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-κB DNA Binding Activity

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Seung Hyun Kim†, Ju Han Song§, Bo Gil Choi§, Hyeoung-Joon Kim‡, and Tae Sung Kim†‡¶

From the †School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, the §College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, and the ¶Genome Research Center for Hematopoietic Diseases, Chonnam National University Hospital, Gwangju 501-757, Republic of Korea

Many sesquiterpene lactone compounds either induce or enhance the cell differentiation of human leukemia cells. However, we reported in a previous study that santonin, a eudesmanolide sesquiterpene lactone, exerts no effects on the differentiation of leukemia cells. In this report, to evaluate the possibility of chemically modifying santonin into its derivatives with differentiation inducing activity, we synthesized a series of santonin derivatives, and determined their effects on cellular differentiation in the human promyelocytic leukemia HL-60 cell system. A diacetoxy acetal derivative of santonin (DAAS) was found to induce significant HL-60 cell differentiation in a dose-dependent manner, whereas santonin in its original form did not. The HL-60 cells were differentiated into a granulocytic lineage when exposed to DAAS. In addition, the observed induction in cell differentiation closely correlated with the levels of NF-κB DNA binding activity inhibited by DAAS. Both Western blot analyses and kinase inhibitor studies determined that protein kinase C, ERK, and phosphatidylinositol 3-kinase were upstream components of the DAAS-mediated inhibition of NF-κB binding activity in HL-60 leukemia cells. The results of this study indicate that santonin can, indeed, be chemically modified into a derivative with differentiation inducing abilities, and suggest that DAAS might prove useful in the treatment of neoplastic diseases.

The majority of cancer cells exhibit deficiencies with regard to their capacity to mature into non-replicating adult cells, thereby remaining in a highly proliferative state. This results in their characteristic tendency to outgrow their normal cellular counterparts. The induction of terminal differentiation represents an alternative approach to the treatment of cancer using conventional anti-neoplastic agents, as cells exposed to chemical or biological differentiation inducers do not undergo the cytodestruction produced by cytotoxic agents. Instead, these cells acquire the phenotypic characteristics of end-stage adult cell forms with no replicative capacity and, ultimately, undergo programmed cell death. Leukemia cells can be induced to undergo terminal differentiation by the administration of a variety of chemical and biological agents, indicating that the malignant state is not an irreversible process. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that caused by cytodestructive agents (1). Human promyelocytic leukemia HL-60 cells are differentiated into monocytic or granulocytic lineages when treated with 1,25-dihydroxyvitamin D3 or all-trans-retinoic acid, respectively (2–4). HL-60 cell cultures have previously been employed as an excellent model system for the in vitro study of cellular differentiation.

Several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic and anti-inflammatory activities (5, 6). The sesquiterpene lactones have also been reported to exert cytostatic and cytotoxic effects against tumor cells (7, 8). Santonin, a sesquiterpene lactone, is commonly found in plants belonging to the Compositae family. α-Santonin is known to be a potent anti-parasitic agent (9). In its α- and β-forms, santonin exhibits anti-pyretic activity in a fashion similar to that of dopamine (10). It is also known to be effective against several species of fungi via the inhibition of spore germination (11). It also exhibits an anti-inflammatory activity in acute inflammatory processes, reminiscent of the effects of non-steroidal anti-inflammatory drugs (12). Santonin derivatives have been shown to exert cytotoxic activity toward KB cells, a human epidermoid nasopharynx carcinoma (13). However, we determined, in a previous study (14, 15), that santonin did not induce the differentiation of HL-60 leukemia cells, although some other sesquiterpene lactones have been reported to induce significant cell differentiation in this cell line.

In this report, we have evaluated the possibility of chemically modifying santonin into derivatives exhibiting differentiation-inducing properties, and have also investigated their underlying mechanisms with regard to HL-60 cell differentiation. Here, we determined that a diacetoxy acetal derivative of santonin (DAAS) profoundly induced cell differentiation via the down-regulation of NF-κB binding activity involved by PKC, PI3K, and ERK.

EXPERIMENTAL PROCEDURES

Materials—The HL-60 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen). The Giemsa staining solution, methanol-free paraformaldehyde, and all other reagents used in this study were obtained from Sigma. Santonin and its derivatives were dissolved in dimethyl sulfoxide to make stock solutions of 100 mg/ml each. These solutions were

1 To whom correspondence should be addressed: School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea. Tel.: 82-2-3920-3416; Fax: 82-2-3290-3921; E-mail: tskim@korea.ac.kr.

