Arch Pharm Res Vol 34, No 2, 191-198, 2011
DOI 10.1007/s12272-011-0202-4

Archives of Pharmacal Research
www.springer.com/12272

Synthesis of C_6-Epimer Derivatives of Diacetoxy Acetal Derivative of Santonin and their Inducing Effects on HL-60 Leukemia Cell Differentiation

Sin Ho Kweon[1], Keun Tae Kim[2], Joon Hee Hong[3], Tae Sung Kim[1], and Bo Gil Choi[2]

[1]School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea, [2]Department of Pharmacy, College of Pharmacy, Chonnam National University, Gwangju 500-757, Korea, and [3]BK21 Project Team College of Pharmacy, Chosun University, Kwangju 501-759, Korea

(Received October 13, 2010/Revised November 23, 2010/Accepted November 23, 2010)

Induction of differentiation is a new and promising approach to leukemia therapy, well illustrated by the treatment of acute promyelocytic leukemia with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid (ATRA). Using combination of either 1,25-$(OH)_2D_3$ or ATRA and chemotherapy, adverse effects 1,25-$(OH)_2D_3$ or ATRA such as hypercalcemic effects have decreased, and long-term survival has improved. In a previous study, we demonstrated that santonin could be chemically modified into a diacetoxy acetal derivative of santonin with strong differentiation-inducing activity. In this study, we further synthesized C_6-epimer derivatives of diacetoxy acetal derivative of santonin and tested their effects on HL-60 cell differentiation. Some of the C_6-epimer derivatives themselves induced increases in cell differentiation. Especially, (11*S*)-3,3-(ethylenedioxy) eudesmano-13-ol-6β-acetate (**7**) was demonstrated to induce differentiation with larger than 80% of the cells attaining a differentiated phenotype. Importantly, **7** strongly enhanced differentiation of HL-60 cells in a dose-dependent manner when combined with either low doses of 1,25-$(OH)_2D_3$ or ATRA. The ability to enhance the differentiation potential of 1,25-$(OH)_2D_3$ or ATRA by **7** may improve outcomes in the therapy of acute promyelocytic leukemia.

Key words: DAAS, 6-Epimer, Differentiation, Leukemia, 1,25-dihydroxyvitamin D_3, All-*trans* retinoic acid

INTRODUCTION

Leukemia can eventually be cured with agents that induce terminal differentiation, presumably with less morbidity than that induced by known cytodestructive agents (Beere and Hickman, 1993). 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and all-*trans* retinoic acid (ATRA) are able to induce a terminal differentiation of leukemic cell-lines, such as HL-60 and U937 cells, and of short-term cultured APL cells isolated from human patients (Breitman et al., 1980; Chomienne et al., 1986). Moreover, ATRA is able to induce complete remission in almost all patients with acute promyelocytic leukemia (APL) through *in vivo* differentiation of APL blasts. Although ATRA can bring about complete remission of APL, treatment with ATRA alone showed severe side effects, including ATRA syndrome (Frankel et al., 1992) and the induction of a secondary resistance to ATRA. Therefore, current attempts to overcome this problem focus on the combination therapy with non-toxic concentrations of either 1,25-$(OH)_2D_3$ or ATRA, along with compounds that have different mechanisms of action, such as paclitaxel (Hershberger et al., 2001), curcumin (Sokoloski et al., 1997), and silibinin (Kang et al., 2001).

Previously, we found that DAAS (Fig. 1), the acetal analogue of α-santonin that contains a non-lactone ring, had remarkable anti-leukemic activities, inducing HL-60 cell differentiation via down-regulation of NF-κB binding activity (Kim et al., 2006a). Also, we have investigated the SAR of DAAS derivatives (Kim et al.,

Correspondence to: Bo Gil Choi, College of Pharmacy, Chonnam National University, Gwangju 500-757, Korea
Tel: 82-62-530-2926, Fax: 82-62-530-2911
E-mail: bgchoi@chonnam.ac.kr

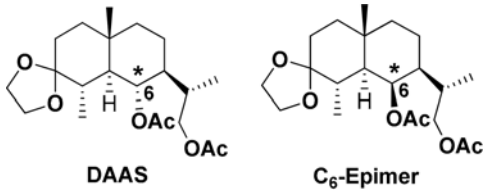


Fig. 1. Chemical structure of DAAS and its C_6 epimer

2006b; Kim et al., 2008a).

In this report, we further synthesized C_6-epimer derivatives of DAAS and investigated their enhancing effects on cellular differentiation of human promyelocytic leukemia HL-60 cells, alone or in combinations of a low dose of 1,25-$(OH)_2D_3$ or ATRA. HL-60 cell-line has been utilized as an excellent model system for studying cellular differentiation *in vitro*. HL-60 cells are differentiated into monocytic or granulocytic lineage when treated with 1,25-$(OH)_2D_3$ or ATRA, respectively (Breitman et al., 1980; Tanaka et al., 1983).

