A Tryptamine Derivative, SST-VEDI-1, Inhibits Apoptosis and Stimulates Mineralization in Osteoblasts

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Abstract. SST-VEDI-1 (VEDI-1) is a new synthetic compound that is synthesized from tryptamine, and has structural similarity to the SSH-BM family of compounds. However, the biological effects of VEDI-1 have yet to be well characterized. A recent report has demonstrated that SSH-BM-type compounds can stimulate osteoblast activity in cultured scales of goldfish. In this study, we examined the effects of VEDI-1 on osteoblastic differentiation as well as its effects on apoptosis, which is known to be closely related to osteoblastic differentiation. We found that VEDI-1 enhanced the formation of mineralized nodules in rat osteoblast cell lines, including ROS17/2.8 cells, and in mouse pre-osteoblast cell lines, including MC3T3-E1 cells, in a dose dependent manner, which was accompanied by increased expression of late osteoblast markers, bone sialoprotein (BSP) and osteocalcin (OC). Furthermore, VEDI-1 inhibited apoptotic cell death and regulated the expression of proteins in the Bcl-2 family. These results suggest that VEDI-1 may facilitate late differentiation of osteoblasts and may have an inhibitory effect on apoptosis.

Key words: SST-VEDI-1, Osteoblast, Mineralization, Apoptosis.

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SST-VEDI-1 (VEDI-1) was synthesized as a novel compound as part of an effort to develop drugs for the treatment of both erectile dysfunction and baldness (Fig. 1a). Its congeners (Fig. 1b) have been isolated and identified as natural products from the seeds of *Rollinia mucosa* (Annonaceae), which is widely distributed in tropical regions of the Americas [1]. The fruit of this plant is edible and is used in folk medicine as a therapeutic agent. At present, VEDI-1 has yet to be found in either the seeds or fruit of *Rollinia mucosa*, although it is possible that VEDI-1 exists as a natural compound. The chemical structure of VEDI-1 is N-nonanoyltryptamine (Fig. 1a). It is named af-

ter the initials of its three inventors and its biological function: Somei, Shigenobu, Tanaka-vasodilator, erectile dysfunction. We have developed two series of new VED compounds and they are classified by the Roman letters I and II [2]. The number I-1 refers to the first compound belonging to the I series. VEDI-1 is synthesized from tryptamine by a synthetic method, which has been reported in a previous paper [2, 3].

Tryptamine is one of the known trace amines [4].

a: SST-VEDI-1 ($n=7$)

Chemical Formula: $C_{19}H_{28}N_2O$

Molecular Weight: 300.44

b: Congeners of SST-VEDI-1
($n = 16, 18, 20, 21, 22, 23$)

Fig. 1. Chemical structure of SST-VEDI-1 (VEDI-1)

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Trace amines are present in the central nervous system at very low concentrations and are believed to have potent sympathomimetic actions [5]. SSH-BM-type compounds are also synthesized from tryptamine, and have the chemical structure 1-benzyl-2,4,6-tribromomelatonin. This family of compounds is named from the initials of their three inventors and their biological function: Somei, Suzuki, Hattori-bone metabolism (and mineralization, matrix, mass, mineral density). VEDI-1 has a similar chemical structure to that of the SSH-BM-type compounds, and these compounds have been demonstrated to stimulate osteoblast activity in cultured scales of goldfish [6]. The teleost scale is a calcified tissue that contains osteoclasts and osteoblasts [7, 8]. Bone matrix components including type I collagen (Col.I), osteocalcin (OC), and osteonectin, are present in these scales as well as in mammalian bone [9-11]. Hydroxyapatite is also found in scales [12]. Teleost scales contain as much as 20% of the body's total calcium and function as an internal calcium reservoir during periods of increased calcium demand, such as during sexual maturation or starvation [13, 14]. Thus, there are many similarities between the teleost scale and mammalian membrane bone, and given the known effects of SSH-BM compounds on scale osteoblast differentiation, we set out to examine the effects of VEDI-1 on osteoblast differentiation in mammalian cells.

