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Synthesis of Diacetoxy Acetal Derivatives of Santonin and their Enhancing Effects on HL-60 Leukemia Cell Differentiation

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Several diacetoxy acetal analogues have been synthesized from santonin and assessed for their ability of inducing or enhancing the differentiation of human HL-60 leukemia cells. The compounds themselves had little effect on HL-60 cell differentiation. However, three analogues, **2a**, **3a**, and **5b**, synergistically enhanced 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]-induced HL-60 cell differentiation when combined with 5 nM of dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], a well-known differentiation inducer. Especially, the compound **5b** profoundly enhanced the 1,25-$(OH)_2D_3$]-induced HL-60 cell differentiation.

Key words: Diacetoxyacetal, Santonin, Leukemia cell differentiation, Dihydroxyvitamin D_3

INTRODUCTION

Leukemia is a cancer that originates in the bone marrow and develops when a leukocyte undergoes transformation into malignant cells. Leukemia cells can be induced to undergo terminal differentiation by a variety of chemical and biological agents, indicating that the malignant state is not irreversible process. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cytodestructive agents (Beere *et al.*, 1993).

Human promyelocytic leukemia HL-60 cell culture has been employed as an excellent model system for studying cellular differentiation *in vitro*. HL-60 cells are differentiated into monocytic lineage when treated with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Breitman *et al.*, 1980; Tanaka *et al.*, 1983). 1,25-$(OH)_2D_3$ is currently under investigation as differentiating agents in a variety of tumor types and seem especially suited for clinical applications. 1,25-$(OH)_2D_3$ is a human hormone with a known physiological function in the control of calcium homeostasis. Differentiation and apoptosis-inducing effects of 1,25-$(OH)_2D_3$ have been demonstrated in neoplastic cells established in culture from these and other tissues (Studzinski *et al.*, 1995), showing that under appropriate conditions, 1,25-$(OH)_2D_3$ can indeed control the growth of these cells. The well-known limitation to the therapeutic use of 1,25-$(OH)_2D_3$ is its hypercalcemic effect. When the hypercalcemia is sufficiently prolonged and severe, widespread calcifications take place in tissues. Current attempts to overcome this problem focus on the combination therapy with nonhypercalcemic concentrations of 1,25-$(OH)_2D_3$ and 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).

Recently, several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic and anti-inflammatory activity (Hehner *et al.*, 1999; Ohnishi *et al.*, 1997). Cytostatic and cytocidal effects of sesquiterpenes against tumor cells have also been reported (Hall *et al.*, 1988; Ross *et al.*,1999).

In this report, we synthesized some analogues of diacetoxy acetal (**1**) and investigated not only their biological effects on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system, but also the effects of combinations of santonin derivatives with 1,25-$(OH)_2D_3$ on HL-60 cell differentiation.

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MATERIALS AND METHODS

Chemistry

Melting points were determined on a Mel-temp II laboratory device and are uncorrected. Nuclear magnetic resonance (NMR) data for ^{1}H-NMR were taken on Varian UNITY *plus* 300 spectrometers and are reported in δ ppm downfield from tetramethylsilane (TMS). The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet. IR spectra (IR) were determined neat or KBr disks on the Jasco FT-IR instrument and are reported in reciprocal centimeters. Thin layer chromatography (TLC) was carried out using precoated plates with silica gel 60F 254 purchased from Merck.

Synthesis of santonin derivatives

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-ol (2)

A solution of (11*S*)-3,3-(ethylenedioxy)eudesmano-13,6-a-diacetate (Simonovic *et al.*, 1963; Kato *et al.*, 1971) (**1**, 7 g, 119.58 mmol) in 5% methanolic KOH (40 mL) was stirred at room temperature for 5 min and then poured into water. The floating solid was extracted with ether. The ethereal solution was washed with water and sat. NaCl aqueous solution and dried over $MgSO_4$. After removal of the solvent the residue was recrystallized from ether-*n*-hexane to give the compound **2** (4.04 g, 60.3%): m.p. 155-158°C (Kato *et al.*, 1971, m.p. 150-152°C); IR(KBr) cm^{-1}: 3539, 1720, 1256; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.82 (3H, d, *J* = 6.6, >CH-C<u>H</u>$_3$), 0.85 (3H, d, *J* = 6.9, >CH-C<u>H</u>$_3$), 0.91 (3H, s, -C-C<u>H</u>$_3$), 2.02 (3H, s, $COCH_3$), 3.46 (2H, d, *J* = 7.2, CH_2OH), 3.94 (4H, m, OCH_2CH_2O), 4.97 (1H, dd, $J_1 = J_2$ =10.2, <u>CH</u>OAc).

