Veratramine modulates AP-1-dependent gene transcription by directly binding to programmable DNA

Fang Bai1,2,3,†, Kangdong Liu4,5,6,7,†, Huiliang Li8,†, Jiawei Wang2, Junsheng Zhu2,7, Pei Hao9, Lili Zhu2, Shoude Zhang2, Lei Shan8, Weiya Ma6, Ann M. Bode6, Weidong Zhang8,*, Honglin Li2,* and Zigang Dong6,7,*

1Faculty of Chemical, Environmental, and Biological Science and Technology, Dalian University of Technology, Dalian 116023, China, 2State Key Laboratory of Bioreactor Engineering, Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China, 3Center for Theoretical Biological Physics, Rice University, Houston, TX 77005, USA, 4Department of Pathophysiology, Basic Medical College, Zhengzhou University, No. 100 Science Road, Zhengzhou, Henan 450001, China, 5Collaborative Innovation Center of Henan Province for Cancer Chemoprevention, Zhengzhou, Henan 450001, China, 6The Hormel Institute, University of Minnesota, Austin, MN, USA, 7China-US (Henan) Hormel Cancer Institute, No.127 Dongmin Road, Zhengzhou 450008, China, 8Department of Natural Product Chemistry, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China and 9Key Laboratory of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT

Because the transcription factor activator protein-1 (AP-1) regulates a variety of protein-encoding genes, it is a participant in many cellular functions, including proliferation, transformation, epithelial mesenchymal transition (EMT), and apoptosis. Inhibitors targeting AP-1 have potential use in the treatment of cancer and other inflammatory diseases. Here, we identify veratramine as a potent natural modulator of AP-1, which selectively binds to a specific site (TRE 5′-TGACTCA-3′) of the AP-1 target DNA sequence and regulates AP-1-dependent gene transcription without interfering with cystolic signaling cascades that might lead to AP-1 activation. Moreover, RNA-seq experiments demonstrate that veratramine does not act on the Hedgehog signaling pathway in contrast to its analogue, cyclopamine, and likely does not harbor the same teratogenicity and toxicity. Additionally, veratramine effectively suppresses EGF-induced AP-1 transactivation and transformation of JB6 P+ cells. Finally, we demonstrate that veratramine inhibits solar-ultraviolet-induced AP-1 activation in mice. The identification of veratramine and new findings in its specific regulation of AP-1 downstream genes pave ways to discovering and designing regulators to regulate transcription factor.

INTRODUCTION

Natural products have historically been invaluable as a source for the discovery and development of a variety of drugs (1). Veratramine, a known natural steroidal alkaloid isolated from plants of the lily family, such as the Veratrum species (2), has been shown to be effective in lowering blood pressure, antagonizing Na+ channel activity, and acting on serotonin (5-HT) with agonist activity (2–4). Importantly, veratramine is structurally similar to the Hedgehog (Hh) pathway modulator, cyclopamine, which prompted our interest in studying whether veratramine has similar pharmacological effects on the Hh pathway.

In this study, veratramine was identified as a downstream modulator of the activation of activator protein-1 (AP-1) by directly binding to the target DNA sequence of AP-1 instead of acting on the Hh signaling pathway. It could inhibit EGF-induced JB6 P+ cell transformation and EGF-induced AP-1 activation in a dose-dependent manner by specifically blocking the binding of AP-1 to its cognate DNA sequence. Furthermore, in an AP-1 transgenic mouse model, veratramine also blocked solar ultraviolet (UV)-
induced AP-1 activation. These results suggest that veratramine might be a potential anticancer candidate acting through different pharmacological mechanisms.