MATERIALS AND METHODS

Chemistry

Melting points were determined on a Mel-temp II laboratory device and are uncorrected. MS spectra were obtained using IT-TOF (Shimazu). Nuclear magnetic resonance (NMR) data for ^{1}H-NMR (300 MHz) and ^{13}C-NMR (125 MHz) was collected on the Varian UNITY spectrophotometer and are reported in δ ppm downfield from tetramethylsilane (TMS). IR spectra (IR) were determined neat or from KBr disks on the Jasco FT-IR instrument and are reported in reciprocal centimeters. Thin layer chromatography (TLC) was carried out using plates precoated with silica gel 60F 254 that were purchased from Merck. Test compounds were synthesized by conventional methods and identified by m.p., IR spectra, ^{1}H-NMR, ^{13}C-NMR and Mass data.

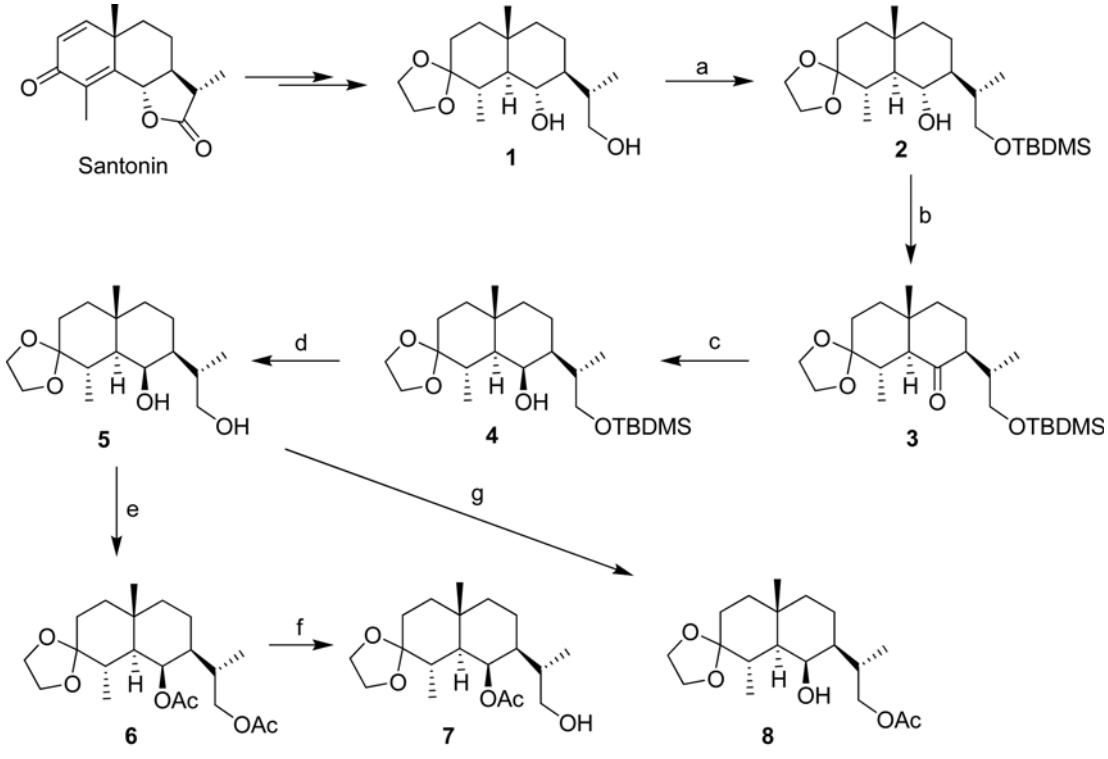
Synthesis

(11*S*)-3,3-(Ethylenedioxy)eudesmano-13, 6α-diol (1)

The known compound **1** was readily synthesized from tetrahydrosantonin using the previously reported procedures. Compound **1**, mp 150-152°C (Kato et al., 1971; mp 149-151°C).

(11*S*)-3,3-(Ethylenedioxy)-13-(*t*-butyldimethylsilyl) oxy-eudesmano-6α-ol (2)

To a solution of **1** (1.50 g, 5 mmol) and imidazole (1.03



Scheme 1. Synthetic pathway of the C_6-epimer derivatives of DAAS.

g, 15 mmol) in dry CH_2Cl_2 (12 mL), *t*-butyldimethylsilyl chloride (0.835 g, 5.54 mmol) was slowly added at −10°C and the mixture was stirred at room temperature for 3 h. After removing solvent, water was added to the reaction mixture, and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtrated, and concentrated *in vacuo*. The residue was chromatographed on silica gel column chromatography (Hexane : EtOAc = 10:1) to give **2** as a colorless oil (710.5 mg, 34.4%). IR (KBr) : 3477 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.78 (3H, d, *J* = 6.9 Hz, CH-C$\underline{H}_3$), 0.82 (3H, s, -CH_3), 0.85 (9H, s, *t*-C(C$\underline{H}_3$)$_3$), 1.62 (1H, m, -C$\underline{H}$-CH_3), 2.14 (1H, m, -C$\underline{H}$-CH_3), 3.46 (3H, m, C$\underline{H}$OH & C$\underline{H}_2$OSi), 3.94 (4H, m, O$\underline{CH_2CH_2}$O).