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-ethanoate (2a)

A solution of the hydroxy-monoacetate (**2,** 0.2 g, 0.6 mmol) in propionic anhydride (1.17 g, 9 mmol) and pyridine (5 mL) was heated at 100 °C over night and extracted with large amounts of ether. The ethereal solution was washed with sat. $NaHCO_3$, water, 2N-HCl, NaCl solution and dried over $MgSO_4$. Removal of the solvent gave the compound **2a** (0.23 g, 96%) : IR(KBr) cm^{-1}: 1735; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.83 (3H, d, *J* = 7.2 Hz, >CH-C<u>H</u>$_3$), 0.87 (3H, d, *J* = 6.9 Hz, >CH-C<u>H</u>$_3$), 0.92 (3H, s, -C-C<u>H</u>$_3$), 1.14 (3H, dd, J_1 = 7.2 Hz, J_2 = 7.8 Hz, $COCH_2C\underline{H}_3$ 2.01 (3H, s, $COCH_3$), 2.32 (2H, q, J_1 = 7.5, $CO\underline{CH}_2CH_3$), 3.95 (6H, m, $O\underline{CH}_2\underline{CH}_2O$, $\underline{CH}_2OCO$), 4.95 (1H, dd, J_1 = 10.05, J_2 = 10.2, <u>CH</u>OAc).

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-benzoate (2b)

Compound **2b** was synthesized from the hydroxy-monoacetate (**2**) in benzoic anhydride and pyridine using method described for compound **2a** (yield 53.6%): m.p. 180-181 °C; IR(KBr) cm^{-1}: 1714; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.84 (3H, d, *J* = 3.3, >CH-C<u>H</u>$_3$), 0.93 (3H, s, -C-CH_3), 0.97 (3H, d, *J* = 6.9, >CH-C<u>H</u>$_3$), 2.02 (3H, s, $COCH_3$), 3.95 (4H, m, OCH_2CH_2O), 4.15 (2H, m, CH_2O), 5.00 (1H, dd, $J_1 = J_2$ = 10.2 Hz, <u>CH</u>OAc), 7.56(3H, m, arom), 8.02 (2H, m, arom).

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-chloroformate (3)

Phosgen solution (2 mL, 1.47 mmol) was added to a solution of **2** (0.5 g, 1.47 mmol) in dry toluene 1 mL at 0°C. The solution was gently shaken at 0°C for 30 min and at room temperature for 2 h. The solution was concentrated under reduced pressure at a temperature not exceeding 60°C and afforded 0.55 g (yield 93%) of brown oil: IR (KBr) cm^{-1}: 1776, 1730; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, d, *J* = 6.9, >CH-C<u>H</u>$_3$), 0.93 (3H, d, *J* = 7.2, >CH-C<u>H</u>$_3$), 0.97 (3H, s, -C-C<u>H</u>$_3$), 2.08 (3H, s, $COCH_3$), 3.93 (4H, m, OCH_2CH_2O), 4.13 (2H, m, CH_2OCO), 5.07 (1H, dd, $J_1 = J_2$ = 10.5, <u>CH</u>OAc).

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-carbamate (3a)

A solution of the chloroformate (**3**, 0.9 mmol) was added to ammonia water (0.9 mL, 4.5 mmol) slowly and with vigorous stirring. The reaction mixture allowed elevating to RT for 2 h. After removal of the solvent the residue was purified by silica gel column chromatography (Hexane: EtOAc=5:1) to give white solid **3a** (0.27 g, 67.3%): m.p. 131-132°C; IR (neat) cm^{-1}: 3446, 3360, 1723; ^{1}H-NMR (300 MHz , $CDCl_3$): d 0.83 (3H, d, *J* = 6.6, >CH-C<u>H</u>$_3$), 0.87 (3H, d, *J* = 6.9, >CH-C<u>H</u>$_3$), 0.92 (3H, s, -C-C<u>H</u>$_3$), 2.02 (3H, s, $COCH_3$), 3.94 (6H, m, OCH_2CH_2O, CH_2OC), 4.66 (2H, s, $CONH_2$), 4.95 (1H, dd, $J_1 = J_2$ = 10.2, <u>CH</u>OAc).