The transcription factor AP-1 is a menagerie of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos, Ma'f and ATF sub-families. AP-1 recognizes either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE,5'-TGACGTCA-3') (5) and regulates expression within the regulatory regions of various genes, thereby controlling cellular proliferation, transformation, differentiation, and apoptosis (6–8). Much evidence indicates that aberrant AP-1 activity is implicated in the pathogenesis of a number of diseases, including tumorigenesis (9–11), inflammation (12–15) and infection (16). Consequently, AP-1 is considered one of the most promising targets for therapeutic cancer intervention along with other transcription factors, such as c-Myc (17), HIF-1 (18), STAT3 (19) and NF-κB (20). Beyond that, the remarkable progress of the development of biomarkers which are used to diagnose and treat cancers (21,22), even provide information regarding the pathways or simulation responses that are involved in the activation of transcription factors, such as NF-κB (23), further pave the way for developing the AP-1 signalling pathway modulators. However, AP-1 is a double-edged sword in tumorigenesis. It can display anti-oncogenic characteristics by inducing apoptosis or it can exert onco-genic activity by signaling and promoting cell survival (24). The final outcome of AP-1 activity in cancer appears to depend on AP-1 dimer composition and on the cellular and genetic context in which it is studied (24). To achieve the aim of therapeutic intervention, many approaches have been explored for modulating various components of the transcription machinery using small molecules (25). Strategies are divided into two principle types: (i) directly targeting the DNA-binding domain or the cognate DNA sequence of a specific transcription factor (11) and (ii) indirectly targeting the co-activators or mediators of transcription (26). Of these two approaches, targeting specific transcription factors by blocking their binding to cognate DNA sequences seems to be more effective and offers several advantages. Specific DNA-binding agents could principally inhibit or affect the function of whole families of transcription factors that share highly conserved DNA sequence domains. This would allow the regulation of only a subset of genes controlled by the given transcription factor without affecting the expression of the entire set of genes. However, the advantages could also be considered drawbacks for DNA-binding drugs, due to the challenges in achieving selectivity for short nucleic acid sequences in the human genome (26). Synthetic polypeptide-based inhibitors that selectively disrupt NF-κB binding by directly binding the DNA sequences, 5'-WGGWWW-3' and 5'-GGGWWW-3', have been recently reported (27). However, potent small molecules, especially potently druggable natural products that can specifically and directly bind the AP-1 target DNA sequence, have not been reported. Consequently, to identify natural active DNA minor groove binders, we performed a virtual screen against a natural product database by specifically targeting the AP-1 target DNA sequence (TRE 5'-TGACTCA-3'). Results identified veratramine and several other active natural products, including compounds 1125 and 1126. Thus, to further understand the effect of veratramine and its active analogues, compounds 1125 and 1126, on AP-1-dependent transcription, RNA-seq was performed on JB6 P+ cells treated with these compounds to measure changes in gene expression. Furthermore, the in vivo and in vitro effects of these compounds on AP-1 activity were also demonstrated.

**MATERIALS AND METHODS**

**Identification of veratramine by virtual screening**

Structure-based virtual screening was conducted using our DNA specific molecular docking method, dNAbinder, to examine an in-house natural product database of ~2000 compounds. The compounds were first ionized and then their most probable tautomeric states were determined with Epik (version 2.0) (28) pH 7.0 ± 2.0. The prepared compounds were geometrically optimized using the OPLS2005 force field implemented in Maestro (29). The X-ray crystal structure of a heterodimer of the bZIP region of c-Fos and c-Jun bound to DNA (PDB code: 1FOS (30)) was used as the virtual-screening-based receptor structure. The c-Fos and c-Jun transcription factors were removed to expose the AP-1 target site on the DNA sequence. The DNA sequence was then prepared with Sybyl (31) to add hydrogens and partial charges. The related parameters were set as default, and only the best-scored binding pose for each compound was retained. The top ranked 500 compounds were kept for visual inspection analysis. Finally, 75 of the 500 compounds were selected as candidate compounds and 35 of the 75 compounds were eventually obtained to perform the bioassay experiments.

