Title: Natural product vibsanine A induces differentiation of myeloid leukemia cells through PKC activation

Authors: Zu-Yin Yu,[1,*] He Xiao,[2,*] Li-Mei Wang,[1] Xing Shen,[1] Yu Jing,[3] Lin Wang,[1] Wen-Feng Sun,[1] Yan-Feng Zhang,[1] Yu Cui,[1] Ya-Jun Shan,[1] Wen-Bing Zhou,[1] Shuang Xing,[1] Guo-Lin Xiong,[1] Xiao-Lan Liu,[1] Bo Dong,[1] Jian-Nan Feng,[2] Li-Sheng Wang,[4] Qing-Liang Luo,[1] Qin- Shi Zhao,[5] and Yu-Wen Cong[1]

Affiliations:

[1]Department of Pathophysiology, Beijing Institute of Radiation Medicine, Beijing, China;

[2]Department of Molecular Immunology, Institute of Basic Medical Sciences, Beijing, China;

[3]Department of Hematology, Chinese PLA General Hospital, Beijing, China;

[4]Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing, China;

[5]Department of Phytochemisty, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China.

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Correspondence: Yu-Wen Cong, Department of Pathophysiology, Beijing Institute of Radiation Medicine, No. 27 Taiping Road, Beijing, China; e-mail: congyw@nic.bmi.ac.cn. Tel/Fax: 86-10-68214653

* These authors contributed equally to this work.

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Abstract

All-*trans* retinoic acid (ATRA)-based cell differentiation therapy has been successful in treating acute promyelocytic leukemia (APL), a unique subtype of acute myeloid leukemia (AML). However, other subtypes of AML display resistance to ATRA-based treatment. In this study, we screened natural, plant-derived vibsane-type diterpenoids for their ability to induce differentiation of myeloid leukemia cells, discovering that vibsanine A potently induced differentiation of AML cell lines and primary blasts. The differentiation-inducing activity of vibsanine A was mediated through direct interaction with and activation of protein kinase C (PKC). Consistent with these findings, pharmacological blockade of PKC activity suppressed vibsanine A-induced differentiation. Mechanistically, vibsanine A-mediated activation of PKC led to induction of the ERK pathway and decreased c-Myc expression. In mouse xenograft models of AML, vibsanine A administration prolonged host survival and inhibited PKC-mediated inflammatory responses correlated with promotion of skin tumors in mice. Collectively, our results offer a preclinical proof of concept for vibsanine A as a myeloid differentiation-inducing compound with potential application as an antileukemic agent.

Introduction

Acute myeloid leukaemia (AML), the most common type of leukemia in adults, is characterized by the proliferation of clonal precursor myeloid cells with arrested differentiation (1). The major therapeutic approach to this disease has been the use of chemotherapeutic agents with associated life-threatening toxicity, 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, has introduced a paradigm for success of cell differentiation therapy for AML (3). In APL, the application of ATRA alone or in combination with anthracycline-based chemotherapy, results in myeloid differentiation of the leukemic blasts and leads to a high rate of complete remission and the long-term survival (3, 4). Unfortunately, ATRA is clinically effective only for the treatment of APL, and most patients usually relapse because of acquired resistance (3). Therefore, the development of new compounds that exploit differentiation pathways is key avenues of investigation for AML patients.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases which are classified into three subgroups: classical (α, βI, βII, and γ), novel (δ, ε, η, and θ) and atypical (ζ and -ι). Activation of classical enzymes (cPKC) depends on Ca^{2+} and diacylglycerol (DAG), while novel enzymes (nPKC) are activated by diacylglycerol (DAG), and atypical enzyme (aPKC) activation takes place independently of calcium or DAG (5, 6). The PKC family of isoenzymes is involved in key cellular processes including cell proliferation, apoptosis, and differentiation (6). There is emerging evidence that activation of PKC plays vital functional roles in the differentiation of hematopoietic cells (7). In particular, a number of PKC activators have

been investigated for their ability to induce differentiation of myeloid leukemia cells with potent antileukemic activity. Phorbol esters are originally the most promising candidates due to their potency (8, 9), but the potent tumor-promoting activity raises concerns about their therapeutic use. Fortunately, naturally occurring bryostatin 1, prostratin, and ingenol-3-angelate are fascinating PKC activators without tumor-promoting activities. Hence, bryostatin 1, a macrolide lactone and a non-selective activator of PKC isozymes, has been examined in clinical trials after showing promising results in cancer xenograft models (10). However, bryostatin 1 shows minimal activity against a few special types of hematological malignancies when it was tested in clinical studies (11-13), and the differentiation-inducing activity of bryostatin 1 upon myeloid leukemia cells still remains inconclusive (14). Recently, the novel PKC-activating agent ingenol-3-angelate has been approved for the topical treatment of actinic keratosis, a premalignant lesion that can progress to invasive squamous cell carcinoma (15). Meanwhile, it has been shown to induce apoptosis and differentiation in a range of myeloid leukemia cell lines and in primary AML blasts (16, 17). Similarly, prostratin, an non-tumor promoting phorbol ester, has been shown to induce differentiation of myeloid leukemia cells in vitro (18, 19), but its therapeutic potential as an anti-HIV drug has been attracting much more attention (18, 20). Therefore, the potential of differentiation therapy with PKC activators in AML has yet to be realized, and novel agents that specifically target PKC with differentiation-inducing activity are still in need of development.

Natural products have long been recognized as an important source of therapeutically effective drugs, and their importance in the prevention and treatment of cancer is becoming increasingly evident. Plants of genus *Viburnum* have proven to be a rich source of vibsane-type diterpenes with piscicidal, plant growth regulatory, and cytotoxic activities (21-23). Vibsane-

type diterpenes, however, are regarded as quite rare natural products because their occurrence has been limited to only two kinds of *Viburnum* plants, *V. awabuki* and *Viburnum odoratissimum* and they have not been extensively studied yet (24). In this work, we describe the identification of a novel differentiation-inducing agent, vibsanine A, an vibsane-type diterpenoid isolated from the leaves of *Viburnum odoratissimum*, by using HL-60 cell line-based phenotype screening. We show that vibsanine A induces differentiation of myeloid leukemia cells by targeting PKC as evidenced by the direct interaction with and activation of PKC by vibsanine A, which leads to the activation of extracellular signal–regulated proteinkinases (ERK) 1 and 2 and consequently to the decreased expression of c-Myc. Moreover, the antileukemic activity of vibsanine A has also been shown in murine AML xenograft models. These results indicate that vibsanine A is a powerful tool for understanding the potential pathophysiological and therapeutic roles of PKC and warrants further evaluation as potential differentiation therapy for myeloid leukemia.

Materials and Methods

Chemicals and reagents

Vibsanine A was isolated from the dried leaves of *Viburnum odoratissimum* as described in Supplementary Methods. Other chemicals and reagents were all from Sigma unless stated otherwise.

Cell culture, morphology, and flow cytometry assay

The human myeloid leukemia cell lines HL-60 and U937 were obtained from China Infrastrucure of Cell Line Resource (Beijing, China) and cultured under recommended conditions. The DNA profiles characteristic of cells was authenticated by STR analysis in 2015. NB4 cells (kindly gifted from Dr. Bingwei 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 ºC, 5% CO2 humidified atmosphere. Cell morphological features and cell-surface differentiation antigens were measured as described (19). Fresh blasts from 11 cases of *de novo* AML patients were obtained with informed consent and study protocols were approved by the Institutional Review Board of Chinese PLA General Hospital.

RNA isolation, real-time quantitative PCR, and transcriptome analysis

Total RNA was isolated by a Trizol kit (Invitrogen). RNA was treated with DNase (Promega). Complementary DNA was synthesized according to manufacturer's instructions. Real-time quantitative PCR was performed with SYBR Green PCR Master Mixture Reagents (TOYOBO) on the CFX96 Touch^{TM} Real-Time PCR Detection system (Bio-Rad). For the microarrays, the NimbleGen 12 × 135K array and the dual-color analysis method were used following the manufacturer's protocol (Roche/NimbleGen).

PKC-GFP translocation assay

293-T cells were seeded onto 40-mm circular glass coverslips, and 24 h later, were transiently transfected with GFP-tagged human PKC-α, β, γ, δ, η, and θ subcloned into pEGFP-N1 plasmid. Translocation of PKC isoforms was visualized by confocal microscopy 48 h after transfection.

