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RESEARCH PAPER

Auranofin promotes retinoic acid- or dihydroxyvitamin D_3-mediated cell differentiation of promyelocytic leukaemia cells by increasing histone acetylation

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Background and purpose: To investigate the molecular mechanism for the effect of auranofin on the induction of cell differentiation, the cellular events associated with differentiation were analysed in acute promyelocytic leukaemia (APL) cells.
Experimental approach: The APL blasts from leukaemia patients and NB4 cells were cotreated with auroanofin and all-*trans*-retinoic acid (ATRA) at suboptimal concentration. The HL-60 cells were treated with auroanofin and a subeffective dose of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$ vit D_3) in combination. The effect of auroanofin was investigated on histone acetylation at the promoter of differentiation-associated genes and expression of cell cycle regulators.
Key results: Treatment with auroanofin and ATRA cooperatively induced granulocytic differentiation of fresh APL blasts isolated from patients and NB4 cells. The combined treatment also increased reorganization of nuclear PML bodies and histone acetylation at the promoter of the RARβ2 gene. Auroanofin also promoted monocytic differentiation of the HL-60 cells triggered by subeffective concentration of 1,25$(OH)_2$ vit D_3. The combined treatment of auroanofin and 1,25$(OH)_2$ vit D_3 stimulated histone acetylation at p21 promoters and increased the accumulation of cells in the G_0/G_1 phase. Consistent with this, the expressions of p21, p27 and PTEN were increased and the levels of cyclin A, Cdk2 and Cdk4 were decreased. Furthermore, the hypophosphorylated form of pRb was markedly increased in cotreated cells.
Conclusions and implications: These findings indicate that auroanofin in combination with low doses of either ATRA or 1,25$(OH)_2$ vit D_3 promotes APL cell differentiation by enhancing histone acetylation and the expression of differentiation-associated genes.
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Keywords: acute promyelocytic leukaemia; auranofin; cell differentiation; histone acetylation; PML body

Abbreviations: APL, acute promyelocytic leukaemia; ATRA, all-*trans* retinoic acid; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; JAK1, janus kinase 1; NBT, nitroblue tetrazolium; PML, promyelocytic leukaemia; pRb, retinoblastoma protein; RAR, retinoic acid receptor; RARE, retinoic acid response element; RPE, R-phycoerythrin; RT-PCR, reverse transcription- polymerase chain reaction; RXR, retinoid X receptor; STAT3, signal transducer and activator of transcription 3; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 1,25$(OH)_2$ vit D_3, 1α, 25-dihydroxyvitamin D_3

Introduction

All-*trans*-retinoic acid (ATRA), which has been used as a therapeutic drug for acute promyelocytic leukaemia (APL), acts by binding to its nuclear receptor, retinoic acid receptor (RAR)/retinoid X receptor (RXR) (Warrell *et al*., 1993). The RAR/RXR heterodimer binds to the retinoic acid response element (RARE) sites of its target genes and acts as a ligand-inducible transcription factor. In the absence of ATRA, the receptor associates with the nuclear corepressor *N*-CoR/histone deacetylase complex, which turns off the transcription. Occupation of the receptor by ATRA displaces the corepressor complex to the transcriptional coactivator complex, which includes histone acetyltransferase and leads to histone acetylation and expression of target genes (Mu *et al*., 1994; Piazza *et al*., 2001).

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APL produces the characteristic promyelocytic leukaemia (PML)–RARα fusion protein through a reciprocal chromosome translocation between the PML gene on chromosome 15 and the RARα gene on chromosome 17 (Lavau and Dejean, 1994). In APL cells, PML–RARα binds to RARE instead of RARα. Because the interaction of PML–RARα fusion protein with the corepressor complex is strong, the corepressor complex is not converted to the coactivator complex, with physiological concentrations of ATRA (Grignani *et al*., 1994; Guidez *et al*., 1998). As a result, the transcriptions of RARα target genes, which are associated with cell differentiation, are constitutively repressed and the differentiation of promyelocytic progenitor cells towards mature cells is not induced (Tallman *et al*., 1997).

A pharmacologically high concentration of ATRA can overcome this failure and induces expression of differentiation-associated genes. For this reason, differentiation-inducing therapy with a high dose of ATRA has been used clinically to treat APL patients (Huang *et al*., 1988). Although ATRA therapy is effective in inducing remission, its major problem is that most relapsed APL patients are resistant to further treatment with ATRA (Degos *et al*., 1990; Douer, 2002).