**Isothermal titration calorimetry (ITC)**

Three sequences of oligonucleotides (the cognate DNA of AP-1 and the random designed decoys DNA) were synthesized and annealed to form double-stranded DNA. The study about the interactions of compound-DNA was performed using Isothermal titration calorimetry (MicroCal iTC200). For the titration of 1125 to DNA, the prepared double-stranded DNA and the compound 1125 were diluted using the buffer (10 mM HEPES, 100 mM NaCl, pH 7.2 and 7.1% DMSO). The concentration of DNA in the cell was 100 μM, and the concentration of 1125 in the syring was 3.57 mM. Similarly, for the interaction study about 1529-DNA, each sequence of DNA was diluted to 1 mM. The prepared compound-DNA was diluted using the buffer (10 mM HEPES, 100 mM NaCl, pH 7.2 and 7.1% DMSO). The concentration of DNA in the cell was 100 μM, and the concentration of 1529 in the syring was 3.57 mM. For the interaction study about 1529-DNA, each sequence of DNA was diluted to 1 mM. The prepared compound-DNA was diluted using the buffer (10 mM HEPES, 100 mM NaCl, pH 7.2 and 10% DMSO). All titration experiments were performed by adding the compound in steps of 2 μl at 25°C. The data were analyzed using Microcal origin software by fitting to a one-site binding model.

**Electrophoretic mobility shift assay (EMSA)**

EMSA assays were conducted using the Lightshift chemiluminescent EMSA kit (Pierce, Shanghai, China). The specific DNAs corresponding to the cognate DNA of AP-1 were synthesized with 5'-biotin labels and annealed to form double-stranded DNA. Reaction solutions contained
2 μl of 10× binding buffer (100 mM Tris-HCl pH 7.5, 500 mM KCl, 10 mM MgCl2, 50 mM DTT, 5 mM EDTA, 50% glycerol), 2.5 μg poly(dI-dC), 100 fmol biotin-labeled probes, different concentrations of the specific compound and ultra-pure water to a total volume of 20 μl. These solutions were incubated at room temperature for 10 min and then 2 μl of 5 mg/ml HeLa nuclear extract (Promega, Beijing, China) were added. The mixtures were then incubated for another 20 min at room temperature. Samples were electrophoresed on a 6% non-denaturing polyacrylamide gel and then transferred to nylon membranes, UV cross-linked, probed with streptavidin–HRP conjugate and incubated with the respective substrate. The results were detected by a chemiluminescence imaging system.

**Luciferase assay to determine AP-1 or NF-κB transactivation**

Confluent monolayers of JB6 P+ cells stably transfected with an AP-1 or NF-κB plasmid were trypsinized and 8 × 103 viable cells suspended in 100 μl of 5% FBS-MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2. When cells reached 80 to 90% confluence, they were cultured in 0.1% MEM at 37°C in a humidified atmosphere of 5% CO2. When cells reached 80 to 90% confluence, they were cultured in 0.1% MEM at 37°C in a humidified atmosphere of 5% CO2. When cells reached 80 to 90% confluence, they were cultured in 0.1% MEM at 37°C in a humidified atmosphere of 5% CO2. When cells reached 80 to 90% confluence, they were cultured in 0.1% MEM at 37°C in a humidified atmosphere of 5% CO2.

Kinase phosphorylation assay

The effects of compounds 586, 1134, 1451, 1460, 1512, 1474 and 1529 on EGF-induced transformation were investigated in JB6 C141 cells as described by Colburn et al. (32). Colonies were counted under a microscope using the Image-Pro Plus software program (Version 6, Media Cybernetics, Silver Spring, MD, USA). Data are shown as means ± S.D. of values obtained from triplicate experiments. The asterisk (*) indicates a significant (P < 0.05) change in the number of colonies as indicated.

**Transcription microarray experiments**

Total RNA was isolated using the TRizol Reagent (Invitrogen, Shanghai, China) following the manufacturer’s instructions. Synthesis of the cDNA target, its hybridization to microarrays and scanning of those arrays was performed using Illumina Whole-Genome Gene Expression Bead Chips (MouseWG-6) and reagents according to the product recommendations (GeneNego Biotechnology (Shanghai) Co., Ltd., Shanghai, China). Each treatment was repeated in triplicate.