2 The abbreviations used are: DAAS, diacetoxy acetal derivative of santonin; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; NBT, nitro blue tetrazolium; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; mAb, monoclonal antibody; JNK, c-Jun NH2-terminal kinase.
Inductive Effect of HL-60 Cell Differentiation by a Santonin Derivative

FIGURE 1. The chemical structures and effects of santonin and its derivatives on HL-60 cell differentiation. A, chemical structures of santonin and its derivatives. B, HL-60 cells were incubated for 72 h with medium alone (M), or with 100 μg/ml of santonin (ST) or its derivatives (compounds 1, 2, 3 and 4). Cell differentiation was assessed via NBT assay. Each value represents the mean ± S.E. (n = 3). *, p < 0.0001, relative to a group incubated with medium alone (M).

diluted by 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.

Synthesis of the Eudesmane Derivatives from Santonin—The eudesmane derivatives of santonin, as shown in Fig. 1A, were synthesized according to the procedure previously described (16, 17). In brief, (11S)-3-oxoeudesmano-13,6α-lactone derivative, which exists in the form of colorless prisms via the acetalization of (11S)-3-oxoeudesmano-13,6α-lactone, was used in the synthesis of colorless prisms via the acetalization of (11S)-3-oxoeudesmano-13,6α-lactone, using ethylene glycol and p-toluenesulfonic acid. The (11S)-3,3-(ethylenedioxy)eudesmano-13,6α-lactone derivative was then reduced to (11S)-3,3-(ethylenedioxy)eudesmano-13,6α-diol (m.p. 150–152 °C) using lithium aluminum hydride, which was acetylated using Ac2O and pyridine to yield the (11S)-3,3-(ethylenedioxy)eudesmano-13,6α-lactone derivative, which appeared as a white solid (m.p. 84–86 °C). The chemical structure of each of the synthesized compounds was identified by analysis of 1H NMR, fourier transform-infrared, and mass spectra.

Determination of Cell Viability and Proliferation—Cell viability was determined using the trypan blue exclusion assay, as previously described (18). Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium assay, as previously described (19). This assay is based on the ability of phagocytic cells to generate superoxide upon stimulation with PMA. For this assay, 2 × 10⁵ cells were harvested via centrifugation, and then incubated with an equal volume of 1% NBT dissolved in phosphate-buffered saline (PBS) containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in darkness. 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.

Morphologic Studies—Single-cell suspensions were prepared, and 2 × 10⁵ cells were loaded into a cyto-funnel and spun at 500 × g in a cytopsin centrifuge. The slides were then fixed with methanol and dried. The slides were stained for 20 min with Giemsa staining solution, and then rinsed in deionized water, air-dried, and observed under a microscope equipped with a camera. The stained cells were assessed with regard to size, regularity of the cell margin, and morphological characteristics of the nuclei.

Immunofluorescent Staining and Cytofluorometric Measurements—Quantitative immunofluorescence measurements were conducted using an Epic XL flow cytograph (Coulter Electronics, Hialeah, FL) equipped with a multiparameter data acquisition and display system, as previously described (20). In brief, single cell suspensions were collected from the various cultures and then washed twice with ice-cold PBS (pH 7.4). Afterward, phycoerythrin-conjugated anti-human CD11b or fluorescein isothiocyanate-conjugated anti-human CD14 monoclonal antibodies (BD Biosciences) were added, followed by incubation at 4 °C for 1 h. After incubation, the cells were washed with PBS and fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was conducted. Background staining was assessed by staining the cells with phycoerythrin- or fluorescein isothiocyanate-conjugated isotype control monoclonal antibodies. One parameter fluorescence histograms were generated by the analysis of at least 1 × 10⁶ cells.

Preparation of Cell Lysates and Western Blot Analysis—The total cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF)) by incubation on ice for 30 min. The total cell lysates were then centrifuged for 10 min at 13,000 × g at 4 °C. Nuclear and cytosolic extracts were prepared from...
the HL-60 cells, as previously described (21). The HL-60 cells were lysed by incubation at 4 °C for 10 min in buffer consisting of 10 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl$_2$, 0.5 mM dithiothreitol, and 0.2 mM PMSF. The cell lysates were centrifuged and the supernatants were stored at −70 °C as cytosolic extracts. After measurement of protein content, the pellets were resuspended in ice-cold buffer consisting of 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl$_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF. After incubation at 4 °C for 20 min, the extracts were centrifuged for 10 min, and the supernatants were collected, aliquoted, and stored at −70 °C as nuclear extracts. The proteins contained in 15 μg of the supernatants were separated out using 10% SDS-PAGE, and then transferred to nitrocellulose membranes using a semi-dry blotting apparatus. The membranes were blocked with 5% nonfat dried milk in TPBS (0.1% Tween 20 in PBS) and probed with appropriate primary and secondary antibodies diluted in TPBS. The immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation—The cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml PMSF) by incubation on ice for 30 min. The cell lysates were incubated overnight in
Protein A coupled with antibody to the p85 regulatory subunit of PI3K. The precipitated proteins were resolved via SDS-PAGE, and evaluated by Western blot analysis.