Binding of [^{3}H]PDBu

The binding affinity of vibsanine A to purified human PKC-α, β, γ, δ, ε, η, and θ isoforms and PKD1 (GenWay Biotech) was determined by competition of [^{3}H]PDBu binding. [^{3}H]PDBu binding was measured as described by Lewin and Blumberg (25).

Kinase assay

The kinase activity of PKC isoforms was assayed using histone III-S as the substrate. PKCs (5–25 ng) were incubated for 5 min at 37°C in a final volume of 75 μl containing 20 mM Tris-HCl,

pH 7.5, 20 μg/μl lipid (POPS:POPC 20:80), 200 μg/ml histone III-S, 100 μM [γ-^{32}P] ATP (100 - 200 cpm/pmol; Amersham), 5 mM $MgCl_2$, and 0.3 mM $CaCl_2$ or 0.1 mM EGTA. The kinase reaction was terminated by the addition of ice-cold 20% trichloroacetic acid and analysed by a filter assay (P81 phosphocellulose filters, Whatman). [^{32}P]-Phosphate incorporation was measured by scintillation counting.

Cellular fractionation

Cells were washed twice in PBS, and then lysed by homogenization in hypotonic lysis buffer in the absence of detergent. Cell debris or nuclear fraction was removed by centrifugation at 1000 g for 10 min at 4 °C. Centrifugation at 100,000 g for 60 min at 4 °C was used to isolate the cytosol (supernatant) and cell membrane (pellet) fractions.

Western Blot analysis

At the end of the desired treatment, total cell lysates preparation and Western blot analysis were performed according to the procedure described before (19).

In vivo xenograft study

Leukemic cells isolated from spleens from leukemic Mll-AF9 transgenic mice (The Jackson Laboratory) were intravenously injected into 6 to 8-week-old male C57BL/6 mice (2.5×10^6 leukemic cells per mouse) 6 hours after sublethal irradiation (totally 4.5 Gy). For HL-60 xenografts, 4 to 6-week-old male NOD/SCID mice were intravenously injected HL-60 cells stably expressing the firefly luciferase (1×10^6 cells per mouse) 6 hours after sublethal irradiation (totally 2.0 Gy). Three days after leukemic cell transplantation, the mice were orally treated with vehicle or vibsanine A. No animals were excluded from analysis and animal groups were not blinded. Animal handling was approved by the Animal Care and Use Committee of Animal Center in Academy of Military Medical Science (AMMS, Beijing, China).

Statistical Analysis

To assess correlation of the transcription profiles of vibsanine A, PMA, and ATRA in HL-60 cells, scatter plots were used and linear relationships were measured. The correlation coefficient, R^2, that indicates the variability of intensity values in one group vs. the other, was calculated using Excel software. Kaplan-Meier survival analysis (SPSS 10.0; SPSS Inc.) was used to compare the survival probabilities of different groups. An unpaired Student's t-test was used to evaluate the difference between two different treatments. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS software.

Results

Vibsanine A triggers differentiation of myeloid leukemia cells

In order to identify novel differentiation-inducing agents, a series of vibsane-type diterpenoids were isolated from the leaves of *Viburnum odoratissimum* (Supplementary Fig. S1A), and screened for their ability to trigger the differentiation of HL-60 cells by detecting the expression level of CD11b, a differentiation marker of myeloid cells. Amongst them, a novel vibsane-type diterpenoid, designated vibsanine A (Fig. 1A), was shown to possess the highest differentiation-inducing activity (Supplementary Fig. S1B). The results showed that vibsanine A treatment increased the percentages of $CD11b^+$ and $CD14^+$ cells in a concentration-dependent manner (Fig. 1B). Vibsanine A-treated HL-60 cells in suspension became adherent to the culture plate and showed substantial changes in morphology, indicative of monocytic differentiation, as evidenced by increased cell size, lower nuclear to cytoplasmic ratio and highly vacuolated cytoplasm (Fig. 1C). Such morphologic and functional differentiation induction could also be observed in cell lines from monoblastic (U937) and promyelocytic (NB4) origin (Supplementary Fig. S2A-C).

Interestingly, the human chronic myelocytic leukemia (CML) K562 cells treated with vibsanine A demonstrated morphologic evidence of megakaryocytic differentiation (Supplementary Fig. S2A) and up-regulated the expression of CD41 and CD61 megakaryocyte cell surface markers (Supplementary Fig. S2D). Finally, induction of myeloid differentiation by vibsanine A in these cells was associated with an inhibition of cell growth and induction of G1 cell cycle arrest (Supplementary Fig. S3).

Next, we determined the potential differentiation-inducing effects of vibsanine A on primary blast cells from 11 cases of AML patients, according to the percentage of CD11b-positive cells. Vibsanine A induced evidence of differentiation in 10 of 11 evaluable AML patient samples, with eight samples responded at doses less than 3 μM (Table 1). We noted that one sample from a patient with M3-AML (patient 1) showed vibsanine A-mediated cell death instead of differentiation, and two samples (patient 5 and 10) exhibited low sensitivity to vibsanine A.

Vibsanine A-induced cell differentiation is dependent on the activation of PKC

We next investigated the effects of other known differentiation-inducing agents, including PMA and ATRA, on the myeloid leukemia cells. Thus, a comparison of the differentiation-inducing activities of vibsanine A with PMA afforded a similar phenotype in the myeloid leukemia cell differentiation assay (Fig. 1B and C, and Supplementary Fig. S2). In contrast, vibsanine A induced a distinct pattern of myeloid leukemia cell differentiation compared with ATRA, producing monocytic differentiation of AML cells (represented by $CD11b^{+}/CD14^{+}$) and megakaryocytic differentiation of CML cells (represented by $CD41^{+}/CD61^{+}$), whereas ATRA specifically induced granulocytic differentiation of AML cells (represented by $CD11b^{+}/CD14^{-}$) (26) (Fig. 1B and C, and Supplementary Fig. S2). By comparing the pattern of genes induced by PMA and by vibsanine A in a nonsupervised transcriptome analysis, we also found a high

similarity between the differentially expressed genes. As early as 6 hours, 89.2% of the genes affected by vibsanine A were co-regulated by PMA, and the gene expression pattern was similarly evident following 24 hours of treatment (88.6%; Fig. 2A, and Supplementary Fig. S4). In contrast, only less than 10% of the genes affected by vibsanine A were modulated by ATRA (Fig. 2A and Supplementary Fig. S4). Consistently, based on global gene expression profiles, correlation analysis demonstrated an extremely strong correlation between vibsanine A and PMA (R^2=0.9733, 0.7672) (Fig. 2B and Supplementary Fig. S4). However, a comparison between the expression profiles of vibsanine A and ATRA yielded a much lower correlation (R^2=0.1262, 0.024) (Fig. 2B and Supplementary Fig. S4). Together, these results suggest that vibsanine A-induced cell differentiation shares high similarity with that induced by PMA.

As a potential differentiating agent, PMA is also a PKC activator and has been demonstrated to induce PKC-dependent differentiation of myeloid leukemia cells (27, 28). Thus, we next investigated whether vibsanine A activated PKC and induced PKC-mediated differentiation. We first studied the functional effects of vibsanine A on PKC activation by using PKC-green fluorescent protein (GFP) fusion protein translocation assay. As shown in Figure 2C, with vibsanine A treatment, classical PKC isoforms including PKC-α, -β, and -γ were translocated almost instantaneously to the plasma membrane, just as it can be seen in PMA-treated cells. Notably, vibsanine A was found to induce a pronounced translocation of novel PKC isoforms including PKC-δ, -η, and -θ simultaneously to the plasma membrane and to the nuclear membrane. However, unlike vibsanine A, PMA caused only the translocation of these isoforms to the plasma membrane. We also observed that treatment with vibsanine A caused a rapid shift of various classical and novel PKCs from the cytosol fraction to membrane fraction in HL-60 cells (Fig. 2D). In contrast, vibsanine A and PMA both showed no effect on the atypical PKC-ζ.