**Solar-ultraviolet-induced AP-1 luciferase activity in vivo**

AP-1 luciferase transgenic mice were identified, grouped, and housed as described previously (33). The mice were developed, propagated and maintained at the Hormel Institute. Mice were maintained under conditions according to guidelines established by Research Animal Resources and the Institutional Care and Use Committee, University of Minnesota. Two weeks after grouping, the basal level of AP-1 luciferase activity was measured by punch skin biopsy. AP-1 luciferase reporter-bearing male and female mice were randomly divided into groups (12 mice/group). Two weeks after the punch biopsy, the mice were treated with veratramine by painting on the back of each mouse (10 μg of veratramine in 200 μl of acetone/mouse). The mice were treated with solar UV (60 kJ/m2 UVA/3.6 kJ/m2 UVB) using a UVA-340 sunlamp (Q-Lab Corporation, Cleveland, OH, USA) 3 h after veratramine treatment. Skin biopsies of equal weight were harvested in 100 μl of lysis buffer (0.1 M potassium phosphate pH 7.8, 1% Triton X-100, 1 mM DTT and 2 mM EDTA) for measurement of luciferase activity at the times indicated. AP-1-dependent luciferase activity in the tissue extract was determined using the luciferase assay reagent from Promega and a luminometer (LuminoSkan Ascent, Labsystems, MD, USA).

**Kinase activity profiling**

The kinase profiling study was conducted using the Caliper assay screening platform (Shanghai ChemPartner Co., Ltd., Shanghai, China). All kinases and other materials were purchased from commercial sources. The compounds were screened in vitro against the different kinases, and staurosporine and PI103 were used as reference compounds. Two concentrations (3 and 10 μM) of the compounds were tested in duplicate on each kinase.

**Statistical analysis**

All quantitative data are expressed as mean values ± S.E. or S.D. as indicated. One-way ANOVA was used for statistical analysis. A probability of P < 0.05 was used as the criterion for statistical significance.
RESULTS

Identification of veratramine from a natural compound database

To identify specific molecules that bind to the AP-1 target DNA sequence (TRE 5′-TGACTCA-3′), virtual screening analysis was performed by searching an in-house natural product database of approximately 2,000 compounds. The virtual screening protocol was assembled based on iDNABinder (http://lilab.ecust.edu.cn/), which is specifically designed to recognize distinct target DNA sequences and identify potential small molecules that bind to this sequence built on a base-sequence-sensitive scoring function (details in Supplemental Information). The top 500 compounds identified by our specific scoring function from the screening solution were inspected visually by considering the following criteria: (i) the binding pose of the candidate compound has to be positioned well into the site of interest (5′-TGACTCA-3′) and (ii) the predicted binding pose of the compound has to form favorable molecular interactions, including hydrogen bonds, hydrophobic interactions, and ionic bonds, with site bases. As a result of the screening, 75 compounds were selected as ‘hits’ and 35 of those compounds were prepared for in vitro testing.

These compounds were evaluated for their effect on AP-1 activity in JB6 P+ cells transfected with an AP-1 reporter plasmid and 18 of the 35 compounds inhibited AP-1 activity (Supplementary Table S1). Additionally, these compounds were evaluated for their effect against NF-κB, the most thoroughly studied transcription factor, to study their binding specificity. The assays were performed with JB6 P+ cells transfected with an NF-κB reporter plasmid. Notably, the compounds had almost no effect on NF-κB activity in vitro (Supplementary Table S1). This result suggests that the 18 active compounds are potentially selective inhibitors of transcription factor AP-1 with little effect on NF-κB.

Specific recognition of AP-1 target DNA sequence

To further investigate the binding specificity and the interactions of compounds with the cognate AP-1 DNA (top panel in Figure 2A), isothermal titration calorimetry (ITC) experiments were performed and two decoy sequences were designed as controls. The first decoy sequence (TRE 5′-TGACTCA-3′), virtual screening experiments were performed and two decoy sequences were designed as controls. The virtual screening protocol was assembled based on iDNABinder (http://lilab.ecust.edu.cn/), which is specifically designed to recognize distinct target DNA sequences and identify potential small molecules that bind to this sequence built on a base-sequence-sensitive scoring function (details in Supplemental Information). The top 500 compounds identified by our specific scoring function from the screening solution were inspected visually by considering the following criteria: (i) the binding pose of the candidate compound has to be positioned well into the site of interest (5′-TGACTCA-3′) and (ii) the predicted binding pose of the compound has to form favorable molecular interactions, including hydrogen bonds, hydrophobic interactions, and ionic bonds, with site bases. As a result of the screening, 75 compounds were selected as ‘hits’ and 35 of those compounds were prepared for in vitro testing.