Mesenchymal cells can differentiate into several specialized cell types including osteoblasts [15]. The developmental process from mesenchymal progenitors to particular cell types can be divided into two stages: commitment and terminal differentiation [16]. This differentiation process is regulated by several cytokines. Bone morphogenetic protein 2 (BMP-2) is one of the most powerful cytokines that promotes the differentiation of mesenchymal cells into osteoblasts *in vitro* and also induces bone formation *in vivo* [17]. BMP-2 exerts its effects through transcriptional regulation of osteogenic genes.

Runt-related transcription factor 2 (Runx2), Osterix (OSX), and Msh homeobox protein 2 (Msx2) are osteogenic transcription factors that are necessary for early and late osteoblast differentiation [18-20]. These osteogenic transcription factors play an essential role in the regulation of osteoblast marker genes such as OC, bone sialoprotein (BSP), and osteopontin (OPN) [21-23]. Regulation by Runx2 is thought to occur when Runx2 binds to an osteoblast-specific cis-acting

element, termed OSE2, in the promoter region of skeletal target genes and regulates its expression [21].

Apoptosis, known as programmed cell death, is closely related to cell differentiation, including osteoblast differentiation. The Bcl-2 protein family plays a prominent role in the regulation of apoptosis. It is well known that the members of this family are implicated in pivotal decision points regarding cell survival thanks to recent functional studies focusing on the importance of mitochondrial events in cell death signaling [24]. This family can be divided into two types: proteins such as Bcl-2 and Bcl-xL that suppress cell death, and others, such as Bak, Bax, and Bad, that appear to promote apoptosis [24].

The study of the regulation of cell differentiation and apoptosis requires *in vitro* models utilizing phenotypically stable cultured cell lines, in which the direct effects on individual cell types can be investigated. Therefore, in this study, three cultured cell lines, ROB-C26, MC3T3-E1, and ROS17/2.8, were used. ROB-C26 is a clonal mesenchymal progenitor-like cell line isolated from newborn rat calvaria [25]. ROB-C26 cells are pre-committed cells and are capable of differentiating into osteoblastic cells with BMP-2-treatment [25]. MC3T3-E1 cells are mouse pre-osteoblast-like cells that can differentiate into mature osteoblasts [26]. ROS17/2.8 cells are rat osteoblast-like osteosarcoma cells that have a mature osteoblast phenotype [27]. MC3T3-E1 cells and ROS17/2.8 cells are committed to the osteoblast lineage and are able to form bone-like nodules during terminal osteoblast differentiation [26, 27].

We found that VEDI-1 enhanced the formation of mineralized bone-like nodules in both ROS17/2.8 cultures and MC3T3-E1 cultures, and that the formation of nodules was accompanied by increased expression of BSP and OC. In addition, VEDI-1 inhibited apoptosis, suppressed Bax, Bak and Bad expression, and induced Bcl-2 expression. These results suggest that VEDI-1 is a stimulator of osteoblast differentiation and an inhibitor of apoptosis *in vivo*.

Materials and methods

Cell culture and reagents

Cells were precultured until they reached confluence in growth media consisting of α -MEM, 10% fetal

bovine serum (FBS) and 1% penicillin-streptomycin. Subsequently, the cells were cultured for the indicated periods of time under the indicated culture conditions in media supplemented with 50 µg/ml L-ascorbate phosphate (Sigma) and 10 mM β-glycerophosphate (Sigma). The culture medium was changed every 2 days. Antibiotics and cell culture media were obtained from Gibco and Wako, respectively. FBS was purchased from Japan Bioserum Co., Ltd. Recombinant hBMP-2 was acquired from R&D Systems. VEDI-1 was synthesized according to the methods described in a previous report [2]. VEDI-1 was dissolved in dimethyl sulfoxide (DMSO) to make a 10^{-3} M stock solution, which was then diluted in culture medium to obtain the desired concentration.

Cell proliferation assay

Cells were seeded onto 96-well plates (1200 cells/well) and pre-cultured until they reached confluence in the growth media. Subsequently, the cells were cultured for the indicated periods under the indicated culture conditions without changing the media. After cells were cultured, we measured the number of cells using a colorimetric cell counting assay kit (Cell counting kit 8, Dojin Co.) according to the manufacturer's instructions.