(11*S*)-3,3-(Ethylenedioxy)eudesmano-6-acetate-13-benzoyl-carbamate(3b)

Compound **3b** was synthesized from the chloroformate(**3**) and benzylamine using method described for compound **3a** (yield 35.97%, white oil): IR(KBr) cm^{-1}: 3348, 1726; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.84 (3H, d, *J*=3.3, >CH-C<u>H</u>$_3$), 0.93 (3H, s, -C-CH_3), 0.97 (3H, *d*, *J*=6.9, >CH-C<u>H</u>$_3$), 2.02 (3H, s, $COCH_3$), 3.85-4.20 (6H, m, OCH_2CH_2O, CH_2O), 4.97 (1H, t, *J*=10.2, <u>CH</u>OAc), 7.43-8.05 (5H, m, arom).

(11*S*)-3,3-(Ethylenedithioxy)eudesmano-13,6α-diol (4)

The compound **4** was synthesized according the literature (Shibata *et al.*, 1986) from the tetrahydrosantonin (yield 32.12%): m.p. 185-186°C (Shibata *et al.*, m.p. 184-186°C); IR (KBr) cm^{-1}: 3401, 2921; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.86 (3H, s, CH_3), 1.35 (3H, d, *J* = 6.6, >CH-C<u>H</u>$_3$), 1.43 (3H, d, *J* = 6.9, >CH-C<u>H</u>$_3$), 3.21 (4H, m, SCH_2CH_2S),

3.50 (3H, m, CHOH, CH_2OH).

(11*S*)-3,3-(Ethylenedithioxy)eudesmano-13,6α-diacetate (4a)

Compound **4a** was synthesized from the diol **4** and Ac_2O and pyridine using method described for compound **2a** (yield 49.5%): m.p. 104-105°C; IR(KBr) cm^{-1}: 1736, 1721; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, d, *J* = 6.9, >CH-CH_3), 0.91 (3H, s, CH_3), 1.14 (3H, d, *J* = 6.6, >CH-CH_3), 2.03 (3H, s, $COCH_3$), 2.06 (3H, s, $COCH_3$), 3.20 (4H, m, SCH_2 CH_2S), 3.89 (2H, dd, J_1 = 3.3, J_2 = 4.2, CH_2OAc), 4.92 (1H, dd, J_1 = J_2 = 10.05, CHOAc).

(11*S*)-3,3-(Propylenedioxy)eudesmano-13,6α-lactone (5)

A mixture of TS (2 g, 7.99 mmol), neopentyl glycol (18.6 g, 178.58 mol) and *p*-toluenesulfonic acid (0.08 g, 0.42 mmol) in dry benzene was refluxed in a flask equipped with a Dean-Stark column for 3 h. Subsequently, the reaction mixture was cooled and washed with sodium bicarbonate solution. The benzene layer was separated and washed with saturated NaCl solution and dried over $MgSO_4$. The residue was purified by column chromatography (Hexane: EtOAc=8:1) to give compound **5** (1.52 g, 69.9%): m.p. 164-165°C; IR (KBr) cm^{-1} :1769; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, s, CH_3), 0.98 (3H, s, CH_3), 1.19 (3H, d, *J* = 6.6, CH_3), 1.23 (3H, d, *J* = 6.6, >CH-CH_3), 3.32 (2H, m, OCH_2C), 3.71 (3H, m, OCH_2C, CHO).

(11*S*)-3,3-(Propylenedioxy)eudesmano-13,6α-diol (5a)

The lactone **5** (1.88 g, 5.59 mmol) dissolved in dry THF (5 mL) was added carefully under stirring to a mixture of lithium aluminum hydride (0.42 g, 11.17 mmol) and dry THF. The reaction mixture was stirred and refluxed for 3 h and then decomposed in the usual way with ethyl acetate and H_2O. The solvent was removed and residue was extracted with ether to obtain crystalline crude product. Crystallization from MeOH gave 1.47 g (yield 77.8%) of the diol m.p. 145-146°C; IR (KBr) cm^{-1}: 3378; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, s, CH_3), 0.85 (3H, s, CH_3), 0.89 (3H, d, *J* = 7.2, >CH-CH_3), 1.18 (3H, s, CH_3), 1.33 (3H, d, *J* = 6.6, >CH-CH_3), 3.31 (2H, m, CH_2OC), 3.47(2H, m, CH_2OH), 3.58 (1H, dd, J_1 = 6.0, J_2 = 10.8, CHOH), 3.71 (2H, dd, J_1 = 11.55, J_2 = 45.6, CH_2OC).