(11*S*)-3,3-(Ethylenedioxy)-13-(*t*-butyldimethylsilyl) oxy-6-keto-eudesmane (3)

Oxalyl chloride (2.0 M, 1.8 mL) was dissolved in dry CH_2Cl_2 (6 mL), then was slowly added to DMSO (0.39 mL) and stirred for 20 min at −78°C. The compound **2** (742.5 mg, 1.8 mmol) dissolved in CH_2Cl_2 (4.5 mL) was added into the prior solution and stirred for 20 min at −78°C. Then, triethyl amine (1.25 mL) was slowly added to the reaction solution and stirred for 20 min at −78°C, and then stirred again at 0°C. The resulting solution was neutralized with saturated $NaHCO_3$ solution and extracted with ethyl acetate (2 × 100 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane : EtOAc = 10:1) to give **3** as oil (401.9 mg, 54.3%): IR (KBr) : 1705 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.77 (3H, d, *J* = 6.9 Hz, CH-C$\underline{H}_3$), 0.82 (3H, s, -CH_3), 0.87 (9H, s, *t*-C(C$\underline{H}_3$)$_3$), 1.08 (3H, d, *J* = 6.6 Hz, CH-C$\underline{H}_3$). 3.46 (2H, m, C$\underline{H}_2$OSi), 3.93 (4H, m, O$\underline{CH_2CH_2}$O).

(11*S*)-3,3-(Ethylenedioxy)-13-(*t*-butyldimethylsilyl) oxy-eudesmano-6β-ol (4)

Diisobutyl aluminium hydride (0.974 mL, 1.0 M DIBALH in CH_2Cl_2) was carefully added to the ketone **3** (400 mg, 0.974 mmol) dissolved in dry CH_2Cl_2 (5 mL) at 0°C and stirred for 1 h at room temperature. Methyl alcohol (1.0 mL) was added to the reaction mixture to stop the reaction and stirred for about 1 h at room temperature until color of the solution was turn into white. The product was filtrated and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane : EtOAc = 15:1) to give **4** as a colorless oil (324 mg, 80.6%), IR (KBr): 3500 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.89 (3H, d, *J* = 4.2 Hz, > CH-C$\underline{H}_3$), 0.90 (9H, s, *t*-C(C$\underline{H}_3$)$_3$), 0.99 (3H, d, *J* = 3.3 Hz, > CH-C$\underline{H}_3$), 1.11 (3H, s, -CH_3), 2.97 (1H, d, *J* = 3.6Hz), 3.43 (2H, m, C$\underline{H}_2$O-Si), 3.96 (4H, m, O$\underline{CH_2CH_2}$O).

(11*S*)-3,3-(Ethylenedioxy) eudesmano-13, 6β-diol (5)

Tetrabutylammonium fluoride (1.05 mL, 1.0 M in THF) was added to the compound **4** (217 mg, 0.526 mmol) in THF (16.6 mL) and stirred for 3 h at room temperature. The solution was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane : EtOAc = 3:1) to give **5** as semisolid (135.3 mg, 86.1%) : IR (KBr) : 3416 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.92 (3H, d, *J* = 6.6 Hz, > CH-C$\underline{H}_3$), 0.98 (3H, d, *J* = 7.2 Hz, > CH-C$\underline{H}_3$), 1.11 (3H, s, -CH_3), 3.57 (1H, dd, *J* = 10.8, 6.6 Hz, C$\underline{H}_2$OH), 3.67 (1H, dd, *J* = 10.8, 3.0 Hz, CH_2OH)), 3.62 (3H, m, C$\underline{H}_2$-OH); 3.95 (4H, m, O$\underline{CH_2CH_2}$O), 4.1 (1H, m, C$\underline{H}$OH); ^{13}C-NMR (125 MHz, $CDCl_3$) : δ 9.81, 15.92, 19.51, 21.75, 31.15, 33.21, 36.82, 38.01, 40.35, 41.63, 47.66, 50.15, 64.94, 65.03, 66.03, 67.04, 111.90 ppm, *m/z* : 298.2 $[M]^+$, 297.2 $[M-1]^+$.

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6β,13-diacetate (6)

A solution of the diol **5** (76.3 mg, 0.24 mmol), in acetic anhydride (4.0 mL) and pyridine (4.0 mL) was heated overnight at 100°C. After removal of the solvent by evaporation *in vacuo*, the residue was purified by silica gel column chromatography (Hexane : EtOAc = 10:1) to give **6** as a colorless oil (49.3 mg, 53.5%) : IR (KBr) : 1734 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, d, *J* = 3.9 Hz, > CH-C$\underline{H}_3$), 0.88 (3H, s, > -CH-CH_3), 1.08 (3H, s, -CH_3), 2.04 (3H, s, COC$\underline{H}_3$), 2.05 (3H, s, COC$\underline{H}_3$), 3.95 (4H, m, O$\underline{CH_2CH_2}$O), 5.36 (1H, s, > C$\underline{H}$-OAc); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 10.08, 14.09, 19.35, 19.41, 20.99, 21.18, 30.90, 33.46, 33.65, 36.73, 40.26, 40.90, 42.75, 50.32, 65.01, 65.02, 67.68, 71.95, 111.46, 170.36, 171.22 ppm, *m/z* 405.2245 $[M+Na]^+$.