Protein Kinase C Activity Assay—The HL-60 cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 50 μg/ml aprotinin by incubation on ice for 30 min. The lysates were then centrifuged for 20 min at 14,000 × g at 4 °C. The proteins contained in 200 μg of the supernatants were then incubated for 2 h with PKC antibody at 4 °C and washed with lysis buffer. The antibody-coupled proteins were centrifuged for 1 min at 5,000 × g and allowed to react with 5 μg of myelin basic protein and 0.5 μl of [γ-32P]ATP in reaction buffer (0.5 mM EGTA, 10 mM MgCl2, 20 mM HEPES (pH 7.4), 50 mM ATP, 2 mM dithiothreitol, 2 mM NaF, and 2 mM sodium orthovanadate) for 30 min at room temperature. The reaction mixture was then analyzed via electrophoresis on a 15% SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from the cells, as previously described (22). An oligonucleotide containing an NF-κB-binding site within the Igκ chain (5′-CCGTT-TAACAGAGGGGCTTCGAG-3′) was utilized as a probe. The labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 μg of nuclear extracts, in 20 μl of binding buffer (10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI–dC), and 1 mM dithiothreitol). The reaction mixture was then analyzed via electrophoresis on a 4% polyacrylamide gel in 0.5 × Tris borate buffer. Specific binding was verified by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides, or CAMP response element-containing oligonucleotides. For supershift assay, 5 μg of nuclear extracts were incubated with antibodies against p50, p52, c-Rel, or p65 subunit of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA.

Overexpression of NF-κB p65—5 × 106 HL-60 cells were transfected with NF-κB p65 DNA or a control empty vector by Bio-Rad Gene Pulser. The voltage was set at 0.3 kV (field strength = 0.75 kV/cm) and the capacitor was set at 500 microfarads in a 0.2-cm gap disposable cuvette. After 24 h, the cells were treated for 24 h with 100 μg/ml of DAAS, or not treated as a control. The cells were collected and the degree of cell differentiation was measured by NBT reduction and cytofluorometric analyses. The expression level of the NF-κB p65 protein was evaluated in transfected HL-60 cells by Western blot analysis.

Statistical Analysis—Student’s t test and one-way analysis of variance followed by the Bonferroni method were employed to determine the statistical significance of differences between values for the various experimental and control groups. A p value of <0.01 was considered to be significant.

RESULTS

Effects of Santonin and Its Derivatives on the Differentiation of HL-60 Cells—HL-60 cells were seeded at a density of 2 × 105 cells/ml, and then treated for 72 h with medium alone, or with 100 μg/ml santonin or its derivatives, (11S)-3-oxoedusmano-13,6-lactone, (11S)-3,3′-(ethylenedioxy)edusmano-13,6-lactone, (11S)-3,3′-(ethylenedioxy)edusmano-13,6-diol, and (11S)-3,3′-(ethylenedioxy)edusmano-13,6-diacetate (DAAS) (Fig. 1A). As shown in Fig. 1B, treatment with 100 μg/ml DAAS induced HL-60 cell differentiation by ~71.9%, whereas neither the original form of santonin nor any of its other derivatives exhibited significant activity.

FIGURE 3. Involvement of PI3K in DAAS-induced HL-60 cell differentiation. A, HL-60 cells were treated for 40 min with various concentrations of PI3K inhibitors (LY 294002 or wortmannin), followed by incubation for 72 h with 100 μg/ml of DAAS. Cellular differentiation was assessed via the NBT assay. Each of the values represents the mean ± S.E. (n = 3). B, HL-60 cells were treated with 100 μg/ml of DAAS for the indicated times. The protein level of PI3K p85 was determined via Western blot analysis. C, HL-60 cells were treated for 10 min with medium alone (M), or with 100 μg/ml of DAAS or santonin (ST). Total p85 and phosphorylated p85 of PI3K were, respectively, determined via Western blot analysis. The experiment was repeated at least twice, with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-Tyr, phosphoryrosine.