(11*S*)-3,3-(Propylenedioxy)eudesmano-13,6α-diacetate (5b)

Compound **5b** was synthesized from the diol **5** and Ac_2O and pyridine using method described for compound **2a** (yield 55.2%); IR (KBr) cm^{-1}: 1731; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, s, CH_3), 0.87 (3H, d, *J* = 6.9, >CH-CH_3), 0.90 (3H, s, CH_3), 1.03 (3H, d, *J* = 6.6, >CH-CH_3), 1.18 (3H, s, CH_3), 2.03 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 3.32 (1H, d, *J* = 11.7, CH_2OC), 3.52 (1H, s, CH_2OC), 3.70 (2H, dd, J_1 = 11.25, J_2 = 44.7, CH_2OC), 3.90 (2H, m, CH_2OAc), 4.95 (1H, dd, J_1 = 10.95, J_2 = 10.2, CHOAc).

Biology

HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.). The synthetic compounds were dissolved in dimethylsulfoxide to make a stock solution of 100 mg/mL. The solutions were diluted at least 1000-fold in the growth medium, such that the final concentration of dimethylsulfoxide had no effect on the differentiation and proliferation of HL-60 cells.

Determination of cell differentiation

HL-60 cell differentiation was assessed by the nitroblue tetrazolium reduction assay as previously described (Collins *et al.*, 1979). This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS containing 200 *n*g/mL of freshly diluted PMA at 37°C for 30 min in the dark. Cytospin slides were prepared and were examined for blue-black nitroblue diformazan deposits, indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

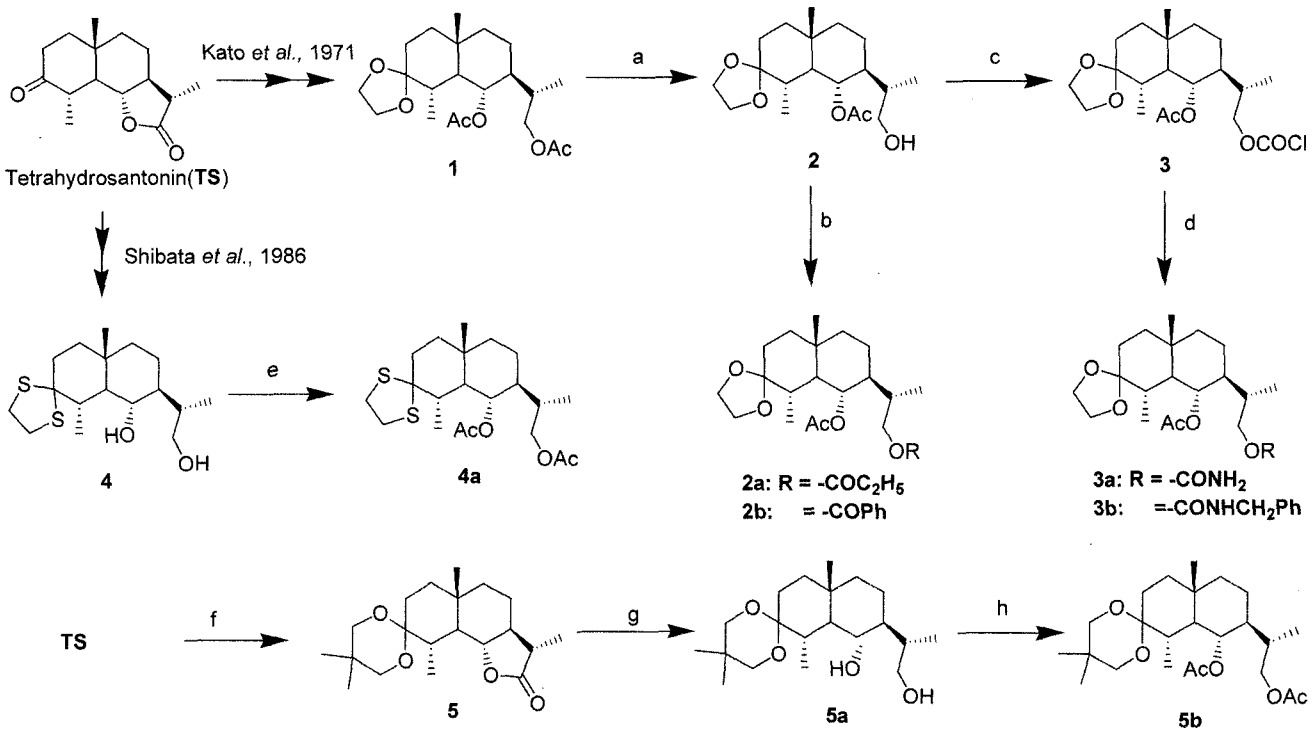
Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between values for various experimental and control groups. A *P* value of <0.05 was considered as significant.