DAPI staining

The cells were seeded at a concentration of 1×10^5 cells/dish in 6 cm culture dishes and precultured until they reached confluence in the growth media. Subsequently, the cells were cultured for the indicated periods under the indicated culture conditions without changing the media. After the cells were cultured, we assessed cell morphology under an inverted light microscope. The cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS, and then incubated with DAPI (1 µg/ml) for 5 min. After washing with PBS, we observed the cells using fluorescent microscopy (Olympus).

Caspase activity

Caspase activity was detected using a Caspase-Glo® 3/7 assay kit (Promega). Briefly, the cells were seeded onto 96-well plates (1200 cells/well) and pre-cultured until they reached confluence in the growth

media. Subsequently, the cells were cultured for the indicated periods under the indicated culture conditions without changing the media. After the cells were cultured, 50 µl of caspase 3/7 reagent was added to each well and incubated for 1 hr on a rotary shaker at room temperature. Luminescence was then measured and recorded for each well.

Alizarin red S staining

Cells were cultured in 12 well plates under the indicated conditions and then cultures were rinsed twice and fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 15 min. Subsequently, Alizarin red S solution was added. Then, following a 15 min incubation, the plates were washed with distilled water.

Ca²⁺ release assay

Cells were cultured in 12 well plates under the indicated conditions. After removing the supernatant, cells were washed with 10 mM Tris-HCl (pH 7.2) solution and 1 N HCl solution was added to each well, followed by incubation until cells were dry. Twenty µl of distilled water was then added to each well, and the amount of Ca²⁺ was determined using the Calcium E-test (Wako Co.) according to the manufacturer's instructions.

Alkaline phosphatase activity

Cells were cultured in 12 well plates under the indicated conditions. Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as the substrate as described by Partridge *et al.* [28]. Protein concentration was also determined using a Bio-Rad protein assay reagent (Bio-Rad), using bovine serum albumin as the standard.

Real time RT-PCR and Western blot analysis

Real-time PCR and western blot analysis were performed as previously described [29]. Sense and antisense primers used for real-time PCR were as follows: Bcl-2 (forward, 5'-TGT CCA GTC AGC TGC A-3'; reverse, 5'-TGA CCC CAC CGA ACT CA-3'), Bcl-xL (forward, 5'-AAG GAT ACA GCT GGA GT-3'; reverse, 5'-CCA TTC ACC GCG GGG CTA TC-3'), Bad (forward, 5'-CTC GCT GGC TCC TGC ACA

CG-3'; reverse, 5'-GCG TCT TCC TGC TCA CTC GG-3'), Bak (forward, 5'-GCT TCA GCC CAC CGC TGG GA-3'; reverse, 5'-CAC GCT GGT AGA CAT ACA GG-3'), Bax (forward, 5'-GGG TGG CAG CTG ACA TGT TT; reverse, 5'-CGC TCA CGG AGG AAG TCC AG-3'), OSX (forward, 5'-GGA GGT TTC ACT CCA TTC CA-3'; reverse, 5'-TAG AAG GAG CAG GGG ACA GA-3'), Msx2 (forward, 5'-CAA GCC CTT CGA GAC CGC C-3'; reverse, 5'-CAG GTA CTG TTT CTG GCG G-3'), Col.I (forward, 5'-TCA GTG GTT TGG ATG GTG CC-3'; reverse, 5'-ACA GCA CCA TCG TTA CCG C-3'). Other primers used were described previously [29]. Messenger RNA expression levels were normalized to the values obtained for β -actin mRNA. The antibodies used for western blotting against Runx2 (sc-10758), OSX (sc-22538), Msx2 (sc-13771), OC (sc-18319) and actin (sc-1615) were obtained from Santa Cruz Biotechnology, and the antibody against BSP (A2-20128) was obtained from American Research Products. As an internal control, an antibody specific for actin was used.