Prolonged treatment with PKC activators such as phorbol esters and other natural products is known to result in down-regulation of different PKC isoforms (29). In leukemia cells, PMA was able to cause down-regulation of the tested classical and novel PKCs except PKC-ε in K562 cells (Supplementary Fig. S5). Vibsanine A, however, only down-regulated PKC-βII in HL-60 cells and PKC-θ in K562 cells, which showed selective activity in down-regulation of PKC isoforms compared with PMA (Supplementary Fig. S5).

To determine the functional role of PKC in vibsanine A-induced AML cell differentiation, we treated HL-60 cells with GF109203X (GFX) and Gö6983, which are blockers of both cPKCs and nPKCs (30). Notably, pretreatment with them completely blocked vibsanine A-mediated cellular differentiation (Fig. 2E and F), indicating that PKC activation is essential for vibsanine A-induced differentiation of AML cells. However, pretreatment with the cPKCs inhibitor Gö6976 (30) and nPKCs inhibitor 5-(3,4-Dimethoxyphenyl)-4-(1H-indol-5-ylamino)-3-pyridinecarbonitrile (31) had no effect (Fig. 2E and F). Finally, PMA showed a similar response to PKC inhibitors as vibsanine A (Supplementary Fig. S6).

Vibsanine A directly interacts with PKC

The identification that the close similarities between vibsanine A and PMA and the activation of PKC involved in the vibsanine A-mediated cell differentiation raised a possibility that vibsanine A might directly interact with PKC. To test this hypothesis, vibsanine A was evaluated for the binding affinity to PKC by using radioligand-binding techniques, where K_i values were measured by competition of [^{3}H]PDBu binding. Saturation analysis of [^{3}H]PDBu binding to various PKC isoforms and PKD1 was first performed and high affinity was demonstrated with the dissociation constant (*Kd*) values (Supplementary Fig. S7). Competition of [^{3}H]PDBu binding assays showed that vibsanine A had Ki values for classic PKC isoforms (cPKCs: PKC-α, β, γ) ranging from 3

nM to 5 nM (Fig. 3A). Similarly, novel PKC isoforms (nPKCs: PKC-δ, ε, η, θ) showed affinity for vibsanine A in the same order although to a lesser extent than cPKC. In the case of PKD1, the Ki value for vibsanine A was 15.82 ± 0.12 nM, much higher than that of PKC isoforms (Fig. 3A).

To gain finer details of the possible binding mode of vibsanine A with the PKC isoforms, we performed molecular docking experiments. It was shown that the PKC-α, β, and ε formed hydrophilic pocket while the PKC-δ formed hydrophobic pocket to interact with vibsanine A (Fig. 3B-E and Supplementary Fig. S8). Compared with the binding energy between PKC and vibsanine A, the complex PKC-ε and vibsanine A was the most stable while the PKC-δ and vibsanine A was the most unstable (Fig. 3F). For the PKC-α, β, and δ, vibsanine A inserted the pocket with the aliphatic side chain, and the ring chain was exposed to the solvent. While vibsanine A interacted with PKC-ε, the whole molecule interacted with the protein. The ring and aliphatic were all involved in the binding.

We next tested whether vibsanine A increased PKC activity in an in vitro kinase assay. The results demonstrated that vibsanine A increased the basal activity of both cPKC and nPKC in a dose-dependent manner with similar potency indicated by EC_{50}s (Fig. 3G and H). However, vibsanine A did not activate PKD1 in such conditions although it was able to bind PKD1 in vitro.

Downregulation of c-Myc expression mediates vibsanine A-induced differentiation

The oncogene c-Myc blocks myeloid differentiation, and its downregulation is critical for myeloid cell differentiation (32). Upon vibsanine A treatment, HL-60 cells showed a strong concentration-dependent loss of protein levels of c-Myc, which was accompanied by an elevation in expression of C/EBPβ and p21 (Fig. 4A), which are transcriptional suppressed by c-Myc and are pivotal for normal myeloid differentiation (33, 34). The reduction of c-Myc

expression by vibsanine A could be regulated at the transcriptional level or at the posttranscriptional level. It was shown that the downregulation of c-Myc protein was detectable within 1 hour of treatment and highly significant from 3 hours of treatment with corresponding decrease in c-Myc mRNA levels (Fig. 4B and C). Previous reports have shown that c-Myc is a very unstable protein with half-life between 20 and 30 minutes and its rapid turnover can be mediated by the ubiquitin-dependent proteasome pathway (35). Thus we examined whether vibsanine A affects c-Myc protein degradation. As shown in Figure 4D, vibsanine A was shown to markedly promote the degradation of c-Myc and shorten its half-life by using a CHX chase assay. Moreover, treatment with proteasome inhibitor MG132 blocked the c-Myc suppression and cellular differentiation induced by vibsanine A (Fig. 4E and F), suggesting the involvement of proteasome-dependent degradation of c-Myc in the vibsanine A-mediated myeloid differentiation. To further evaluate whether suppression of c-Myc confers the differentiation-inducing effects of vibsanine A, we generated HL-60 cells in which the c-Myc cDNA was ectopically expressed, which resulted in the mRNA but not the protein levels of c-Myc that were dramatically elevated but both significantly resistant to vibsanine A-induced suppression (Fig. 4G and H). Ectopic c-Myc expression conferred complete resistance to myeloid differentiation and G1 cell cycle arrest induced by vibsanine A (Fig. 4I and Supplementary Fig. S9). Consistently, induction of c-Myc antagonized the upregulation of C/EBPβ and p21 at both mRNA and protein levels by vibsanine A (Fig. 4G and H). We next sought to confirm the functional role of c-Myc in the vibsanine A-mediated cellular differentiation using another AML cell line NB4 with relative low endogenous expression of c-Myc (Supplementary Fig. S10). As expected, infected NB4 cells showed increased expression of the c-Myc protein, which led to the inhibition of downregulation of c-Myc with slight decrease of upregulation of C/EBPβ and p21

and consequently to the cell differentiation induced by vibsanine A (Supplementary Fig. S10). These results, collectively, support an important role for downregulation of c-Myc in vibsanine A-induced differentiation of AML cells.

Vibsanine A-induced c-Myc downregulation is mediated by the PKC/ERK signaling pathway

Next, we addressed how vibsanine A-mediated PKC activation decreased c-Myc expression. It has been well documented that MAPKs, especially MEK/ERK/MAP kinase signaling pathway, play an important role in myeloid differentiation (36, 37). In line with these reports, vibsanine A induced the phosphorylation of Raf-1, MEK, and ERK in a dose-dependent manner, with marked increases in p38 and JNK phosphorylation (Fig. 5A). However, only inhibitor of ERK, but not of JNK and p38, abrogated vibsanine A-induced cellular differentiation (Fig. 5B and C). Pretreatment with the ERK inhibitor U0126 blocked ERK phosphorylation and antagonized vibsanine A-induced upregulation of C/EBPβ and p21 (Fig. 5D). Importantly, inhibition of ERK activation with U0126 effectively reversed the vibsanine A-induced suppression of c-Myc expression levels (Fig. 5E), which might be mediated by inhibition of the dephosphorylation of Ser62 by vibsanine A since Ser62 phosphorylation at c-Myc has been shown to be crucial for its protein stability (38). Moreover, pretreatment with GFX blocked vibsanine A-induced Raf-1, MEK, and ERK activation and effectively antagonized upregulation of C/EBPβ and p21 protein levels and downregulation of c-Myc protein levels (Fig. 5D and E). These results indicate that reduction of c-Myc expression by vibsanine A is at least in part a downstream effect of PKC-activated ERK proteins.

Vibsanine A prolongs survival of mouse xenograft models of AML

To investigate the *in vivo* effects of vibsanine A, we intravenously transplanted leukemic blasts from Mll-AF9 transgenic mice into sublethally irradiated C57BL/6 recipients to generate AML mice. All of these mice rapidly developed an AML phenotype with anemia, high white blood cell (WBC) count, and hepatosplenomegaly (Supplementary Table S1). In mice treated with vibsanine A, the level of WBC count increase was depressed. Moreover, mice treated with vibsanine A clearly had a survival benefit over vehicle-treated mice (Fig. 6A). To confirm the in vivo efficacy of vibsanine A, we further intravenously transplanted HL-60 cells stably expressing the firefly luciferase gene into sublethally irradiated NOD/SCID mice to generate a mouse xenograft model of AML. In this model system, bioluminescent imaging showed a decelerated tumor progression in vibsanine A-treated mice (Fig. 6B). Median overall survival of vibsanine A-treated animals was significantly longer than control animals (Fig. 6C). Finally, vibsanine A administration was well tolerated, and no significant weight loss was observed (Supplementary Fig. S11). Together, these results demonstrate that vibsanine A has antitumor efficacy in mouse xenograft models of AML leukemia.