(11*S*)-3,3-(Ethylenedioxy)eudesmano-13-ol-6β-acetate (7)

A solution of diacetate **6** (111.3 mg, 0.29 mmol) in 5% methanolic KOH (0.6 mL) was stirred at room temperature for 5 min and then poured into water. The floating solid was removed through filtration and extracted with ether. The ethereal solution was washed with brine, dried over $MgSO_4$ and concentrated with under reduced pressure. The residue was purified by silica gel column chromatography (Hexane : EtOAc = 3:1) to give **7** as colorless plate (76.5 mg, 77.5%) : mp 92~98°C; IR (KBr) : 3448 cm^{-1}, 1733; ^{1}H-NMR (300 MHz, $CDCl_3$) : δ 0.86 (3H, d, *J* = 6.6 Hz, > CH-C$\underline{H}_3$), 0.89 (3H, d, *J* = 7.5 Hz, > CH-C$\underline{H}_3$), 1.07 (3H, s, -CH_3), 2.04

(3H, s, COC$\underline{H}_3$), 3.48 (1H, dd, J = 10.5, 4.8 Hz, C$\underline{H}_2$-OH), 3.60 (1H, dd, J = 10.5, 5.4 Hz, C$\underline{H}_2$-OH), 3.87 (4H, m, O$\underline{CH_2CH_2}$O), 5.40 (1H, br s, $\underline{CH}$OAc); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 10.03, 14.30, 19.43, 19.74, 21.22, 30.97, 33.44, 36.65, 36.85, 40.33, 41.05, 42.81, 50.16, 65.02, 66.88, 72.34, 111.48, 170.84 ppm, m/z : 342.2430 $[M+1]^+$, 341.2394 $[M]^+$.

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6β-ol-13-acetate (8)

A solution of the diol **5** (78.5 mg, 0.263 mmol) in acetic anhydride (1.0 mL) and pyridine (2.0 mL) was stirred at room temperature for 1 h. After removal of the solvent by evaporation *in vacuo*, the residue was purified by silica gel column chromatography (Hexane : EtOAc = 4:1) to give **8** as a colorless oil (72.6 mg, 81.1%) : IR (KBr) : 3521, 1731 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) : δ 0.91 (3H, d, J = 3.9 Hz, > CH-C$\underline{H}_3$), 1.00 (3H, d, J = 4.2 Hz, > CH-C$\underline{H}_3$), 1.09 (3H, s, -CH_3), 2.06 (3H, s, COC$\underline{H}_3$), 3.95 (4H, m, O$\underline{CH_2CH_2}$O), 4.02 (H, dd, J = 3.0, 6.2 Hz, C$\underline{H}$OH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 9.85, 15.78, 19.37, 20.38, 21.00, 31.11, 32.96, 34.13, 36.67, 40.27, 41.26, 45.26, 50.10, 64.94, 65.03, 67.83, 68.34, 111.81, 171.26 ppm, m/z : 342.2435 $[M+1]^+$, 341.2399 $[M]^+$.

Biology

HL-60 cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium supplemented with 10% FBS (Gibco BRL). DAAS and its derivatives were dissolved in dimethyl sulfoxide to make stock solutions of 100 μg/mL each. These solutions were diluted at least 1000-fold in the growth medium, such that the final concentration of dimethyl sulfoxide would exert no effects on the differentiation and proliferation of HL-60 cells. *p*-Nitroblue tetrazoliumchloride was purchased from the USB Co. 1,25-$(OH)_2D_3$, all-*trans* retinoic acid (ATRA) and other reagents were purchased from the Sigma Chemical Co.

Determination of cell differentiation

The degree to which the HL-60 cells had differentiated was assessed via nitro blue tetrazolium reduction assay. This assay is based on the ability of phagocytic cells to generate superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested via centrifugation, and then incubated with an equal volume of 0.1% NBT dissolved in phosphate buffered saline containing 200 ng/mL of freshly diluted PMA at 37°C for 30 min in dark. Cytospin slides were prepared and examined for blue-black nitro blue diformazan deposits, considered to be indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed in each of the experiments.

Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay. Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μL of MTT (5 mg/mL) was added to each well in 96-well plates. After incubation for 4 h at 37°C, the crystals of viable cells were dissolved with 100 μL of 0.04 N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic XL flow cytofluorograph equipped with a multi-parameter data acquisition and display system. Briefly, a single-cell suspension was collected from the various cultures and washed twice with ice-cold phosphate buffered saline (pH 7.4). Afterwards, phycoerythrin (PE)-conjugated anti-human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (Becton Dickinson) were added, followed by incubation at 4°C for 1 h. After incubation, the cells were washed with PBS and were fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was performed. Background staining was determined by staining the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. One parameter fluorescence histograms were generated by analyzing at least 1×10^4 cells.