To further determine the effects of DAAS on the differentiation of human leukemia cells, the HL-60 cells were treated with varying amounts of DAAS. As shown in Fig. 2A, DAAS significantly induced HL-60 cell differentiation in a dose-dependent manner. This DAAS-induced cell differentiation was verified by morphologic studies. As shown in Fig. 2B, the Giemsa-stained undifferentiated control HL-60 cells treated with the medium alone were identified predominantly as promyelocytes, exhibiting round and regular cell margins, large nuclei, and very little cytoplasm, suggesting that these cells were highly active in DNA synthesis and proliferated rapidly. The DAAS-treated cells exhibited morphological alterations, including irregular cell margins. As the concentrations of DAAS increased, we noted significant reductions in cell size, denser chromatin, and an increased cytoplasm to nuclear ratio, all of which suggested that the rate of DNA synthesis was decreasing. The DAAS-induced cell differentiation was also verified by cytofluorometric analysis, in which the expression of specific surface antigens on the HL-60 cells was assessed. CD11b is a cell surface marker for differentiation into either monocytes or granulocytes (23). As shown in Fig. 2C, DAAS treatment resulted in an increase in the number of CD11b-positive cells exhibiting high fluorescence intensities. These results clearly indicated that DAAS itself was an inducer of differentiation in the HL-60 cells.

To determine the differentiation pathway that HL-60 cells have taken after the DAAS treatment, the cells were incubated with 0–100 μg/ml of DAAS, after which cytofluorometric analysis was conducted, using mAb for the monocytic surface antigen CD14. The CD14 antibody reacts with a glycosyl phosphatidylinositol-anchored single chain glyco-
Inductive Effect of HL-60 Cell Differentiation by a Santonin Derivative

To investigate the reason why DAAS, not santonin, can induce the differentiation of HL-60 cells, we evaluated any involvement of several signaling molecules related to cell differentiation. First, previous studies have provided evidence that PI3K activity plays a crucial role in the differentiation of HL-60 cells (26, 27). To determine whether or not PI3K was involved in DAAS-induced HL-60 cell differentiation, HL-60 cells were treated with the specific PI3K inhibitors, 1-(5-isoquinolinesulfonyl)-2-(3-furo-4,3,2-de-indeno-4,5-h)-2-benzopyran-3,6,9-trione (wortmannin) or 2-(4-morpholino)-8-phenyl-1(4H)-1-benzopyran-4-one (LY294002), in the presence of DAAS. Both of these PI3K inhibitors were determined to exert a dose-dependent inhibitory effect on DAAS-induced HL-60 cell differentiation (Fig. 3A). To further characterize the involvement of PI3K in DAAS-induced HL-60 cell differentiation, the levels of p85, a regulatory subunit of PI3K, were determined. As shown in Fig. 3B, the levels of PI3K p85 were elevated after 10 min of treatment with DAAS. Furthermore, DAAS treatment also appeared to induce an increase in the levels of phosphorylated PI3K in HL-60 cells, whereas santonin itself did not. The PKC protein levels achieved peak levels between 10 and 30 min, followed by a gradual decrease. In addition, in an attempt to determine the PKC isoforms induced by DAAS, HL-60 cells were treated with DAAS and the protein levels of various PKC isoforms were determined via Western blot analysis, using mAbs for each of the PKC isoforms. In this report, we focused on the conventional PKC isoforms such as α, βI, βII, and γ, as these PKC isoforms are known to be the most abundantly expressed in leukemia cells, and the expression levels have been correlated closely with cell differentiation in HL-60 cells (29). As shown in Fig. 4D, DAAS treatment resulted in an increase in the protein levels of PKCβI, PKCβII, and PKCγ in the HL-60 leukemia cells, but the protein level of PKCα did not change.