Other differentiation-inducing agents have been investigated. Among them, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2$ vit D_3), a physiologically active form of vitamin D_3, induces differentiation of myeloid leukaemic cells along the monocyte/macrophage lineage (Abe *et al*., 1981). The biological response to $1,25(OH)_2$ vit D_3 is mediated through its nuclear vitamin D receptor, a ligand-inducible transcription factor (Kato, 2000). However, clinical trials of high doses of $1,25(OH)_2$ vit D_3 for treating leukaemia have been limited because of the hypercalcemic side effect and incomplete cell differentiation (Evans, 1988).

A major goal in therapeutic strategies for treating patients with APL is to achieve terminal differentiation and to solve the problems of drug resistance and harmful side effects. One useful strategy is combined treatment with ATRA or $1,25(OH)_2$ vit D_3 at low doses, which does not induce toxicity, along with another drug, so that the two drugs act synergistically.

Auranofin (2, 3, 4 and 6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*-(triethylphosphine) gold) is a lipophilic gold compound, used to treat rheumatoid arthritis, based on its anti-inflammatory property (Blodgett *et al*., 1984; Borg *et al*., 1988). The drug inhibits the production of proinflammatory cytokines such as interleukin-1β and tumour necrosis factor-α through inactivation of nuclear factor-κB (Jeon *et al*., 2000), and blocks interleukin-6 signalling by inhibiting phosphorylation of janus kinase 1 (JAK1) and signal transducer and activator of transcription 3 (STAT3) (Kim *et al*., 2007). We found recently that auroanofin has a novel antileukaemic activity in NB4 cells, inducing apoptosis at relatively high concentrations (1–2 μM) and cell differentiation at lower concentrations (0.3–0.5 μM) by acting synergistically with a physiological concentration of ATRA (5 nM) (Kim *et al*., 2004; Park and Kim, 2005).

To investigate the molecular mechanisms underlying the stimulatory effect of auroanofin on APL cell differentiation, we used auroanofin in combination with low doses of ATRA or $1,25(OH)_2$ vit D_3 to treat primary APL cells isolated from patients and the APL cell lines (NB4 and HL-60) and then analysed the histone acetylation at the promoter regions of differentiation-associated genes.

Methods

Cell culture and treatment

This study was approved by the Institutional Review Board of the Catholic University of Korea, Seoul. Fresh APL blasts were isolated from the bone marrow of four patients bearing leukaemia at St Mary's Hospital, Seoul (Table 1); all patients signed a written consent form to donate the bone marrow for research. After gradient centrifugation using histopaque-1077, the monocytes were eliminated by removing the adherent cells. The non-adherent leukaemic cell fraction was used as a source of fresh APL blasts. NB4 cells and HL-60 cells were maintained at 37 °C in 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 2 mM L-gluatmine, 10 mM Hepes, 100 UmL^{-1} Penicillin, 100 $\mu g\,mL^{-1}$ Streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco Life Technology, Gaithersburg, MD, USA).

To investigate the effect of auroanofin on granulocytic differentiation, the fresh APL blasts and NB4 cells were seeded (1×10^6 cells) in 10 cm dishes and incubated for 4–5 days in medium containing auroanofin (0.3–0.5 μM) and ATRA (10 nM). To analyse monocytic differentiation, 2×10^6 HL-60 cells were incubated for 3 days in the presence of auroanofin (0.5 μM) and $1,25(OH)_2$ vit D_3 (3 nM).

Evaluation of cell differentiation

Induction of cell differentiation was evaluated by measuring the morphological changes, nitroblue tetrazolium (NBT) reduction and expression of surface antigen markers (CD11b and CD14). To observe morphological changes, the incubated cells were collected on glass slides by cytospin centrifugation. Cells were fixed in methanol, stained with

Table 1 Clinical and haematological characteristics of patients

Sample no.	*Diagnosis*	*FAB*	*Age/sex*	*WBC* ($\times 10^9 L^{-1}$)	*% leukaemic cells*	*Karyotype*
1	APL	M3	63/M	93.96	91	46, XY, t(15;17)(q22;q21)[20]
2	APL	M3	38/M	18.04	83	46, XY, der(4) t(4;8)(p22;q22) t(8;11)(q24.1;q23), t(15;17) (q22;q21) [8]/48, XY, + 8x2, t(15;17) (q22; q21)[12]
3	APL	M3	37/M	1.06	48	46, XY, t(15;17)(q22;q21)[20]
4	APL	M3	12/F	1.65	18	46, XX, t(15;17)(q22;q21)[20]

Abbreviations: APL, acute promyelocytic leukaemia; FAB, French–American–British classification at diagnosis; WBC, white blood cells.

