the possibility of using lower doses of Ara-C that may have increased efficacy and more tolerability. Cell-cycle analysis of HL-60 cells treated with Ara-C + prostratin revealed a significant accumulation of cells in the G1 phase, which may correlate with the ability of prostratin (at an active concentration) to induce myeloid cell differentiation through a G1 cell-cycle block. However, this combination showed no enhanced effect on apoptosis of AML cells, further indicating a synergistic activity of Ara-C with prostratin in induction of differentiation. Furthermore, prostratin can synergize with other agents such as decitabine that alone induces weak differentiation as well as VD3 which though a potent differentiation compound leads to toxic hypercalcemia at the doses required for its activity as a single agent. As expected, we also found that ERK/MAPK and PKC pathways are involved in the differentiation process induced by these combinations. Prostratin’s ability to synergize with these agents possibly relates to the fact that it induces differentiation through a different mechanism.

Taken together, we propose that prostratin has the potential to be a differentiation inducer or in combination with other anticancer agents, such as Ara-C for the treatment of AML. The results of the present study provide a rationale for further preclinical and clinical evaluation of prostratin for its potential as an AML therapeutic.

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Conflict of interest

None declared.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.10.018.

References