Mitogen-activated protein kinases (MAPKs) are downstream elements in the PKC signaling pathway of cell differentiation (30, 31). To determine the involvement of the ERK, a MAPK, in DAAS-induced cell differentiation, HL-60 cells were treated with 2-(2’-amino-3’-methoxy-phenyl)-oxanaphthalen-4-one (PD 98059), a specific ERK inhibitor, in the presence of DAAS. PD 98059, a synthetic compound, inhibits the ERK pathway by preventing the activation of ERK kinase by c-Raf (32). As shown in Fig. 5A, PD 98059 inhibited DAAS-induced HL-60 cell differentiation. In contrast, SP 600125, a JNK MAPK-specific inhibitor, did not inhibit DAAS-induced cell differentiation. The levels of pERK were observed to increase in a time-dependent manner for up to 30 min (Fig. 5B). Moreover, inhibitors for PKC and PI3K inhibited the ERK activation stimulated by DAAS (Fig. 5, C and D). Therefore, PI3K, PKC, and ERK all appear to be involved in DAAS-induced cell differentiation.
NF-κB Is Active in HL-60 Leukemia Cells and the Inhibition of NF-κB Activity by DAAS May Be Involved in the Induction of HL-60 Cell Differentiation—To ascertain whether or not the levels of DAAS employed in this study were capable of inhibiting NF-κB DNA binding activity in unstimulated HL-60 leukemia cells, we analyzed nuclear extracts of HL-60 cells for NF-κB activity via an EMSA, using a probe specific to the NF-κB DNA-binding motif. As shown in Fig. 6, the HL-60 cells exhibited relatively high levels of NF-κB DNA binding activity under untreated control conditions. This binding was specific because it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, nonspecific oligonucleotide. A 24-h exposure to DAAS induced a marked reduction in this NF-κB DNA binding activity in a concentration-dependent manner, but santonin itself exerted no significant effects on NF-κB binding activity (Fig. 6, A and B).

To determine the effects of inhibitors of PI3K, PKC, and ERK on NF-κB activation in the DAAS-treated HL-60 cells, the cells were treated with 100 μg/ml of DAAS, followed by treatment with each of the inhibitors, after which the levels of NF-κB DNA binding activity were determined via NF-κB EMSA. As shown in Fig. 6C, the PI3K extracts of HL-60 cells for NF-κB activity via an EMSA, using a probe specific to the NF-κB DNA-binding motif. As shown in Fig. 6, the HL-60 cells exhibited relatively high levels of NF-κB DNA binding activity under untreated control conditions. This binding was specific because it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, nonspecific oligonucleotide. A 24-h exposure to DAAS induced a marked reduction in this NF-κB DNA binding activity in a concentration-dependent manner, but santonin itself exerted no significant effects on NF-κB binding activity (Fig. 6, A and B).

To determine the effects of inhibitors of PI3K, PKC, and ERK on NF-κB activation in the DAAS-treated HL-60 cells, the cells were treated with 100 μg/ml of DAAS, followed by treatment with each of the inhibitors, after which the levels of NF-κB DNA binding activity were determined via NF-κB EMSA. As shown in Fig. 6C, the PI3K
inhibitor markedly recovered the NF-κB binding activity, which had been attenuated as the result of DAAS treatment. In addition, the PKC and ERK inhibitors also resulted in an increase in NF-κB binding activity, whereas the JNK inhibitor exerted no such effects. These results indicate that PI3K, PKC, and ERK might be upstream components involved in the DAAS-associated inhibition of NF-κB binding activity.

To ascertain the specificity as well as the identity of NF-κB complex in HL-60 cells, EMSA was conducted in HL-60 cells with antibodies against the typical NF-κB subunits p50, p52, p65, or c-Rel, or with excess amounts of unlabeled NF-κB oligonucleotide. Incubation of the nuclear extract in unstimulated HL-60 cells with an antibody against either p50 or p65 shifted the band with the higher molecular weight, whereas an antibody against either p52 or c-Rel did not (Fig. 7A). The bands of NF-κB complex completely disappeared when HL-60 cells were treated with DAAS (Fig. 6), indicating that the NF-κB complex inactivated by DAAS was indeed NF-κB existing as a heterodimer of p50 and p65 subunits.

Furthermore, in an attempt to understand the mechanism underlying the inhibitory effects of DAAS on NF-κB activation, Western blot analysis was performed to examine the degradation of the inhibitory factor IκBα and the nuclear translocation of the functionally active subunit p65. Treatment of HL-60 cells with DAAS clearly decreased the level of NF-κB p65 in the nucleus and increased the level of IκBα in the cytosol, whereas santonin had no effect, indicating that DAAS inhibited the degradation of IκBα and the translocation of p65 to the nucleus (Fig. 7B).

Overexpression of NF-κB p65 Reduces the DAAS-induced Differentiation of HL-60 Cells—To assess whether a forced expression of NF-κB p65 is capable of inhibiting the DAAS-induced maturation of tumoral myeloid precursors along the granulocytic lineage, HL-60 cells were transfected with an expression plasmid containing the cDNA of NF-κB p65.
p65, and then treated with DAAS. The degrees of cell differentiation were determined.