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In silico and EMSA characterization of the veratramine binding mode

To characterize the site-specific mode of veratramine binding to the AP-1 target DNA sequence, virtual screening was used to predict the binding mode of veratramine. Results indicated that veratramine could fit well into the minor groove of the AP-1 DNA sequence with a well-shaped complementary conformation (Figure 2B). Veratramine formed hydrophobic contacts with bases A35 and C34 and hydrogen bonds between its hydroxyl groups and bases A35, T7 and C34. Thus, veratramine binds to the AP-1 DNA cognate sequence by specifically recognizing the -AC- subsequence.

To further examine the binding of AP-1 to its consensus recognition site, we conducted electrophoretic mobility shift assays using HeLa cell nuclear extracts. Two bands were detected by chemiluminescence (Figure 2C). The uppermost band was the AP-1-specific DNA complex and the lower band corresponded to the free biotin-labeled specific DNA probe. A 100-fold concentration of specific or nonspecific (NS) unlabeled probe was used to illustrate the binding specificity of AP-1 with its cognate DNA sequence. The formation of the AP-1/DNA complex was effectively inhibited by increasing the concentration of veratramine (compound 1529; left panel) or compound 1125 (right panel).

Veratramine suppresses EGF induced AP-1 transactivation and EGF-induced JB6 P+ cell transformation

To investigate the effect of veratramine on AP-1 in cells, we performed an AP-1 transactivation assay using JB6 P+ cells stably transfected with a luciferase reporter gene or cells stably transfected with the NF-κB luciferase reporter gene as a control. Approximately 70% of the EGF-induced AP-1 transactivation activity was inhibited by 2.5 μM veratramine in a dose-dependent manner (Figure 3A). NF-κB transactivation was not significantly affected by any concentration of veratramine. These results indicated that veratramine might exert its anticancer effects by selectively inhibiting AP-1. Blocking tumor promoter-induced AP-1 activation can inhibit neoplastic transformation in JB6 cells. (35) We therefore investigated whether inhibiting AP-1 with veratramine could affect EGF-induced cell transformation. Results indicated that veratramine inhibited EGF-induced JB6 cell transformation in a dose-dependent manner (Figure 3B).

Genome-wide effects of veratramine on EGF-induced gene expression

Because veratramine effectively inhibited EGF-induced AP-1 transactivation and JB6 P+ cell transformation, RNA-seq experiments were conducted to establish the global modulating effects of veratramine (1529) or its analogues (compounds 1125 or 1126) on the EGF-inducible JB6 P+ cell transcriptome. Genes with a log2 normalized change of >2-fold and adjusted P-values of 0.05 or less were considered to be significantly and differentially expressed. Results revealed a total of 529 out of 22,310 genes with a significant change in expression after treatment with 10 μM veratramine or 20 μM compound 1125 or 1126 compared...
Figure 1. Binding isotherm obtained from ITC show the specific binding interactions of AP-1 cognate DNA with veratramine (compound 1529, upper three subfigures) and compound 1125 (lower three subfigures). (A) The chemical structures of veratramine and compound 1125. (B) ITC data for the titration of veratramine (upper panels) and compound 1125 (lower panels) into three types of DNA sequences (from left to right, AP-1 cognate DNA; AP-1 mutant DNA; and randomly-designed DNA). The upper portion in each panel shows the change in enthalpy per injection of veratramine (compound 1529) or 1125 into a corresponding DNA sequence. The lower portions indicate the concentration normalized heat form titration at the molar ratio of compounds.
Figure 2. Veratramine (compound 1529) selectively binds and inhibits AP-1-dependent activity, but does not affect NF-κB. (A) The three sequence of DNA templates used for the EMSA and the luciferase assays, they are: cognate sequence of AP-1 (top panel, the AP-1 binding site is highlighted in red color), reported mutant target DNA sequence of AP-1 (middle panel, mutation is highlighted in italic and AP-1 binding site is in red), and randomly designed decoy sequence (bottom panel); (B) computational analysis shows that veratramine inserts into the minor groove of the AP-1 DNA sequence. (C) EMSA results show the inhibition of the transcription factor/DNA complex formation by veratramine or compound 1125. A 100-fold concentration of specific or nonspecific (NS) non-labeled probe (relative to the biotin-labeled specific DNA probe) was used to illustrate the specificity of the protein/DNA complexes. Oligonucleotides were incubated alone (lanes labeled ‘AP-1 probe’), with HeLa nuclear extracts in the absence (lanes labeled ‘DMSO’) or the presence of increasing concentrations of the compound as specified at the top of the lane.