Statistical analysis

The results are expressed as the mean ± S.E. of three independent experiments. Student's t-test was used to determine statistical significance of difference between values for various experimental and control groups.

RESULTS AND DISCUSSION

Synthesis of C_6-epimer derivatives of DAAS

To make C_6-epimer derivatives of DAAS, the diol derivative **1** obtained from the santonin was used as a starting material. After the primary OH group of diol **1** was protected with TBDMSCl, secondary hydroxy group of the compound was oxidized by oxalyl chloride and DMSO to give ketone derivative **3**. The compound

3 was reduced by DIBALH to give the 6β-ol **4** selectively. After removing the protecting group from the compound **4**, the diol **5** was acetylated to give diacetate **6** or monoacetate **8** according to reaction temperature, respectively**.** Also partial hydrolysis of **6** by alkali solution produced **7**. The nuclear Overhauser enhancement (NOE) experiments with **1** and **5** confirmed these assignments. As expected, NOE enhancements were found between the cis-oriented hydrogens. Upon irradiation of C_4-CH_3, weak NOE patterns were observed at the proximal hydrogens of compound **5** [C_6-C*H*-] *vs* those of compound **1** [C_6-C*H*-].

Effect of DAAS derivatives on HL-60 cell differentiation

To determine the effect of DAAS derivatives on HL-60 cell differentiation, the HL-60 cells were treated with 100 μg/mL of each of the DAAS derivatives, and the numbers of differentiated cells, as measured by nitroblue tetrazolium (NBT) positivity, were determined. As a control, the cells were treated with media alone. As shown in Fig. 2A, **7** by itself strongly enhanced the degree of cell differentiation in a concentration-dependant manner. The effects were maximal at 100 μg/mL of **7**, with greater than 71% of the treated cells attaining a differentiated state. For all treatment except **6**, cells' viability was greater than 95% throughout the incubation period. The compound **6** is highly toxic to HL-60 cells, as demonstrated by the trypan blue exclusion assay (data not shown).

Cytofluorometric analysis of ATRA and sesquiterpene lactone-treated HL-60 cells was performed using fluorophore-conjugated antibodies for two cell surface antigens, CD11b and CD14, which are differentially expressed on myeloid cells differentiating into granulocytic and monocytic lineages. CD11b a general myeloid differentiation marker, is expressed on both monocytes and granulocytes (Kansas et al., 1990), while CD14 is a specific monocytic differentiation marker (van der Schoot et al., 1987). As shown in Fig. 3 (*upper panels*), treatment with **7** resulted in an increase in the number of CD11b-positive cells exhibiting high fluorescence intensities. In contrast, the treated cells showed little staining with anti-CD14 monoclonal antibody (Fig. 3, *bottom panels*). These results indicate that the effective **7** stimulated HL-60 cell differentiation along the granulocytic pathway.

SAR of C_6-epimer derivatives of DAAS on HL-60 cell differentiation

We earlier reported that activity order was DAAS (Diacetyl) > C_{13}-monoacetyl >> C_6-monoacetly ≈ Diol (**1**) in DAAS derivatives (Kim et al., 2008a). But in its C_6-epimer series, activity order showed some different fashion from DAAS. First the diol, **5** also has considerable action whereas its epimer **1** showed little action in DAAS series. Interestingly C_6-monoacetyl, **7** was the most active in HL-60 cell differentiation whereas its 6-epimer did not show any action. Unexpectedly, the C_6-epimer of DAAS showed cytotoxic action in 100 μg/mL. Accordingly, C_6 β-OH of C_6-Epmer series seems to be more effective in drug-receptor interaction than C_6 α-OH of DAAS series. Monoacetylation of its 6β hydroxyl group is required for optimal activity but acetylation of C_{13} -OH group seems not favorable in activity.

Effect of the compound 7 on 1,25-$(OH)_2D_3$- and all-*trans* RA-induced HL-60 cell differentiation

A major goal in the treatment of acute myeloid leukemia (AML) is to achieve terminal differentiation and to prevent drug resistance and side effects.