Vibsanine A inhibits PMA-induced inflammatory responses in mouse skin

PMA is the most widely studied tumor promoter and exerts tumor promoting, inflammatory, and hyperplastic activity in mouse skin (6, 39). We next compared the hyperplastic effects of vibsanine A and PMA in mouse skin model. A dose of 7.5 nmol PMA induced strong hyperplasia on the back skin of CD-1 mice, whereas even the highest test concentration (2.5 μmol) of vibsanine A did not cause significant epidermal hyperplasia at various time posttreatment (Supplementary Fig. S12). Furthermore, the topical application of vibsanine A, prior to that of PMA application to mouse skin resulted in inhibition in the induction of epidermal hyperplasia in a dose-dependent manner (Fig. 6D). Consistently, vibsanine A

significantly reduce the level of skin edema induced by PMA (Supplementary Fig. S12). Together, these results revealed an evident inhibitory effect of vibsanine A on PMA-mediated inflammatory responses.

Discussion

In the present study, we provided the first demonstration that vibsanine A, a vibsane-type diterpene, exhibited potent differentiation-inducing activity in myeloid cell lines and primary patient–derived blasts with antileukemic effect on mouse xenograft models. Importantly, we identified vibsanine A as a potent and competitive activator of PKC in biochemical, structural, and cellular analysis and established a key role for PKC signaling in vibsanine A-induced myeloid differentiation.

The principal molecular mechanism drawn from the experiments reported here links the signaling pathways activated by vibsanine A and by PMA. A comparison of the differentiation-inducing activities of vibsanine A with PMA afforded a very similar phenotype in cellular differentiation assay and gene expression analysis. Vibsanine A activated PKC with inducing cellular translocation of various PKCs to the plasma membrane and/or the nuclear membrane, which is one hallmark of PKC activation (40). Conversely, pharmacological inhibition of PKC blocked vibsanine A-induced cellular differentiation, suggesting a predominant role of PKC in the differentiation. Prolonged activation of PKCs with phorbol esters may cause their downregulation，and different PKC ligands may drive different patterns of PKC localization as well as downreguation. For example, bryostatin 1 translocated PKC-δ predominantly to the nuclear membrane and inversely prevented PKC-δ translocation to the plasma membrane and its downregulation by PMA (29, 41). We found that vibsanine A showed selectivity to a degree in

downregulation of the PKC isozymes as compared with PMA. Vibsanine A also caused simultaneous translocation of nPKCs to the plasma membrane and the nuclear membrane, whereas PMA induces only translocation to the plasma membrane in PKC-GFP translocation assay. The distribution of PKC isoforms in different cellular compartments determines the functions of these isoforms by presenting them to different substrates and interaction partners. Therefore, different patterns of localization and/or downregulation of PKC isoforms could contribute to the differing biological response of vibsanine A compared with the other PKC ligands, such as we indeed observed for inflammatory responses.

Classical PKC ligands such as phorbol esters, Ingenol 3-angelate, and prostratin serve as structural mimetics of DAG and bind to the C1 domains of PKC. In this paper, the competitive inhibition of PKC by vibsanine A with respect to ligands for the phorbol ester binding site, and the ability of vibsanine A to compete with [^{3}H]PDBu for binding, raised a possibility that vibsanine A interacts specifically with the phorbol binding C1-domain. In line with this assumption, molecular docking studies revealed that vibsanine A interacted with the C1 domains of PKC. A highly favorable pattern of hydrophilic or hydrophobic interactions with the expected entropy gain due to conformational restriction appears to contribute to its high binding affinity. The observation that the binding energy obtained from the models do not always corroborate with the experimental binding data indicate the fact that both the proteins and ligands can undergo conformational changes in solutions. Although the detailed structural determinants for vibsanine A binding to PKCs remains to be investigated, our results definitely identify vibsanine A as a potent PKC activator. Furthermore, as an 11-membered ring compound, vibsanine A bears significant structural differences relative to other PKC activators including phorbol esters, bryostatins, ingenols, et al. Most of these known PKC activators are highly complex in their

chemical structure, which makes a great obstacle in structural modification. Obviously, vibsanine A shows a less constrained structure than them, and the first total synthesis of vibsanine A has been achieved (42). Thus, structural optimization to screen and improve the activity of such vibsane-type compounds is practicable.

c-Myc is an oncogenic transcription factor, and it is found deregulated in about half of human tumors and appears frequently associated with tumor progression in solid tumors and leukemia (43, 44). One of the most relevant activities of c-Myc in carcinogenesis is the impairment of cell differentiation (45), and repression of c-myc is required for terminal differentiation of many cell types, including myeloid cells (46, 47). Recently, we have presented an essential role for c-Myc suppression in the differentiation-inducing effects of prostratin as a PKC activator (19). In light of these observations, it was intriguing that c-Myc was rapidly down-regulated at transcriptional and posttranscriptional levels by vibsanine A treatment, and the fact that block of the degradation of endogenous c-Myc and the enforced ectopic expression of c-Myc both effectively antagonized vibsanine A-mediated differentiation strongly supported the role of decreased c-Myc in vibsanine A-induced AML cell differentiation. Our further investigations showed that repression of c-Myc was mediated by PKC-dependent activation of ERK/MAP kinase signaling pathway, which has been widely shown to mediate terminal differentiation in various lineages of hematopoietic cells (36, 37). We conclude that vibsanine A induces AML cell differentiation by activating PKC, followed by ERK activation, which leads to c-Myc downregulation.

PKCs have been shown to play a complex role in tumorigenesis, which is initially recognized from the tumor promoting activity of phorbol esters in mouse skin (6). As potent PKC activators, phorbol esters evoke diverse and dramatic effects on mouse skin, including tumor promotion, inflammation and hyperplasia (39). However, prostratin, a non-tumor-promoting phorbol ester,

has been shown to inhibit above effects induced by PMA with identified antipromoting activity (48). Similarly, vibsanine A was shown to have no inflammatory activity with an evident inhibitory effect on PMA-mediated inflammatory responses. Induction of hyperplasia and its maintenance upon repeated treatment have proved so far to correlate best with tumor promoting activity in the mouse skin model system (49). In light of this, our findings therefor supported the preliminary conclusion that vibsanine A is not tumor promoting but antipromoting. Indeed, Antal et al. have recently established that PKC isozymes generally function as tumor suppressors, indicating that therapies should focus on restoring, not inhibiting, PKC activity (50). In this context, classic PKC agonists such as PMA, which showed tumor-promoting effects, and Bryostatin-1, which failed as a therapeutic, all downregulate PKC and likely result in the inhibition of PKC activity. Conversely, vibsanine A activated PKC with less downregulation of them in leukemia cells, suggesting a promising clinical potential (50). Overall, the potent antileukemic activity and the absence of inflammatory activity with predicted antipromoting potential with vibsanine A is encouraging. These particularities highlight vibsanine A and related vibsanine diterpenes as a family of natural products worthy of further investigation in the efforts toward safe and efficient differentiation therapy of AML.

Supplementary information is available at journal´s website.

Conflict of interest

The authors declare no conflict of interest.

Authors' Contributions

Conception and design: Z.Y. Yu, H. Xiao, Y.Y. Cong

Development of methodology: Z.Y. Yu, H. Xiao, L.M. Wang, X. Shen, L. Wang, W.F. Sun, Y.F. Zhang, Y. Cui, Y.J. Shan, and W.B. Zhou

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Jing, S. Xing, G.L.Xiong, X.L. Liu, and Q.L. Luo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z.Y. Yu, H. Xiao, B. Dong, J.N. Feng, Y.Y. Cong

Writing, review, and or revision of the manuscript: Z.Y. Yu, L.S. Wang, Y.Y. Cong

Study supervision: Y.W. Cong

Other (Provided vital new reagents): Q.S. Zhao

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Figure Legends

Figure 1. Differentiation induction activity of vibsanine A on HL-60 cells.

(A) Chemical structure of vibsanine A.