Figure 3. Veratramine (compound 1529) suppresses AP-1 activity and EGF-induced JB6 P+ cell transformation. (A) Veratramine selectively suppresses AP-1-dependent transactivation activity, but not NF-κB transactivation. (B) JB6 P+ cells were treated with EGF and increasing concentrations of veratramine and incubated for one week in a 37°C, 5% CO2 incubator. Cells were harvested and colonies counted using a microscope and the Image-Pro Plus software program (Version 6, Media Cybernetics, Silver Spring, MD) software program.
Veratramine inhibits the downstream signalling pathway of AP-1

To examine the mechanisms of veratramine’s effect on AP-1, most genes from this signalling pathway were collected, and the magnitude of their change in expression induced by each inhibitor was examined by gene profiling (Figure 4B). Expression changes were substantially below 2-fold in most cases for all three compounds, excluding a significant induction of Il16 and some genes in the clustered group 100 and the repression of Meti7a1 and Ili1ra1 and Tesk1 in group 117 (Figure 4B). With the competitive binding of veratramine on the AP-1 site, the expression of AP-1 family proteins, including Myc, Jun, FosB and Fos clustered in groups 99 and 100, were significantly (~3.0-fold) or relatively (~1.5-fold) increased, as expected for positive feedback. These changes in expression led to T cell activation by inducing the expression of its modulator, Il16 (Figure 4B). Alternatively, the stimulation of Il16 can lead to the phosphorylation of c-Jun and p38 MAPK (36). Thus, the increasing expression of Il16 is likely the result of positive feedback of the AP-1 signaling pathway. Additionally, another transcription factor, Egr1, was up-regulated. Although the AP-1 proteins were activated, their downstream target genes, including CDKs, Il5 and AP-1 inducible MMPs, such as Mmp3, whose overexpression correlates with carcinogenesis, remain unaffected or even suppressed (Figure 4C and D), indicating that the downstream genes in the AP-1 signaling pathway were inhibited by veratramine. However, other MMPs genes, such as Mmp2, Mmp24 and Mmp28, which do not contain TATA box, are not affected by AP-1 and do not belong to the AP-1 downstream, therefore, their expression should not be affected by the veratramine, but may be altered by their upstream genes. We also performed significance-calculating analyses by using gene set enrichment analysis (GSEA) (37,38) and the results are shown in Supplementary Figure S1. The enrichment plot (Supplementary Figure S1A) and the relative expression heat map (Supplementary Figure S1B) again confirmed the repression of AP-1 downstream genes, such as Cd14, Mmp9, Il6, Nudt1, Ets1, Timp1 (Metalloproteinases1), Nr4a2, Maf1, etc., while the AP-1 genes, such as, Fos, Jun and Myc etc. are activated by the modulation of veratramine.

Veratramine may not affect the MAPK pathways or other kinases

To determine whether AP-1 activity is involved in the regulation of MAPKs expression in response to various stimuli, we analyzed the activity of MAPKs and expression of genes in the MAPKs signaling pathways (Figure 4E). Except for the AP-1 family genes, only three genes (Sera1, Map4k1 and Pla2g5) in groups 175 and 179 were up-regulated >2.0-fold after treatment with veratramine or compound 1125; whereas the expression of the majority, including MPKs, was not affected (Supplementary Table S2). The increased expression level of Map4k1 is likely correlated with the activation of c-Myc (39). In contrast to veratramine and compound 1125, compound 1126 affected the expression of genes in groups 169, 170 and 173 differently, resulting in easily detectable up- or down-regulation. Overall, the EGF-induced cytosolic MAPKs signal cascades are not affected by veratramine or compound 1125. The activation of AP-1 by veratramine probably occurs through positive feedback for the downstream regulation of the AP-1 signaling pathway.