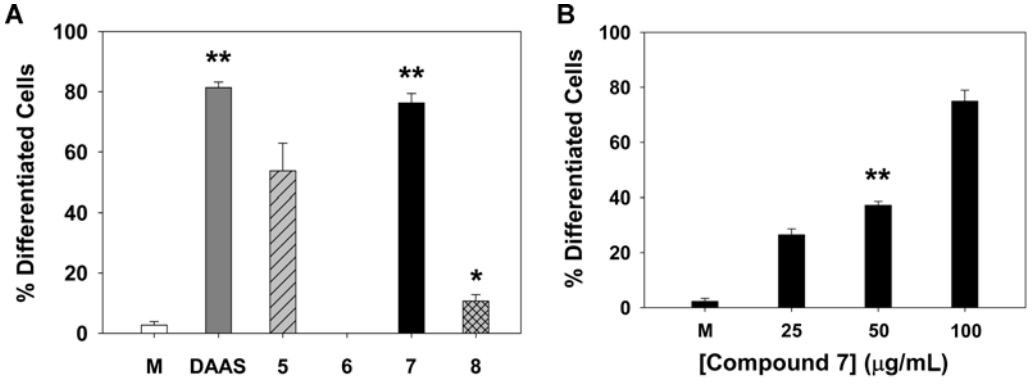


Fig. 2. Effects of compound **7** on HL-60 leukemia cell differentiation. HL-60 leukemia cells were incubated for 72 h with media alone, 100 μg/mL of DAAS derivatives, or with various concentrations of **7**. Afterward, the cellular differentiation was determined by the NBT reduction assay. Each of the value represents the mean ± S.E. (n = 3), *$p < 0.01$, **$p < 0.001$; relative to an untreated group (M).

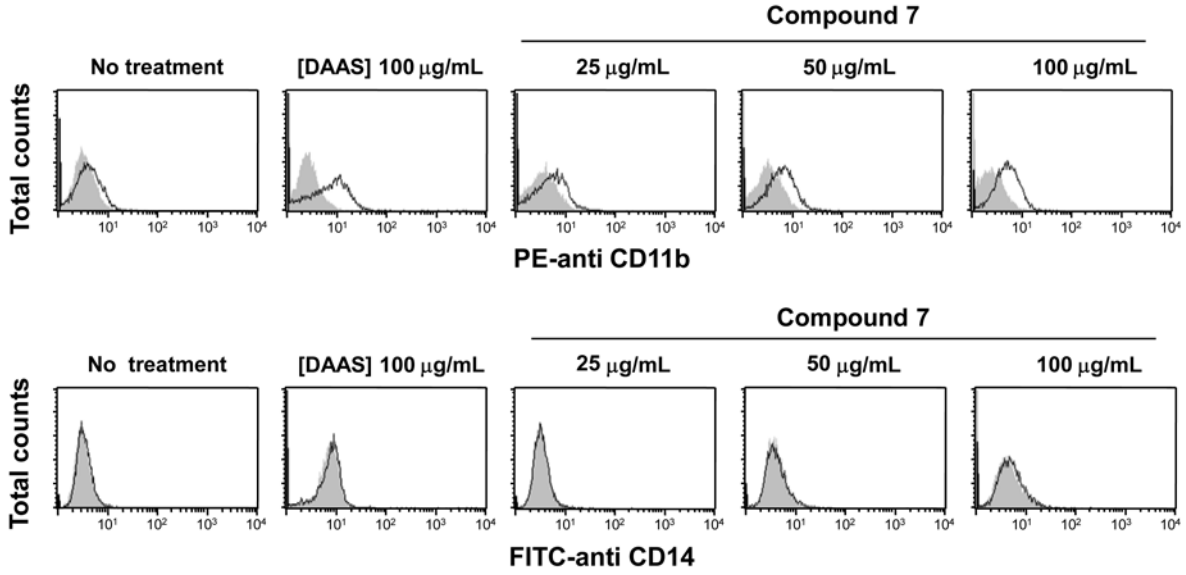


Fig. 3. Cytofluorometric analysis of **7**-induced HL-60 cell differentiation using mAbs for a differentiation marker CD11b and a monocytic marker CD14. HL-60 cells were treated for 72 h with media alone, or with various concentrations of **7**, after which cytofluorometric analysis was performed using PE-conjugated anti-CD11b mAb or FITC-conjugated anti-CD14 mAb (unshaded area), or each isotype control mAb (shaded area). The data are representative of three independent experiments.

Combined treatment with low doses of ATRA or 1,25-$(OH)_2D_3$ that do not induce toxicity with another drug is one useful strategy for the treatment of AML. To determine the effect of **7** on ATRA- and 1,25-$(OH)_2D_3$-induced cell differentiation, HL-60 cells were treated with **7** in combination with either low (nontoxic) doses of ATRA (50 nM) or 1,25-$(OH)_2D_3$ (5 nM), and cellular differentiation was assessed by the NBT assay. As shown in Fig. 4, **7** strongly potentiated ATRA- and 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation in a concentration-dependent manner.

In the present study we have demonstrated that **7**, C_6-epimer derivative of DAAS, potentiates 1,25-$(OH)_2D_3$- and ATRA-induced differentiation in HL-60 promyelocytic leukemia cells that are widely used as a model system for differentiation studies. HL-60 cells were synergistically differentiated into monocytes or granulocytes when treated with the C_6-epimer **7** in combination with either 1,25-$(OH)_2D_3$ or ATRA. Many previous studies have shown some chemical combinations which exerted an additive or synergistic effect on HL-60 cell differentiation. These combinations include plant-derived compounds and ATRA or 1,25-$(OH)_2D_3$ (Kim et al., 2008b), arsenic trioxide and ATRA (Wang and Chen, 2008), COX inhibitors and 1,25-$(OH)_2D_3$ (Jamshidi et al., 2008), histone deacetylase inhibitors and RA (Savickiene et al., 2006), and AM-80 and ATRA (Ishida et al., 2004).