Luciferase assay for the (OSE2)x6-Luc construct

A promoter construct fused to firefly luciferase, (OSE2)x6-Luc, was kindly provided by Dr. Gerard Karsenty. A plasmid, pGL3-Basic (Promega), was used as a negative control. ROS17/2.8 cells were seeded on 6 well plates at 90% confluence and were then incubated for 18 hr. Two micrograms of (OSE2)x6-Luc or pGL3-Basic were transfected into the cells using lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, a luciferase assay was performed using a Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. The luciferase activity of the (OSE2)x6-Luc and pGL3-Basic-transfected cells was normalized to the values obtained for Renilla luciferase activity as an internal control.

cAMP assay

Cells were seeded onto 96-well plates (1200 cells/well) and pre-cultured until they reached confluence in the growth media. Subsequently, the cells were cultured for the indicated periods of time under the indicated culture conditions without changing the media. After cell culture, cAMP levels were detected using a cAMP-GloTM assay kit (Promega) according to the manufacturer's instructions.

Statistical analysis

Results are presented as the means $\pm$ SD of triplicate cultures and the difference was assessed by a Student's t-test. Significant differences ($p < 0.05$) are shown by the indicated marks.

Results

Effects of VEDI-1 on apoptosis

To investigate the effects of VEDI-1 on starvation-induced apoptosis, confluent ROS17/2.8 cells were cultured in the absence or presence of VEDI-1 without changing the media for the indicated numbers of days, and the cell number was then counted as an O.D. Results for the cell counting assay are shown in Figure 2A. Cell numbers in the 10^{-11} M and 10^{-10} M VEDI-1-treated cultures and in the untreated control cultures rapidly decreased from day 3 to day 5. However, the decrease in cell number was smaller in 10^{-10} M VEDI-1-treated cultures compared to untreated control cultures. In contrast, cell numbers in the 10^{-7} - 10^{-9} M VEDI-1-treated cultures decreased slightly from day 3 to day 5. Inhibition of the decrease in cell number in VEDI-1-treated cultures was confirmed by observation under a light microscope (Fig. 2A, right panel). Furthermore, under a fluorescence microscope, untreated control cells exhibited the morphological features of early apoptotic cells, including bright nuclear condensation indicated by DAPI staining. In contrast, nuclear condensation was rarely observed in 10^{-9} M VEDI-1-treated cultures (Fig. 2B).

Effects of VEDI-1 on apoptotic signaling pathways

Next, to determine which apoptotic signaling pathways were influenced by VEDI-1, we investigated the activity of caspase3/7. After cells were cultured under starvation conditions, caspase3/7 activity increased significantly in the untreated control cultures. However, caspase3/7 activation was suppressed by VEDI-1-treatment in both ROS17/2.8 and MC3T3-E1 cultures (Fig. 3).

To investigate the effects of VEDI-1 on the mRNA expression of Bcl-2 family members, we performed real-time PCR, and the results are shown in Figure 4. mRNA expression of Bad, Bak, and Bax was de-

Fig. 2. Effects of VEDI-1 on starvation-induced apoptosis

(A) Changes in numbers of ROS17/2.8 cells. We cultured ROS17/2.8 cells with the indicated concentrations of VEDI-1 for the indicated numbers of days under the conditions described in the Materials and Methods section. After culture, we determined the number of surviving cells by measuring O.D. and observing cells under a light microscope (right panel). (B) Nuclear morphological changes in ROS17/2.8 cells. We cultured cells under the same conditions indicated in A for 5 days. We then performed fluorescent staining of nuclei by DAPI on VEDI-1-treated or untreated cells. Cells were visualized using a fluorescence microscope. Arrowheads indicate nuclear condensation.

Fig. 3. Effects of VEDI-1 on caspase3/7 activity

We cultured ROS17/2.8 cells and MC3T3-E1 cells with or without VEDI-1 (10^{-9} M) for the indicated numbers of days and then measured caspase3/7 activity at each time point. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cells at each time point].