(B) Quantification of CD11b and CD14 expression in HL-60 cells treated with vibsanine A (0.2, 1, 5, and 10 μM), PMA (10 nM), or ATRA (1 μM) for 72 h. Data represent mean ± SD of three independent experiments.

(C) Phase-contrast observation (top) and Giemsa's staining morphology (bottom) were detected in HL-60 cells after treatment for 72 h with vibsanine A (10 μM), PMA (10 nM), or ATRA (1 μM). Images were acquired with an Olympus BX-53 microscope (Olympus, Tokyo, Japan). The results are representative of three independent experiments. Scale bars are 20 μm.

Figure 2. The role of PKC activation in the vibsanine A-mediated differentiation.

(A) HL-60 cells were treated for 6 h with vibsanine A (10 μM), PMA (10 nM), and ATRA (1 μM), after which total RNA was isolated and microarray analysis was performed according to the manufacturer's protocol. The areas of the Venn diagram represent the number of genes that were significantly regulated by only one compound or by both compounds amongst vibsanine A, PMA, and ATRA. The percentages of co-regulated genes in vibsanine A-treated HL-60 cells are indicated. The regulated genes were identified by statistical analysis to show significant gene expression ($p < 0.05$, one-way ANOVA) and by a 2-fold cutoff with differential expression in the absence and presence of chemicals.

(B) Average difference scatter correlation graphs. The transcription profiles of vibsanine A, PMA, and ATRA were compared in HL-60 cells, and the Pearson rank correlation coefficient of each pair is indicated.

(C) Translocation of green fluorescent protein (GFP)-tagged protein kinase C (PKC) isoforms in 293-T cells. 293-T cells expressing GFP-tagged PKC-α, β, γ, δ, η, and θ were treated with 5 μM vibsanine A or 10 nM PMA for 45 min. Images taken at 0 and 5 min are shown. Data represent confocal images obtained from a representative experiment. The results are representative of three independent experiments. Scale bars are 20 μm.

(D) Translocation of various PKC isoforms in HL-60 cells. HL-60 cells were treated with vibsanine A or PMA for 20 min. Cell lysates were prepared and fractionated into soluble fraction and particulate. Equal amounts of these fractions were resolved by SDS-PAGE and Western blot analysis using the respective specific antibodies. The results are representative of three independent experiments.

(E, F) After pretreatment with and without 4 μM GFX, 4 μM GÖ6983, 1 μM GÖ6976, or 10 μM 5-(3,4-Dimethoxyphenyl)-4-(1H-indol-5-ylamino)-3-pyridinecarbonitrile for 1 h, HL-60 cells were treated with 10 μM vibsanine A for 48 h. Giemsa's staining morphology (E) and CD11b expression (F) were detected. Data represent mean ± SD of three independent experiments. *$P < 0.01$ versus vibsanine A-treated cells. The results in panel **E** are representative of three independent experiments. Scale bars are 20 μm.

Figure 3. Biochemical analysis of PKC activation by vibsanine A.

(A) Binding of vibsanine A to different protein kinase C (PKC) isoforms. Binding affinities of vibsanine A to PKC-α, β, γ, δ, ε, η, θ, and PKD1 were measured in the presence of phosphatidylserine by inhibition of [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding. Data represent mean ± SD of three independent experiments.

(B-F) Binding model of vibsanine A into PKC-α (B), β (C), δ (D), and ε (E). The key residues of PKC (green lines) interact with vibsanine A (pink balls and sticks) was indicated. The binding energy (kCal/mol) between PKC isoforms and vibsanine A (F) were analyzed with distance geometry and computer graphics technology.

(G, H) Kinase activity of different PKC isoforms induced by vibsanine A with indicated concentrations. Activation of purified human PKC-α, β, γ, δ, ε, η, and θ was assayed by measuring the incorporation of ^{32}P from ATP into PKC substrate (histone III-S) in the presence of 20 μg/μl lipid (POPS:POPC 20:80), 5 mM $MgCl_2$, and 0.3 mM $CaCl_2$ (G) or 0.1 mM EGTA (H) for 5 min at 37°C. Mean values ± SE of triplicate measurements from a single experiment are shown. Two additional experiments showed similar results.

Figure 4. The role of c-Myc in vibsanine A-induced differentiation.

(A) HL-60 cells were treated with indicated concentrations of vibsanine A for 24 h. After treatments, total cell lysates were prepared and examined by Western blot analysis using the respective specific antibodies.

(B, C) HL-60 cells were treated with 10 μM vibsanine A for 0, 1, 3, 6, and 12 h. The protein levels and mRNA levels of c-Myc were examined by Western blot analysis (B) and real-time PCR (C), respectively.

(D) HL-60 cells were incubated with 100 μM CHX in the presence or absence of 10 μM vibsanine A. Total cell lysates were obtained at the indicated time points and examined by Western blot analysis (Left panel). c-Myc signal was quantified by densitometry, normalized relative to actin signals (Right panel).

(E, F) HL-60 cells were incubated with 10 μM vibsanine A for 3, 6 (E), and 24 (F) h in the presence or absence of 10 μM MG132. Total cell lysates were obtained and examined by Western blot analysis (E). Quantification of the percentage of $CD11b^+$ cells were determined by flow cytometric analysis (F).

(G) c-Myc, C/EBPβ, and p21 mRNA levels in HL-60 cells transduced with empty control vector or the Myc cDNA as determined by real-time PCR. Cells were treated for 24 h with 10 μM vibsanine A. Results were normalized to GAPDH, with the relative mRNA level in untreated cells transduced with empty control vector set to 1. *$P < 0.01$ versus untreated cells transduced with empty control vector.

(H) 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 24 h with 10 μM vibsanine A.

(I) Quantification of the percentage of $CD11b^+$ cells in HL-60 cells transduced with empty control vector or the Myc cDNA. Cells were treated with vibsanine A for 48 h at the indicated concentrations. Data represent mean ± SD of three independent experiments. All experiments were repeated three times with similar results.

Figure 5. The PKC-ERK pathway in the downregulation of c-Myc by vibsanine A.

(A) HL-60 cells were treated with indicated concentrations of vibsanine A 15 min. After treatments, total cell lysates were prepared and examined by Western blot analysis using the respective specific antibodies.

(B, C) After pretreatment with and 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 10 μM

vibsanine A for 48 h. Giemsa's staining morphology (B) and the percentage of $CD11b^+$ cells (C) were measured. Data represent mean ± SD of three independent experiments. *P < 0.01 versus vibsanine A-treated cells. Scale bars in panel B are 20 μm.

(D, E) HL-60 cells were pretreated with or without 4 μM GFX or 10 μM U0126 for 1 h followed by cotreatment with or without 10 μM vibsanine A in the presence of the inhibitor for 24 h (D) or the indicated times (E). After treatments, total cell lysates were prepared and analyzed by Western blot analysis using the respective specific antibodies. All experiments were repeated three times with similar results.

Figure 6. In vivo activity of vibsanine A

(A) Kaplan-Meier survival curves of control and treated mice with AML. Secondary recipient mice were transplanted with splenocytes from a primary leukemic mouse and then treated 3 days later with vibsanine A (200 mg/kg, n = 4) or vehicle (oil, n = 4) daily by oral gavage for 5 days each week for 28 days. *P < 0.01 against vehicle-treated mice.

(B, C) In vivo efficacy of vibsanine A in a HL-60 xenograft model. NOD/SCID mice were injected with luciferase-labeled HL-60 cells and treated with vibsanine A (200 mg/kg, n = 8) or vehicle (oil, n = 8) as described in (A). Serial bioluminescence images (B) of representative mice receiving vibsanine A or vehicle are shown. A plot of overall survival probability (C) was estimated with the Kaplan–Meier method. *P < 0.01 against vehicle-treated mice.

(D) Inhibitory effect of vibsanine A on epidermal hyperplasia caused by PMA in CD-1 mice. Mice were treated with indicated concentrations of vibsanine A followed 30 min later with or without 8.1 nmol PMA. The animals were killed at 72 h after treatment and skin biopsies were processed for hematoxylin and eosin staining. Representative pictures are shown. Images were

acquired with an Olympus BX-53 microscope (Olympus, Tokyo, Japan). Scale bars are 50 μm. All experiments were repeated twice with similar results.