As expected, the level of NF-κB p65 was substantially increased in the transfected HL-60 cells (Fig. 8A). Importantly, the transfection of HL-60 cells with an NF-κB p65 plasmid significantly reduced the levels of HL-60 cell differentiation induced by DAAS, as demonstrated by NBT reduction assay (Fig. 8B) and cytofluorometric analysis antibody against CD11b, a surface marker of differentiation (Fig. 8C). These results indicate that the overexpression of NF-κB p65 inhibits the differentiation inducing activity of DAAS in HL-60 cells.

DISCUSSION

In this study, we determined that a DAAS significantly induced the differentiation of HL-60 promyelocytic leukemia cells into granulocytes, whereas santonin itself exerted no such effects. This result demonstrates that the chemical modification of santonin to a diacetoxy acetal derivative confers the ability to induce the differentiation of human promyelocytic leukemia cells. Treatment of HL-60 cells with Me₂SO or retinoic acid, and granulocyte-colony stimulating factor-induced myeloid differentiation can be induced by agents including PMA, 1,25-dihydroxy vitamin D₃, or sodium butyrate (2, 3, 17, 33).

The induction of differentiation in HL-60 cells requires the activation of a variety of signal transduction pathways, including the PI3K (24), PKC (34, 35), and MAPK pathways (36). Previous reports demonstrated the involvement of the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/ERK/MAPK pathway in PMA-, retinoic acid-, and granulocyte-colony stimulating factor-induced myeloid differentiation. In our study, inhibitors of PI3K, PKC, and ERK also resulted in a significant reduction in DAAS-induced HL-60 cell differentiation, thereby indicating that PI3K, PKC, and ERK may be, at least in part, involved in the induction of HL-60 cell differentiation by DAAS.

Importantly, inhibitors of PI3K and PKC exerted inhibitory effects on DAAS-stimulated ERK activation (Fig. 5C), which suggests that PI3K and PKC may be upstream components of ERK activation induced by DAAS. However, the JNK MAPK inhibitor, SP 600125, had no effect on DAAS-induced HL-60 cell differentiation (Fig. 5).

In previous studies, we and others (37, 38) demonstrated that interference with NF-κB activation appears to be a common feature of agents that enhance differentiation in HL-60 cells. The results of the NF-κB EMSA conducted in this study showed that unstimulated HL-60 leukemia cells exerted relatively high levels of NF-κB activity, a finding consistent with Ref. 37. Constitutively high levels of NF-κB activity have been detected in a variety of tumor cells, because aberrant NF-κB expression has been associated with both oncogenesis and carcinogenesis (39).

Furthermore, we previously reported that the inhibition of this NF-κB activity sensitized the HL-60 cells to several differentiation-inducing agents, mostly notably dihydroxyvitamin D₃ (14). In the present study, we demonstrated that NF-κB was constitutively activated in untreated HL-60 cells, which significantly decreased upon addition of DAAS in a concentration-dependent manner (Fig. 6, A and B). Moreover, we showed that this NF-κB was comprised of p50 and p65 subunits (Fig. 7). The levels of cellular differentiation induced by DAAS were closely correlated with the inhibitory levels of NF-κB binding activity, suggesting that NF-κB inhibition might be involved in DAAS-induced HL-60 cell differentiation. Furthermore, the inhibitors of PI3K, PKC, and ERK resulted in a recovery of the NF-κB binding activity inhibited by DAAS treatment, suggesting that PI3K, PKC, and ERK may be involved in the NF-κB inhibition inherent to DAAS-induced HL-60 cell differentiation (Fig. 9). In future studies, the involvement of other signaling molecules, such as caspase (40), should also be investigated in DAAS-treated HL-60 cells.

It remains unclear as to why DAAS induces HL-60 cell differentiation, and other derivatives do not. However, it is known that several of the identified differentiation inducers harbored esters (41, 42). Some research groups have synthesized several esters of differentiation inducers for increasing the ability of cell differentiation (43–45).

In conclusion, we have demonstrated that DAAS induces HL-60 cell differentiation via the PI3K, PKC, and ERK pathways, as well as the inhibition of NF-κB. These findings suggest that DAAS might eventually prove useful in treatment of neoplastic diseases.

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Inductive Effect of HL-60 Cell Differentiation by a Santonin Derivative

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