Fig. 4. Messenger RNA expression of Bcl-2 family members

We cultured ROS17/2.7 cells with or without VEDI-1 (10^{-9} M) for 3 days and then performed real-time PCR. [Mean $\pm$ S.D. ($n = 3$). ($P < 0.05$). *Different from untreated control cells].

creased in VEDI-1-treated cells compared to the untreated control cells. In contrast, Bcl-2 expression increased in VEDI-1-treated cells compared to the untreated control cells. No differences were observed with respect to Bcl-xL mRNA expression between VEDI-1-treated cells and untreated control cells.

Effects of VEDI-1 on osteoblast differentiation

To analyze the effects of VEDI-1 on osteoblast differentiation, we compared the mineralization levels of VEDI-1-treated cultures and untreated control cultures. We first examined the dose- and time-dependence of the effects of VEDI-1 on ROS17/2.8 cells. We found that the number of Alizarin red S-positive mineralized bone-like nodules was increased in 10^{-9} M and 10^{-10} M VEDI-1-treated cultures compared to untreated control cultures (Fig. 5A). However, VEDI-1 (10^{-7} M)-treated cultures had a slightly decreased number of mineralized nodules compared to untreated cultures (Fig. 5A). Similarly, Ca^{2+} levels were increased by VEDI-1 treatment in a dose-dependent manner (Fig. 5B). 10^{-11} M VEDI-1 seemed to have a small effect on the formation of mineralized nodules, but there was not a statistically significant difference in Ca^{2+} levels (Fig. 5A and B).

Our time-dependence experiment revealed that Alizarin red S-positive mineralized nodules were detected at low levels in both the 10^{-9} M VEDI-1-treated

cultures and the untreated cultures on day 8 (Fig. 5C). After day 8, the number of mineralized nodules increased rapidly in the VEDI-1-treated cultures compared to the untreated cultures. We confirmed our Alizarin red S staining results with a Ca^{2+} release assay. We saw that Ca^{2+} release from the mineralized nodules was increased in VEDI-1 treated-cultures compared to the untreated control cultures from Day 10 to Day 16 (Fig. 5D).

Next, to determine whether VEDI-1 stimulates the formation of mineralized nodules in other cell types, we cultured MC3T3-E1 and ROB-C26 cells in the absence or presence of VEDI-1 for the indicated numbers of days, and then performed Alizarin red S staining and a Ca^{2+} release assay. The levels of mineralized nodules and Ca^{2+} were increased in the VEDI-1-treated cultures compared to the untreated control cultures on Day 21 and Day 28 in MC3T3-E1 cultures as well as in ROS17/2.8 cultures (Fig. 6A and B). However, in ROB-C26 cultures, VEDI-1 failed to induce the formation of mineralized nodules, although they were induced in BMP-2-treated cultures (Fig. 6C). Furthermore, BMP-2-induced mineralized nodule formation was suppressed by treatment with VEDI-1 in ROB-C26 cultures. Ca^{2+} measurements were consistent with the results of the Alizarin red S staining (Fig. 6C and D).

To further investigate the effects of VEDI-1 on the early phase of osteoblast differentiation in each cell

Fig. 5. Effects of VEDI-1 on mineralization in ROS17/2.8 cells

(A) Dose-dependent effects of VEDI-1 on the formation of mineralized bone-like nodules in ROS17/2.8 cultures. We cultured cells with the indicated concentrations of VEDI-1 for 12 days and then performed Alizarin red S staining. (B) Quantitation of calcium deposition. We cultured cells under the same conditions indicated in A. We then measured calcium contents of the cell layers using the o-cresolphthalein complexone method. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cultures at each time point]. (C) Time-dependent effects of VEDI-1 on the formation of mineralized nodules. We cultured ROS17/2.8 cells with or without VEDI-1 (10^{-9} M) for the indicated numbers of days and then performed Alizarin red S staining. (D) Quantification of calcium deposition. We cultured cells under the same conditions indicated in C, and then measured calcium contents. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cultures at each time point].

culture, we measured ALP activity. ROS17/2.8 cells, MC3T3-E1 cells, and BOB-C26 cells were cultured under the indicated conditions and then ALP activity was measured. ALP activity was increased by VEDI-1 treatment during the early phase in ROS17/2.8 (Days 4 and 8) and MC3T3-E1 (Days 7 and 14) cultures (Fig. 7). In contrast, in ROB-C26 cultures, ALP activity was decreased in cultures treated with a combination of BMP-2 and VEDI-1 compared to cultures treated with BMP-2 alone (Fig. 7).