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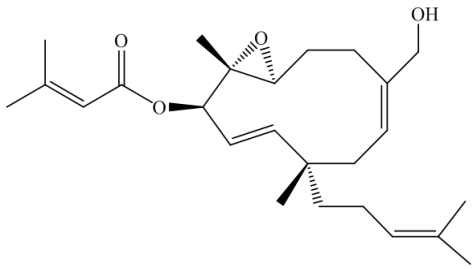
Table 1. Patient data and response to vibsanine A or ATRA

NO.	Sex	Age (years)	FAB (subtype)	Cytogenetic	Blasts %	WBC ($\times 10^9$/L)	CD11b-positive cells %				
							DMSO	vibsanine A (μM)			ATRA
								1	3	10	
1	M	14	M3	46, XY, PML/RARα$^+$	89	1.58	0.3	1.9	2.3	1.7	12.7
2	F	46	M4	AML/ETO$^+$	44	9.12	20.6	29.8	36.2	37.1	27.7
3	F	68	M0	BCR/JAK2$^+$	78	10.53	0.9	19.4	22.6	27.0	13.8
4	M	73	M4	ND	87	129.2	16.2	24.8	29.1	39.2	17.6
5	F	53	M2	FLT3-ITD$^+$	84	24.99	10.1	12.0	16.5	72.7	ND
6	F	14	M4	ND	56	61	35.1	40.1	60.3	62.3	34.3
7	F	53	M5	47, XX	96	84.54	25.2	40.4	44.1	63.9	32.5
8	M	66	M5	BCR/ABL$^-$	81	18.37	35.8	79.0	85.9	90.3	ND
9	M	25	M2	ND	96	59.32	16.5	37.9	59.9	63.6	7.2
10	M	55	M2	ND	85	22.5	2.2	3.4	11.7	34.8	ND
11	M	24	M5	MLL$^-$	92	149	10.1	24.4	42.0	48.0	25.6

FAB indicates French–American–British classification. ND indicates not done.

A



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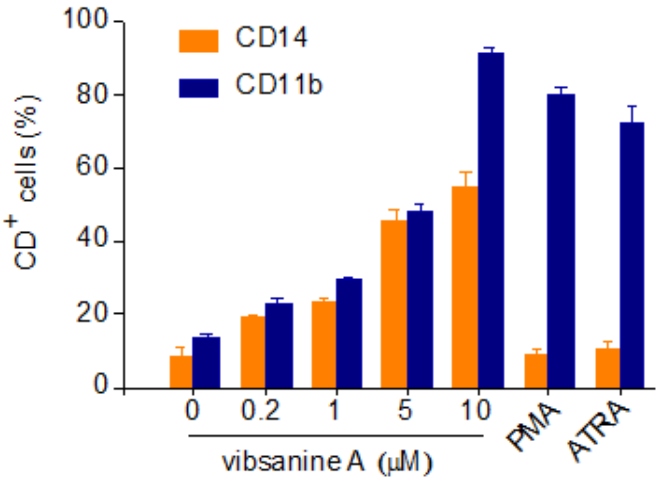

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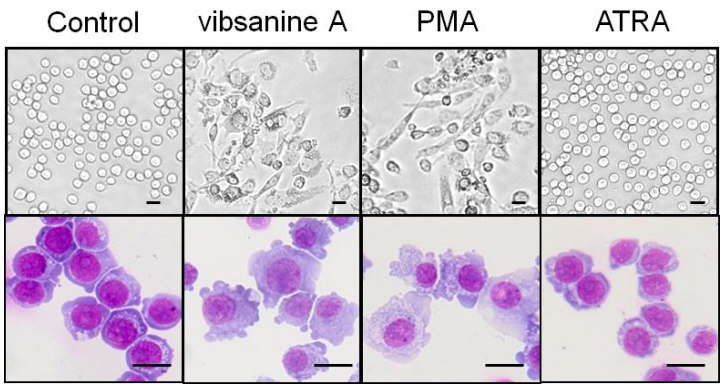


Figure 1

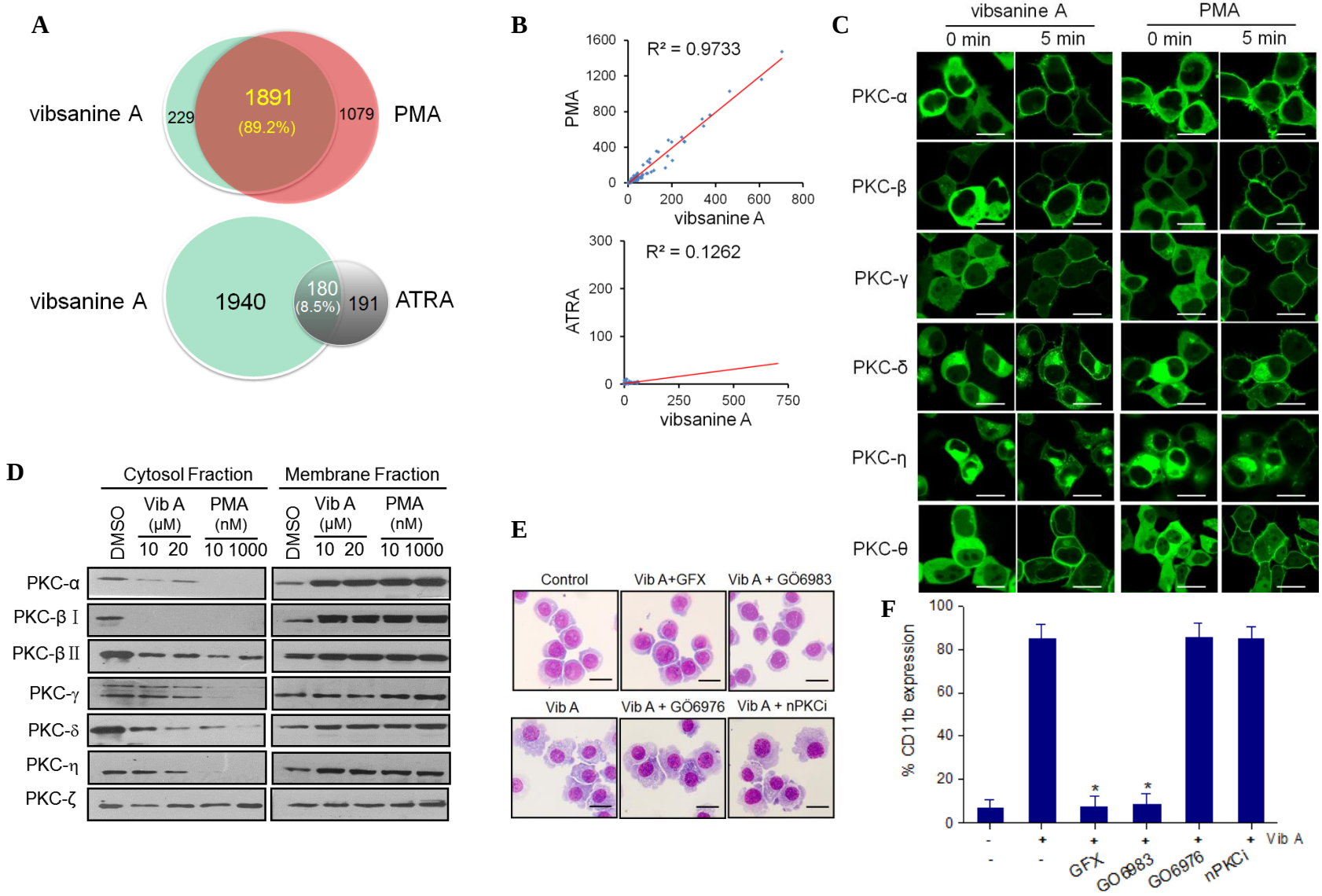
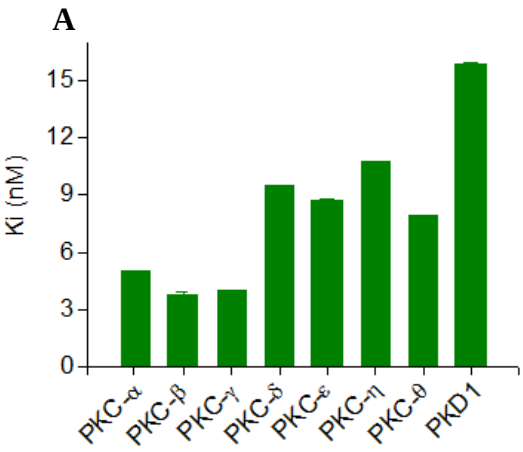
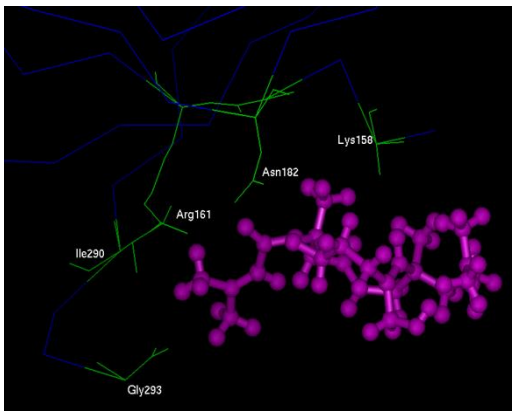
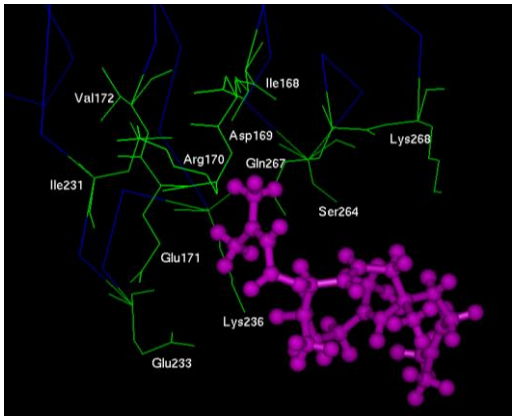