The mechanism by which **7** potentiates 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation is not clear. However, it is believed that 1,25-$(OH)_2D_3$ and ATRA mediate biological responses such as cell differentiation as a result of their interaction with nuclear receptors to regulator gene transcription (Si et al., 2007) and with a putative cell membrane receptor to

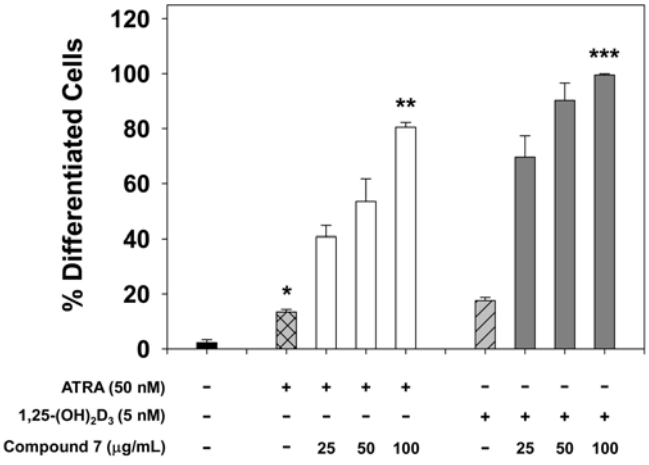


Fig. 4. Enhancing effects of compound **7** on HL-60 cell differentiation in combination with 1,25-$(OH)_2D_3$ or ATRA. HL-60 cells were treated for 72 h with media alone, 50 nM ATRA or 5 nM 1,25-$(OH)_2D_3$ alone, or with 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA in the presence of various concentrations of **7**. Afterward, the cellular differentiation was determined by the NBT reduction assay. Each of the values represents the mean ± S.E. (n = 3). *$p < 0.001$, relative to the untreated group; **$p < 0.001$, relative to the group treated with 50 nM ATRA alone; ***$p < 0.001$, relative to the group treated with 5 nM 1,25-$(OH)_2D_3$ alone.

generate rapid non-genomic effects, including the opening of voltage-gated calcium and chloride channels (Norman et al., 2002) and the activation of protein kinase C, mitogen-activated protein kinase and phosphatidylinositol 3-kinase (Pan et al., 1997; Marcinkowska et al., 2006; Hughes et al., 2008).

1,25-$(OH)_2D_3$ and some of its analogues are also used for the treatment of psoriasis (Mathieu and Jafari, 2006). ATRA has been used for the treatment of leukemia patients (Degos and Wang, 2001). The results presented here suggest that treatment of patients with combinations of **7** and 1,25-$(OH)_2D_3$, or **7** and ATRA may produce a greater therapeutic response than 1,25-$(OH)_2D_3$ or ATRA alone, possibly with less toxicity.

In conclusion, **7**, a C_6-epimer derivative of DAAS, potentiates 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation. These results suggest a possible use of the C_6-epimer derivatives of DAAS in the treatment of leukemic diseases.

ACKNOWLEDGEMENTS

This study was financially supported by Chonnam National University in 2008. We would like to thank to staffs of the Korea Basic Science Institute for NMR and Mass measurements.

REFERENCES

Beere, H. M. and Hickman, J. A., Differentiation: a suitable strategy for cancer chemotherapy. *Anticancer Drug Des.*, 8, 299-322 (1993).

Breitman, T. R., Selonick, S. E., and Collins, S. J., Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. U. S. A.*, 77, 2936-2940 (1980).

Chomienne, C., Balitrand, N., Cost, H., Degos, L., and Abita, J. P., Structure-activity relationships of aromatic retinoids on the differentiation of the human histiocytic lymphoma cell line U-937. *Leuk. Res.*, 10, 1301-1305 (1986).

Degos, L. and Wang, Z. Y., All *trans* retinoic acid in acute promyelocytic leukemia. *Oncogene*, 20, 7140-7145 (2001).

Frankel, S. R., Eardley, A., Lauwers, G., Weiss, M., and Warrell, R. P. Jr., The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann. Intern. Med.*, 17, 292-296 (1992).

Hershberger, P. A., Yu, W. D., Modzelewski, R. A., Rueger, R. M., Johnson, C. S., and Trump, D. L., Calcitriol (1,25-dihydroxycholecalciferol) enhances paclitaxel antitumor activity in vitro and in vivo and accelerates paclitaxel-induced apoptosis. *Clin. Cancer Res.*, 7, 1043-1051 (2001).

Hughes, P. J., Lee, J. S., Reiner, N. E., and Brown, G., The vitamin D receptor-mediated activation of phosphatidylinositol 3-kinase (PI3Kalpha) plays a role in the 1alpha, 25-dihydroxyvitamin D_3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cell lines. *J. Cell. Biochem.*, 103, 1551-1572 (2008).