To further investigate the effect of veratramine on other AP-1-activation-related upstream kinases, we performed kinase assays either with EGF alone (10 ng/ml) or EGF plus veratramine (1529, 20 μM) treatment. The results indicate that the AP-1 related upstream kinases, including JNKs, ERKs, p38, Msks and Hsp27 (Figure 5A and B), were not affected by veratramine (1529) treatment. Furthermore, Western blot analysis revealed that AP-1-activation related kinases, including ERKs, Msks, Rsk2, c-Fos, c-Jun and CREB, were not affected by treatment with different concentrations of veratramine (1529; Figure 5C). These results further verify that blocking AP-1 activity by veratramine does not occur through its upstream kinases. In addition, the inhibitory effects of veratramine against critical AP-1 related up-stream kinases were investigated by kinase activity profiling. Results indicated that these kinases may not be obviously affected by veratramine (Figure 5D), which again indicates that veratramine is a modulator that acts on the downstream signaling of AP-1.

Veratramine does not act on the Hh signaling pathway

The Hh pathway is an essential signaling pathway in embryonic development and is critical for maintaining tissue polarity and stem cell populations (40,41). Deregulated Hh pathway activity can consequently result in the hereditary developmental disorder, holoprosencephaly (42). In contrast, hyperactivation of this pathway is linked to many extracutaneous cancers (43). In the absence of Hh ligands, the unbound Patched 1 (Ptc1) protein acts catalytically to suppress the activity of Smoothened (Smo), a key player in Hh signal transduction, by preventing its localization to the cell surface. Once Ptc1 is bound to active Hh ligands, the inhibition of Smo by Ptc1 is released, allowing Smo to signal downstream, eventually leading to activation of Gli family transcription factors (Gli2 and Gli3) to regulate target
Figure 4. Profiling of the AP-1 pathway using the RNA-seq global transcriptome to assess changes in gene expression in JB6 P+ cells treated with veratramine (1529) or the analogue compound 1125 or 1126. All ratios are normalized to the EGF-induced state. (A) Changes in the gene expression profile of the whole genome set (top left). Significant differences in gene expression JB6 P+ cells treated with veratramine or its analogues exposed to veratramine or its analogues (top right; log2 expression change > 2.0 fold after treatment with any compound). Venn diagrams showing the overlap among the 529 genes activated (bottom left) or repressed (bottom right) by treatment with compounds. (B) Profiling of the changes in gene expression in the AP-1 pathway. (C, D) Individual normalized changes in MMP and CDK gene expression. (E) Profiling of changes in gene expression in the MAPK signaling pathway. Data were analyzed by hierarchical clustering (Euclidian distance and average linkage).
Veratramine (compound 1529) has no effect on EGF-induced MAPK pathways. (A) JB6 cells were cultured with 0.1% FBS/MEM and starved for 24 h. The cells were treated or not treated with compound 1529 (20 μM) for 2 h and then cells were stimulated or not stimulated with 10 ng/ml of EGF for 30 min. Protein extracts (500 μg) were prepared and used for phospho-MAPK array analysis. Array spots were visualized using an ECL detection kit. (B) The density of each duplicated array spot in 'a' was measured as described in Materials and Methods. The graph shows the normalized change in EGF-induced phosphorylation of JNKs, ERKs, p38, MSK and HSP27 compared with untreated control (i.e. a value of 1). Data are shown as an average of duplicate samples. (C) The important protein components related to AP-1 were verified by Western blotting. The phosphorylation of c-Fos and c-Jun and their upstream kinases were not affected by veratramine. Each experiment was repeated three times and representative blots are shown. (D) Percent of inhibition by veratramine (10 or 3 μM) against 17 kinases.

gene expression (44,45). The first Hh pathway inhibitor to be identified was the plant-derived steroidal alkaloid, cyclopamine, which binds directly to the transmembrane helices of Smo and blocks cellular responses to Hh signaling (46,47). The discovery of cyclopamine has created exciting new prospects for molecular targeted therapies and prevention of human cancers associated with Hh signaling (48). However, cyclopamine has limited potential as an orally bioactive therapeutic agent due to its instability and concentration sensitivity in modulating Hh signaling (41). A relatively low concentration of cyclopamine can specifically inhibit Hh signaling, but a high dose can cause cell death without affecting Hh target gene expression (5).