Effects of VEDI-1 on osteogenic transcription factors and osteoblast markers

We next sought to investigate the effects of VEDI-1

on the expression of osteogenic transcription factors (Runx2, OSX, and Msx2) and osteoblast markers (BSP, OC, OPN, and Col.I). We cultured ROS17/2.8 cells in the presence or absence of VEDI-1, and then performed real-time PCR and western blotting. We detected no effects of VEDI-1 on mRNA expression or protein expression of Runx2, Msx2, OPN, or Col.I (Fig. 8A and B). However, mRNA and protein expression of BSP and OC was increased in VEDI-1-treated cells compared to untreated control cells by Day 12, although no difference was detected on Day 1 (Fig. 8A and B). In contrast, OSX mRNA and protein expression were slightly decreased in VEDI-1-treated cells compared to untreated control cells (Fig. 8A and B).

Next, in order to examine whether VEDI-1 plays a

Fig. 6. Effects of VEDI-1 on mineralization in MC3T3-E1 cells and ROB-C26 cells (A and C) Time-dependent effects of VEDI-1 on the formation of mineralized nodules. We cultured MC3T3-E1 cells with or without VEDI-1 (10^{-9} M) for the indicated numbers of days. We also cultured ROB-C26 cells with or without VEDI-1 (10^{-9} M) in the presence or absence of BMP-2 (100ng/ml) for the indicated numbers of days. After culture, we performed Alizarin red S staining. (B and D) Quantification of calcium deposition. We cultured both cell lines under the same conditions indicated in A and C, respectively, and then measured calcium contents. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cultures at each time point].

role in the regulation of Runx2 transcriptional activity, we performed a luciferase assay using the (OSE2)x6-Luc construct. The luciferase assay showed that the activity of (OSE2)x6-Luc in VEDI-1-treated cells was similar to that in untreated control cells (Fig. 8C).

Effects of VEDI-1 on cAMP

To determine whether VEDI-1 regulates osteoblast differentiation by activating the cAMP pathway, we next analyzed the formation of cAMP. We cul-

tured ROS17/2.8 cells with multiple concentrations of VEDI-1 or no treatment for the indicated periods of time and then measured cAMP. The cAMP levels in cultures treated with a low concentration of VEDI-1 (10^{-11} M- 10^{-9} M) were similar to those of untreated control cultures (Fig. 9). However, cAMP levels in cultures treated with a higher concentration of VEDI-1 (10^{-7} M and 10^{-8} M) were lower than in the untreated control cultures during the early culture phase (from 1 to 12 hr) (Fig. 9).

Fig. 7. Effects of VEDI-1 on ALP activity

We cultured ROS17/2.8 cells and MC3T3-E1 cells with or without VEDI-1 (10^{-9} M) for the indicated numbers of days. We also cultured ROB-C26 cells with or without VEDI-1 (10^{-9} M) in the presence or absence of BMP-2 (100 ng/ml) for the indicated numbers of days. After culture, ALP activity was measured at each time point. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated controls at each time point, **Different from both untreated controls and from cells treated with VEDI-1 alone at each time point, †Different from cells treated with BMP-2 alone at each time point].

Discussion

We have demonstrated that VEDI-1 inhibits apoptotic cell death and promotes the formation of mineralized bone-like nodules in osteoblasts.

First, we investigated the effects of VEDI-1 on apoptosis. A cell counting assay showed that VEDI-1 suppressed starvation-induced cell death at concentrations of 10^{-7} M- 10^{-10} M in ROS17/2.8 cultures (Fig. 2A and B). In addition, VEDI-1 inhibited nuclear condensation and suppressed caspase activity. Nuclear condensation is one of the most typical apoptotic characteristics [30]. Furthermore, caspase3 is the most prevalent caspase within cells and is responsible for the majority of apoptotic effects [31]. Taken together, these findings suggest that VEDI-1 inhibits apoptotic

cell death.