Ishida, S., Shigemoto-Mogami, Y., Shinozaki, Y., Kagechika, H., Shudo, K., Ozawa, S., Sawada, J., Ohno, Y., and Inoue, K., Differential modulation of PI3-kinase/Akt pathway during all-trans retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis. *Biochem. Pharmacol.*, 68, 2177-2186 (2004).

Jamshidi, F., Zhang, J., Harrison, J. S., Wang, X., and Studzinski, G. P., Induction of differentiation of human leukemia cells by combinations of COX inhibitors and 1,25-dihydroxyvitamin D_3 involves Raf1 but not Erk 1/2 signaling. *Cell Cycle*, 7, 917-924 (2008).

Kang, S. N., Lee, M. H., Kim, K. M., Cho, D., and Kim, T. S., Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by silibinin: involvement of protein kinase C. *Biochem. Pharmacol.*, 61, 1487-1495 (2001).

Kansas, G. S., Muirhead, M. J., and Dailey, M. O., Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. *Blood,* 76, 2483-2492 (1990).

Kato, K., Hirata, Y., and Yamamura, S., Syntheses of shyobunone and related sesquiterpenes. *Tetrahedron*, 27, 5987-5992 (1971).

Kim, S. H., Song, J. H., Choi, B. G., Kim, H. J., and Kim, T. S., Chemical modification of santonin into a diacetoxy acetal form confers the ability to induce differentiation of human promyelocytic leukemia cells via the down-regulation of NF-kB DNA binding activity. *J. Biol. Chem.,* 281, 13117-13125 (2006a).

Kim, S. H., Chung, S. Y., Kim, T. S., and Choi, B. G., Synthesis of diacetoxy acetal derivatives of santonin and their enhancing effects on HL-60 leukemia cell differentiation. *Arch. Pharm. Res.,* 29, 40-45 (2006b).

Kim, S. H., Chung, S. Y., Kim, Y. W., Kim, T. S., and Choi, B. G., Synthesis of DAAS derivatives and their enhancement of HL-60 leukemia cell differentiation. *Arch. Pharm. Res.,* 31, 300-304 (2008a).

Kim, S. H., Danilenko, M., and Kim, T. S., Differential enhancement of leukaemia cell differentiation without elevation of intracellular calcium by plant-derived sesquiterpene lactone compounds. *Br. J. Pharmacol.*, 155, 814-825 (2008b).

Marcinkowska, E., Garay, E., Gocek, E., Chrobak, A., Wang, X., and Studzinski, G. P., Regulation of C/EBPbeta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D_3. *Exp. Cell Res.*, 312, 2054-2065 (2006).

Mathieu, C. and Jafari, M., Immunomodulation by 1,25-dihydroxyvitamin D_3: therapeutic implications in hemodialysis and renal transplantation. *Clin. Nephrol.*, 66, 275-283 (2006).

Norman, A. W., Bishop, J. E., Bula, C. M., Olivera, C. J., Mizwicki, M. T., Zanello, L. P., Ishida, H., and Okamura, W. H., Molecular tools for study of genomic and rapid signal transduction responses initiated by 1 alpha,25 $(OH)_2$-vitamin D_3. *Steroids*, 67, 457-466 (2002).

Pan, Q., Granger, J., O'Connell, T. D., Somerman, M. J., and Simpson, R. U., Promotion of HL-60 cell differentiation by 1,25-dihydroxyvitamin D_3 regulation of protein kinase C levels and activity. *Biochem. Pharmacol.*, 54, 909-915 (1997).

Savickiene, J., Treigyte, G., Borutinskaite, V., Navakauskiene, R., and Magnusson, K. E., The histone deacetylase inhibitor FK228 distinctly sensitizes the human leukemia cells to retinoic acid-induced differentiation. *Ann. N. Y. Acad. Sci.*, 1091, 368-384 (2006).

Si, J., Mueller, L., Schuler, A., Simon, J., and Collins, S. J., The retinoic acid receptor/CaMKII interaction: pharmacologic inhibition of CaMKII enhances the differentiation of myeloid leukemia cells. *Blood Cells Mol. Dis.*, 39, 307-315 (2007).

Sokoloski, J. A., Shyam, K., and Sartorelli, A. C., Induction of the differentiation of HL-60 promyelocytic leukemia cells by curcumin in combination with low levels of vitamin D_3. *Oncol. Res.*, 9, 31-39 (1997).

Tanaka, H., Abe, E., Miyaura, C., Shiina, Y., and Suda, T., 1(a),25-dihydroxyvitamin D_3 induces differentiation of human promyelocytic leukemia cells (HL-60) into monocytic macrophages, but not into granulocytes. *Biochem. Biophy. Res. Commun.*, 117, 86-92 (1983).

Van der Schoot, C. E., von dem Borne, A. E., and Tetteroo, P. A., Characterization of myeloid leukemia by monoclonal antibodies, with an emphasis on antibodies against myeloperoxidase. *Acta Haematol.*, 78, 32-40 (1987).

Wang, Z. Y. and Chen, Z., Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood*, 111, 2505-2515 (2008).