Figure 2

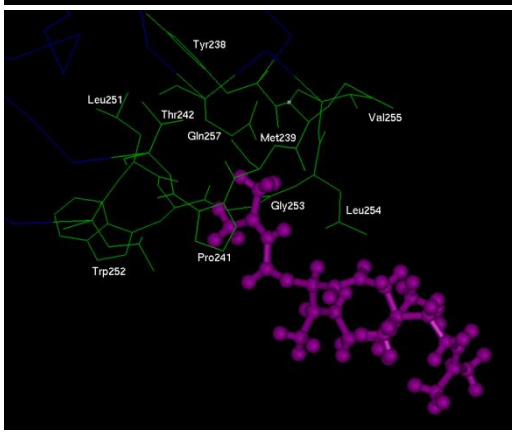


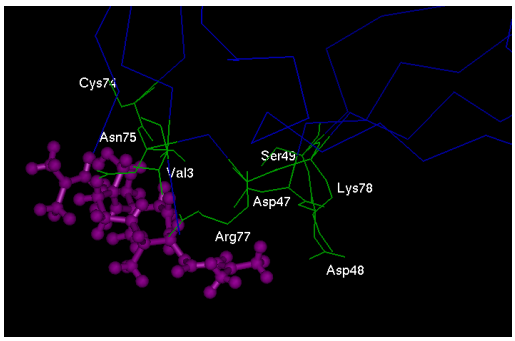


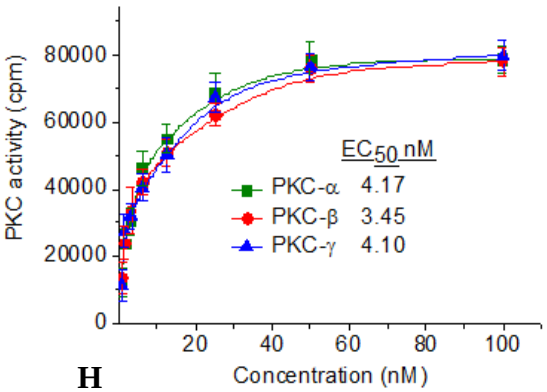


F

vibsanine A/PKC complex	Van der Waals energy	Electric energy	Total energy
Vib A/PKC-α	-16.63	-12.86	-29.49
Vib A/PKC-β	-13.34	-10.45	-23.79
Vib A/PKC-δ	-12.71	-9.03	-21.74
Vib A/PKC-ε	-21.87	-16.09	-37.96