Giemsa solution and photographed. For NBT reduction, the cells (1×10^6) were washed with serum-free RPMI medium and incubated at 37 °C in 0.25 mL of the medium containing NBT (1 mg mL^{-1}) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) of 5 μg mL^{-1}. After incubation for 30 min, the cells were lysed by the addition of dimethyl sulphoxide solution containing 0.04 M HCl. The dissolved formazan was quantified by measuring the absorbance at 570 nm.

Flow cytometric analysis

A direct immunofluorescence staining technique was used to detect cell surface markers. Briefly, primary APL cells from patients were treated with auroanofin or ATRA or both, and HL-60 cells were treated with auroanofin or 1,25(OH)$_2$ vit D$_3$ or both. The cells were washed twice with buffer A (phosphate-buffered saline; PBS, 0.1% sodium azide and 1% heat-inactivated fetal bovine serum), and an aliquot (1×10^6 cells) was resuspended in buffer A and incubated on ice for 30 min with R-phycoerythrin (RPE)-conjugated monoclonal mouse antihuman CD11b antibody for primary APL cells or with fluorescein isothiocyanate-conjugated monoclonal mouse antihuman CD14 antibody for HL-60 cells. As an isotype control, the cells were incubated with RPE-conjugated mouse IgG1. The incubated cells were washed again, fixed with 1% paraformaldehyde, resuspended in 500 μL of buffer A containing propidium iodide, and analysed using an FACScan flow cytometer (BD Biosciences, San Diego, CA, USA).

Immunostaining of PML

Cytospin preparations of leukaemic cells were fixed with cold methanol at 4 °C for 10 min. The slides were washed three times with PBS containing 1% fetal bovine serum and incubated overnight with antihuman-PML antibody at 4 °C. The cells were washed with PBS and incubated for 1 h with Cy3-conjugated rabbit antimouse IgG.

Cell cycle progression

The HL-60 cells treated with auroanofin or 1,25(OH)$_2$ vit D$_3$ or both were washed with cold PBS, pelleted and fixed with 80% ethanol in PBS. The cells were resuspended in 1 mL of PBS containing 50 μg mL^{-1} propidium iodide and 100 μg mL^{-1} DNase-free RNase A and incubated at 37 °C with agitation for 30 min. The propidium iodide -stained cells were analysed using an FACScan flow cytometer.

Western blot analysis

Cells were washed twice with PBS and lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 10 μg mL^{-1} aprotinin and 5 μg mL^{-1} leupeptin for 20 min on ice. The nuclear extracts were prepared by the method described earlier (Kim *et al.*, 2007). Equal protein amounts of total cell lysates or nuclear extracts were separated on a 12% SDS-polyacrylamide gel and analysed with various human antibodies. Antibodies against p21, p27, PTEN, cyclin A, cyclin-dependent kinase 2 (Cdk2), Cdk4, PML and hypophosphorylated retinoblastoma protein (pRb) were used as the primary antibodies. The proteins of interest were visualized using an enhanced chemiluminescence-based detection system (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