Cyclopamine is a known inhibitor of the Hh signaling pathway (49). To determine whether veratramine interacts with Smo and affects the Hh signaling pathway in the same manner as cyclopamine (Figure 6A), gene expression profiling of the Hh signaling pathway after treatment with veratramine (1529) or its analogues was conducted (Figure 6B). Results indicated no obvious perturbation in gene expression of this signaling pathway, except for the enhancement of the Myc and Boc transcription factors. This result supports the hypothesis that veratramine acts as a downstream modulator of AP-1 and does not interfere with the cytosolic...
Veratramine suppresses solar UV-induced AP-1 activity in vivo

Ultraviolet (UV) irradiation activates AP-1 through MAPK signaling pathways, which play an important role in UV-induced skin inflammation and carcinogenesis. Agents that block AP-1 activation show promise for use in protecting against UV-induced inflammation and skin carcinogenesis. To further examine the effect of veratramine (1529) on AP-1 activity in vivo, AP-1 activities were measured in solar UV-irradiated AP-1-luciferase transgenic mice. The results indicate that pretreatment of mouse dorsal skin with veratramine (1529) before UV exposure effectively and markedly suppressed solar UV induced AP-1 activation at 36 h after solar UV exposure (Figure 7).

DISCUSSION

Because the gene promoters recognized by any particular transcription factor could have different sequences, targeting a specific promoter sequence by a small molecule might allow the regulation of only a subset of genes controlled by the given transcription factor and might at least partially avoid any unexpected side effects. The current study introduces a binding-site-specific downstream modulator of AP-1, veratramine, which was identified in a virtual screening of an in-house natural product database. Veratramine, long considered a natural product of interest, has been reported to have anti-thrombotic activity and serotonin-agonist activity (3). Moreover, this compound has been reported to be a potential signal transduction inhibitor for treating cancers4, but its direct binding target was not determined until now. An important aspect of this work is the discovery of veratramine as a specific natural small molecule inhibitor, which selectively recognizes and binds the AP-1 DNA target sequence (5′-TGACTCA-3′). This discovery exemplifies the efficiency of our iDNABinder protocol in the design of specific DNA-binding agents, which challenges the selectivity for short nucleic acid sequences that may be abundant in the human genome. Furthermore, a series of in vitro experiments indicates that veratramine acts on the downstream signaling of AP-1, but does not interfere with the EGF-induced cytosolic MAPK signaling cascades or other AP-1 activation-related kinases. This indicates that the gene modulation occurs only on a subset of genes controlled by AP-1.

To explore the anti-cancer effects of veratramine, its ability to suppress EGF-induced AP-1 transactivation and transformation was evaluated in JB6 P+ cells. As expected, the AP-1 dependent transactivation could be effectively suppressed by veratramine in a dose-dependent manner. However, the NF-κB dependent transactivation was not affected by treatment with veratramine. Moreover, cell transformation also could be effectively inhibited by veratramine. These findings demonstrate a selective inhibition of the AP-1-dependent signaling pathway by veratramine.

Although veratramine has the same scaffold as the Smo inhibitor cyclopamine, a completely different pharmacological mechanism was confirmed in this study. Veratramine selectively and directly binds with a specific site of the target DNA sequence of AP-1 instead of acting on the Hh signaling pathway; thus, it may not have the same teratogenicity and toxicity as cyclopamine. Finally, our study clearly demonstrated that veratramine effectively suppresses SUV-induced AP-1 activation in vivo. All these findings indicate that veratramine is a potentially valuable anticancer agent that should be further explored and developed.

AVAILABILITY

DNA-specific docking program iDNABinder is an open source and can be requested by email to Prof. Honglin Li (hlli@ecust.edu.cn).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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