RESULTS AND DISCUSSION

Chemistry

As shown in Scheme 1, the diacetoxy acetal derivative (**1**) was readily synthesized from the santonin using the previous reported procedure (Ando *et al.*, 1987: Kato *et al.*, 1971). Partial hydrolysis of the compound **1** using 5% KOH gave monoacetoxy acetal (**2**), which was esterified using propionic anhydride or benzoic anhydride and pyridine to give propionic ester (**2a**) and benzoic ester (**2b**). Monoacetoxy acetal (**2**) was transformed to chloroformate (**3**), which was sequentially carbamylated using addition of NH_4OH to give the carbamate (**3a**), having NH_2 group instead of CH_3 group of diacetoxy acetal (**1**). Dithioacetal (**4**), a sulfur analogue of the compound **2** was synthesized from thioketalization of tetrahydrosantonin



Reaction and reagents
a) 5%-KOH b): i) propionic anhydride/pyridine, Rt. ii) benzoic anhydride/pyridine c) $COCl_2$ d): i) NH_4OH, ii) benzylamine
e) Ac_2O/pyridine f) 2.2-dimethylpropanediol, TsOH g) $LiAlH_4$ h) Ac_2O/pyridine

Scheme 1. Synthetic pathway of target compound

using ethane dithiol and toluenesulfonic acid, and sequentially its reduction by lithium aluminum hydride, and acetylation by Ac_2O and pyridine gave (11*S*)-3,3-(ethylenedithioxy)-eudesmano-13,6α-diacetate (**4a**). Similarly diacetoxy acetal using 2,2-dimethylpropane diol instead of ethylene glycol *via* acetalization, reduction by $LiAlH_4$ and acetylation was synthesized according to the method as described above.

Effect of the synthesized santonin derivatives on HL-60 cell differentiation

HL-60 cells were seeded at a density of 2×10^5 cells/mL, and the cells were treated with medium alone, or treated for 72 h with 20 μg/mL of each of the diacetoxy acetal analogues. As shown in Table I, the treatment with 20 μg/mL of compound **1** induced HL-60 cell differentiation approximately by 30.9%, whereas the other derivatives had little or no effect on cell differentiation.

In order to whether or not the santonin derivatives enhance HL-60 cell differentiation when combined with low doses of 1,25-$(OH)_2D_3$, HL-60 leukemia cells were treated with various concentrations of synthetic compounds in combination with 5 nM of 1,25-$(OH)_2D_3$, and the cellular differentiation was assessed by a nitroblue tetrazolium reduction assay. As shown in Fig. 1, the addition of the compounds **1**, **2a**, **3a**, and **5b** to cultures exposed to a suboptimal concentration of 1,25-$(OH)_2D_3$ (5nM), which by itself caused a relatively low level of differentiation, resulted in a marked increase in the degree of cell differentiation, whereas the compounds **2b** and **4a** had no effects on leukemia cell differentiation. Especially, the compound **5b** significantly enhanced 1,25-$(OH)_2D_3$-induced cell differentiation. The compounds **2a**, **3a**, or **5b** synergistically enhanced 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation, though the compounds themselves had little effect on HL-

Table I. Effects of santonin derivatives on HL-60 cell differentiation. HL-60 cells were treated for 72 h with medium alone (M) or with 20 μg/mL of santonin derivatives. The cell differentiation was assessed by the NBT assay. Each value represents the mean ± s.e. mean (n=3).

Compound (20 μg/mL)	% Differentiated cells
	Mean ± s.e. mean
M	3.0 ± 1.5
1	*30.9 ± 2.8
2a	9.3 ± 2.0
2b	2.1 ± 0.3
3a	10.6 ± 1.6
3b	ND[a]
4a	5.6 ± 0.6
5b	17.3 ± 1.8

*$P<0.001$, relative to an untreated group (M). [a]ND, not detected.