The sensitivity of cells to apoptotic stimuli is often regulated by the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family members [24], and in this study, the inhibition of apoptosis by VEDI-1 was accompanied by an increase in anti-apoptotic Bcl-2 mRNA expression and a decrease in the expression of Bak, Bax, and Bad mRNA, which are pro-apoptotic members of the Bcl-2 family [24] (Fig. 4). On the basis of this evidence, we suggest that VEDI-1 inhibits apoptosis via a Bcl-2 family-dependent signaling pathway.

In a previous paper, it was determined that 60 to 90% of murine osteoblasts die by apoptosis, and then the rest become osteocytes or lining cells *in vivo* [32]. Glucocorticoid is a well-known inducer of apoptosis in osteoblasts [33]. Glucocorticoid-induced apop-

Fig. 8. Effects of VEDI-1 on the expression and transcriptional activity of osteogenic genes

(A) mRNA expression of osteogenic transcription factors (Runx2, Osterix, and Msx2) and late osteoblast markers (BSP, OC, OPN, and Col.1). We cultured ROS17/2.8 cells with or without VEDI-1 (10^{-9} M) for the indicated numbers of days and then performed real-time PCR. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cells at each time point]. (B) Expression of Runx2, Osterix, Msx2, BSP, and OC proteins. We cultured ROS17/2.8 cells under the same conditions indicated in A and then performed western blotting. Actin was used as an internal control. (C) Transcriptional activity of Runx2. We transfected a promoter construct, (OSE2)x6-Luc, into ROS 17/2.8 cells. After 24 hr, cells were treated or not treated with VEDI-1 (10^{-9} M) for an additional 24 hr, and then luciferase activity was determined. A vector, pGL-Basic (Basic), was used as a negative control.

Fig. 9. Effects of VEDI-1 on cAMP activity

We cultured ROS17/2.8 cells with multiple concentrations of VEDI-1 for the indicated periods of time and then measured cAMP activity at each time point. [Mean $\pm$ S.D. ($n = 3$; $P < 0.05$). *Different from untreated control cells at each time point].

tosis is a regulator of the expression of Bcl-2 family members [34]. Therefore, it is possible that VEDI-1 may inhibit glucocorticoid-induced apoptosis through a Bcl-2 dependent mechanism. However, estrogen protects primary osteocytes against glucocorticoid-induced apoptosis by preventing the translocation of glucocorticoid receptors to the nucleus [35]. One recent report has demonstrated that one of the SSH-BM-type compounds induces the expression of estrogen receptor (ER) mRNA [6]. We have also confirmed in our laboratory that VEDI-1 induces the expression of ER mRNA [Mikami *et al.* unpublished data]. Therefore, another possible explanation is that VEDI-1 may enhance bone formation by inhibiting glucocorticoid-induced osteoblast apoptosis through an estrogen-dependent signaling pathway *in vivo*.

Second, we investigated the effects of VEDI-1 on the formation of mineralized nodules. Alizarin red S staining revealed that treatment with VEDI-1 (10^{-11} M– 10^{-9} M) enhanced the formation of mineralized nodules in ROS17/2.8 cultures in a dose-dependent manner (Fig. 5). However, 10^{-7} M and 10^{-8} M VEDI-1 failed to increase nodule formation, although they were able to block apoptosis. Given that 10^{-10} M and 10^{-9} M VEDI-1 do block apoptosis, it appears that the anti-apoptotic and differentiation-inducing effects of VEDI-1 *in vitro*

are separate.