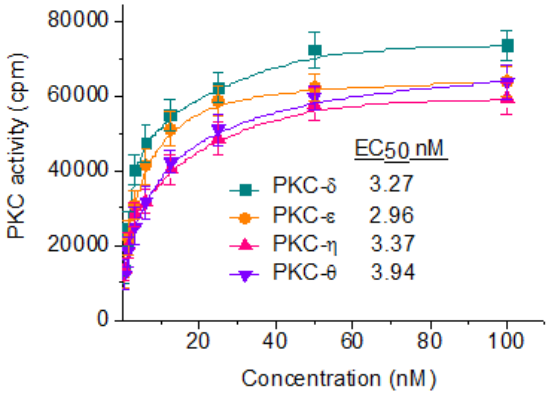


Figure 3

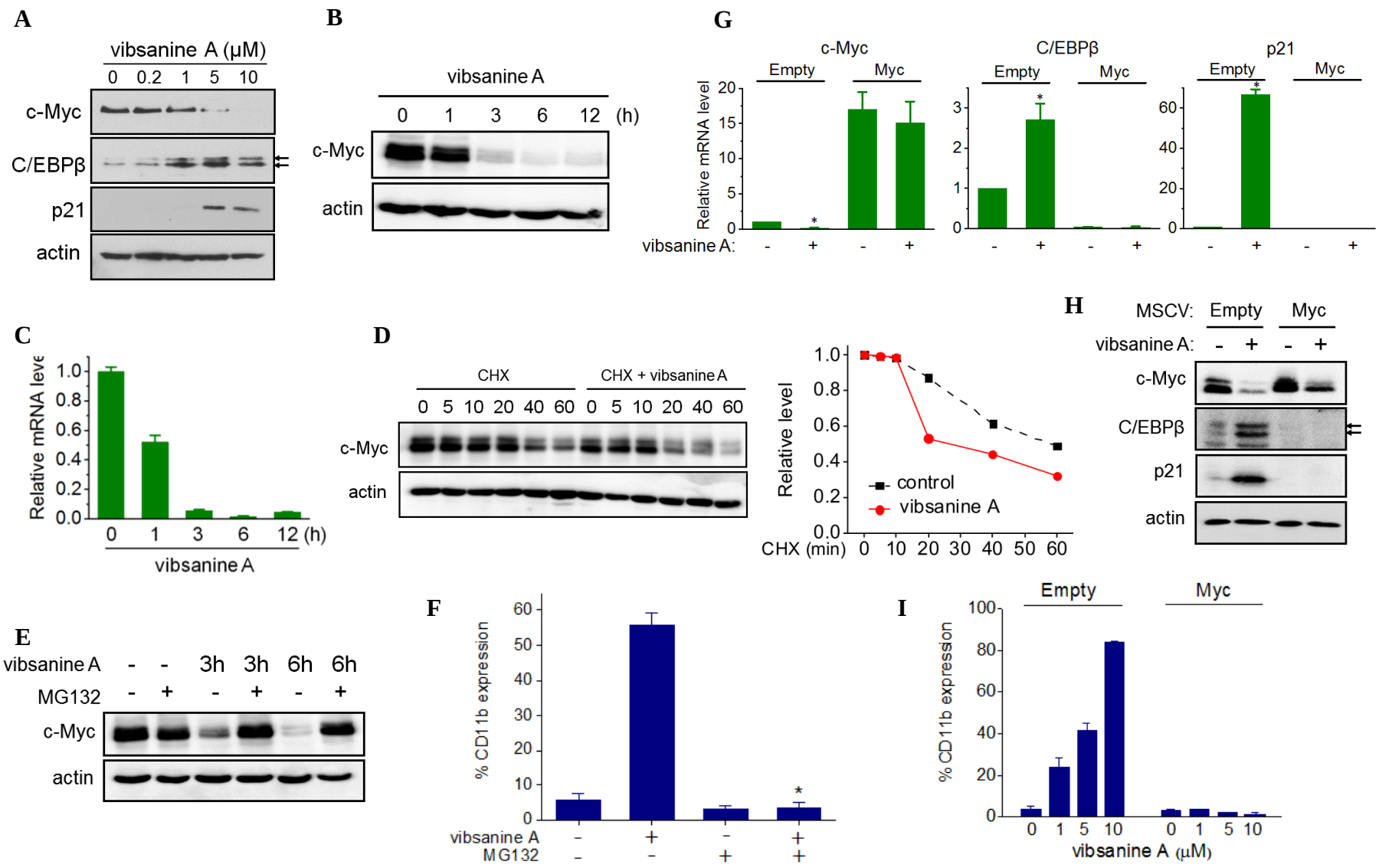


Figure 4

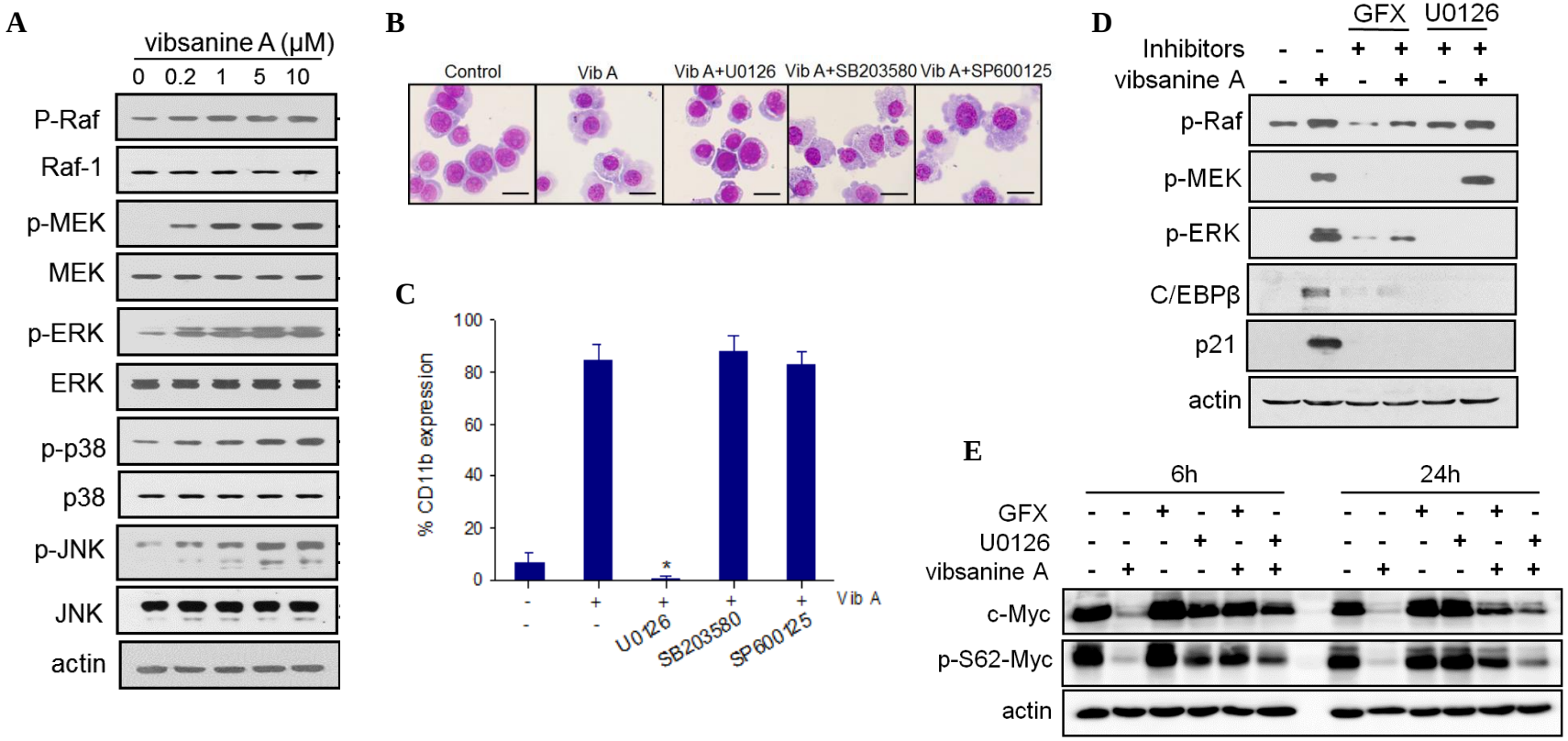


Figure 5

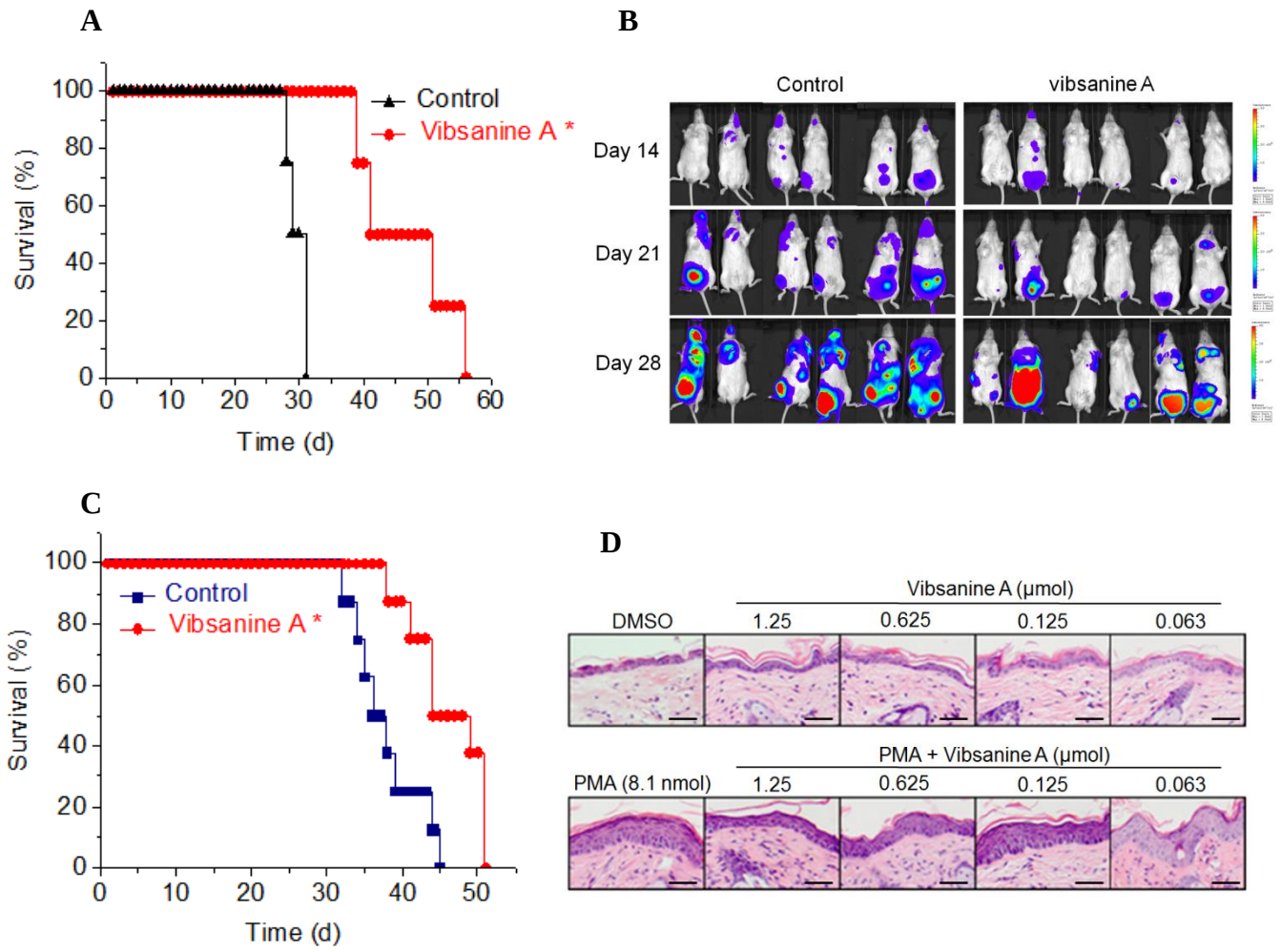


Figure 6

Cancer Research
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Natural product vibsanine A induces differentiation of myeloid leukemia cells through PKC activation

Zu-Yin Yu, He Xiao, Li-Mei Wang, et al.

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