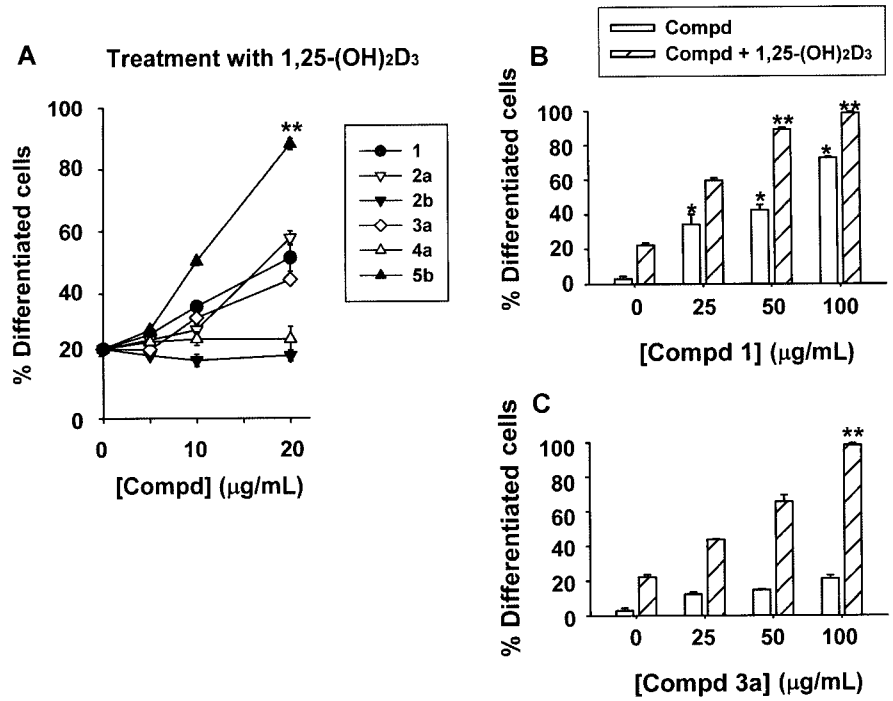


Fig. 1. Effects of diacetoxy acetal analogues on 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation. HL-60 cells were treated for 72 h with 5 nM 1,25-$(OH)_2D_3$ in combination with various concentrations (0-20 μg/mL) of compounds **1**, **2a**, **2b**, **3a**, **4a**, and **5b** (*A*). HL-60 cells were treated for 72 h with 5 nM 1,25-$(OH)_2D_3$ alone or in combination with various concentrations (0-100 μg/mL) of compound **1**(*B*) or **3a**(*C*). The cellular differentiation was assessed by the NBT assay. Each value represents the mean ± s.e.mean (n=3). *$P<0.05$, relative to an untreated group. **$P<0.05$ relative to the 1,25-$(OH)_2D_3$ treated group.

60 cell differentiation. Moreover, as shown in Fig. 1B and C, despite of the similar potency of differentiation-inducing activity on 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation with the compound **1**, and the compound **3a** did not affect the activity in HL-60 cell differentiation.

Many previous studies have shown some chemical combinations, which exert an additive or synergistic effect on HL-60 cell differentiation (Hershberger *et al.*, 2001; Kang *et al.*, 2001; Sokoloski *et al.*, 1997). Here, we reported the combined effects of the diacetoxy acetal derivatives of santonin with 1,25-$(OH)_2D_3$ affects on HL-60 cell differentiation. The compounds **1**, **2a**, **3a**, and **5b** potentiated the induction of HL-60 cell differentiation treated with 1,25-$(OH)_2D_3$, whereas the compounds **2b** and **4a** had no effects on leukemia cell differentiation. The compound **5b** is a most effective enhancer of HL-60 cell differentiation when combined with 1,25-$(OH)_2D_3$.

The mechanism by which the compound **5b** potentiates 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation is not clear. However, the results presented here suggest that treatment of patients with combinations of **5b** and 1,25-$(OH)_2D_3$ may produce a greater therapeutic response than 1,25-$(OH)_2D_3$ alone, possibly with less toxicity. Clinical studies are needed to evaluate this possibility, especially at concentrations of the compound **5b** that do not have known side effects.

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