Increased mineralized nodule formation by VEDI-1 treatment was observed in both mature osteoblast ROS17/2.8 cultures and in pre-osteoblast MC3T3-E1 cultures (Figs. 5 and 6). However, in mesenchymal progenitor ROB-C26 cultures, no mineralized nodules were observed regardless of VEDI-1 treatment, although BMP-2 was able to induce nodule formation in these cells (Fig. 6). Furthermore, BMP-2-induced nodule formation was suppressed by VEDI-1 treatment in ROB-C26 cultures. ALP activity was similarly affected by VEDI-1 treatment (Fig. 7). These results suggest that the stimulatory effects of VEDI-1 on the formation of mineralized nodules depend not only on the concentration of VEDI-1 but also on the degree of cell differentiation. The development of mesenchymal progenitors into particular cell types can be divided into two stages: commitment and terminal differentiation [16]. ROS17/2.8 and MC3T3-E1 cells already have been committed to becoming osteoblasts and are able to form mineralized nodules during terminal osteoblast differentiation [26, 27]. In contrast, ROB-C26 cells are pre-commitment cells [25], which require commitment to the osteoblast lineage prior to the formation of mineralized nodules. On the basis of this evidence, we hypothesize that VEDI-1 facilitates the formation of mineralized nodules in terminal osteoblast differentiation, but is unable to commit progenitor cells to becoming osteoblasts or to inhibit the commitment to the osteoblast lineage.

Third, we attempted to determine whether VEDI-1 affected the expression of osteogenic genes. The results of real-time PCR and western blot analysis showed that mRNA and protein expression of BSP and OC were increased in VEDI-1-treated cells on Day 12 (Fig. 8). However, these expression levels were not changed on Day 1 of VEDI-1 treatment (Fig. 8). This may indicate that VEDI-1 regulates BSP and OC expression indirectly. The expression of osteoblast marker genes, including BSP and OC, is regulated by Runx2, Osterix, and Msx2 [21–23]. However, VEDI-1 failed to increase the expression of these transcription factors or Runx2 transcriptional activity. Therefore, we believe that VEDI-1 indirectly increases BSP and OC expression in a Runx2, Osterix, and Msx2-independent manner, which is followed by enhancement of the formation of mineralized nodules.

VEDI-1 is synthesized from tryptamine, which is a trace amine [2,3]. A family of G protein-cou-

pled receptors (GPCRs) has been reported to be trace amine-associated receptors (TAAR1) [4,5]. Some amines derived from thyroid hormone decarboxylation and deiodination, known as thyronamines, have been shown to be endogenous compounds that stimulate cAMP production via activation of TAAR1 [36]. Cyclic AMP signaling regulates both osteogenic and apoptotic events [37, 38]. VEDI-1 and thyronamines have a common basic chemical structure, thus we set out to determine whether VEDI-1 affects cAMP production in osteoblasts. However, contrary to our expectations, 10^{-11} M- 10^{-9} M VEDI-1, which exhibited a positive effect on mineralization, had no effect on cAMP production (Fig. 9). However, 10^{-7} M and 10^{-8} M VEDI-1 inhibited cAMP production (Fig. 9). This may indicate that high concentrations of VEDI-1 can inhibit terminal osteoblast differentiation through a cAMP-dependent signaling pathway via TAAR1. Unfortunately, we have no direct evidence indicating that VEDI-1 binds to TAAR1. Therefore, it is important to note that our results do not rule out the possibility that VEDI-1 regulates osteoblast differentiation in a TAAR1-cAMP independent manner.

The LD₅₀ for VEDI-1 was established to be more than 80 mg/kg in a male ddY mouse and no side effects have been seen. Based on this finding, experiments directed toward breeding and cashmere growth promotion in goats were carried out successfully in Inner Mongolia, China for 2 years [39]. In these ex-

periments, the VEDI-1 dose range used was 10-0.1mg/60 kg a day. In this study, VEDI-1 had positive effects on mineralization at a lower concentration (10^{-11} M- 10^{-9} M) [39]. On the basis of these findings, it is possible that VEDI-1 could be used as an anti-osteoporosis agent, although the results of further *in vitro* and *in vivo* studies are needed to demonstrate whether VEDI-1 has clinical utility for the enhancement of bone formation.

In conclusion, VEDI-1 enhanced terminal osteoblast differentiation, as indicated by mineralization, in a dose-dependent manner. In addition, VEDI-1 inhibited apoptosis, which was accompanied by regulation of the expression of Bcl-2 protein family members. Our studies have characterized the activity of VEDI-1, and suggest that VEDI-1 is an important target for future studies on osteoblast differentiation and apoptosis.

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