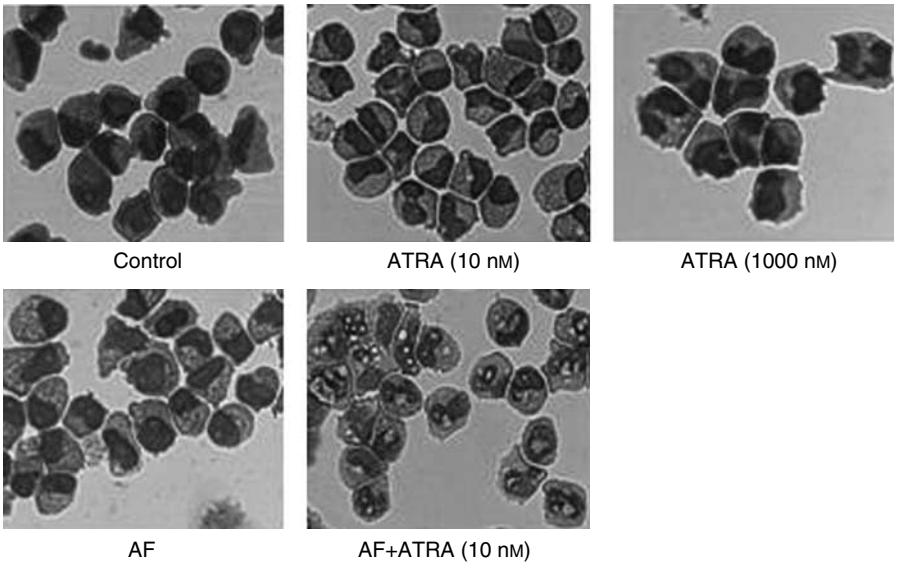

Figure 1 Morphological changes in acute promyelocytic leukaemia (APL) cells treated with auroanofin (AF) and all-*trans*-retinoic acid (ATRA). APL cells were isolated from the bone marrow of patients with leukaemia and the cells were treated with AF (0.5 μM) and ATRA (10 nM) or both for 5 days. The cells were collected onto a slide glass, fixed and stained with Giemsa solution. The stained cells were observed on a microscope and photographed (× 400). The APL cells treated with ATRA (1000 nM) were used as a positive control for granulocytic differentiation. The control cells in the figure denote cells treated with ethanol used as vehicle. The results shown here are representative of four separate experiments using APL cells isolated separately from four leukaemia patients.

RNA isolation and reverse transcription PCR (RT-PCR)
Total RNA was extracted using RNA STAT-60 solution according to the manufacturer's instruction (TEL-TEST, Friends-wood, TX, USA). A unit of 1 μg of total RNA was reverse transcribed for cDNA using Molony Murine Leukaemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA). The reaction was carried out at 42 °C for 1 h. The cDNAs for p21 and RARβ2 were amplified using specific primers (p21: sense 5′-CCGTGTTCTCCTTTTCCTCTCTCC-3′, antisense 5′-GAAAGATCTACTCC CCCATCATATACC-3′, RARβ2: sense 5′-AACGCGAGCGATCCGAGCAG-3′, antisense 5′-ATTTG TCCTGGCAGACGAAGCA as follows: 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min. The PCR products were separated on 1.2% agarose gel and stained with ethidium bromide.

Chromatin immunoprecipitation (ChIP) assay
A ChIP assay was performed using a ChIP assay kit according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, 2×10^6 cells were treated with auroanofin and ATRA or auroanofin and 1,25(OH)$_2$ vit D$_3$ and fixed with 1% formaldehyde for 10 min. The cells were lysed in cell lysis buffer for 10 min on ice. The lysates were sheared by sonication and then the sonicated cell supernatants were precleared for 2 h at 4 °C with salmon sperm DNA-saturated protein A agarose. A small amount of each sample was reserved for using as an input DNA control in PCR analysis. Samples were divided into two fractions and immunoprecipitated with antiacetyl histone H3-specific antibody and with antihuman IgG-negative control antibody. The protein–DNA complex was treated with 1% SDS in 0.1 M $NaHCO_3$ and incubated at 65 °C for 4 h to reverse the cross-links. The DNA was extracted, and the promoter regions of RARβ2 and p21 were amplified with the specific primers by PCR. The primers were: RARβ2, sense 5′-TCCTGGGAGTTGGTGATGTCAG-3′, antisense 5′-AAAC CCTGCTCGGATCGCTC-3′; and p21, sense 5′-GCACTCTGG AGGAGGACACA-3′; antisense 5′-GCCAGCTCTCGCACT CTGT T-3′.

Statistical analysis
Student's *t*-test and one-way ANOVA were used to analyse the differences between values obtained in the various experimental and control conditions; $P<0.05$ was considered significant.

Materials
The NB4 cell line was kindly provided by the Korean Leukaemia Cell and Gene Bank in the Catholic University of Korea (Seoul, Korea) and the HL-60 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Auroanofin was purchased from Alexis (Lausen, Switzerland). ATRA, 1,25(OH)$_2$ vit D$_3$, Histopaque-1077, Giemsa solution, NBT, PMA, propidium iodide, Triton X-100, sodium deoxycholate, phenylmethylsulphonyl fluoride, leupeptin and aprotinin were purchased from Sigma Chemical Co. (St Louis, MO, USA).

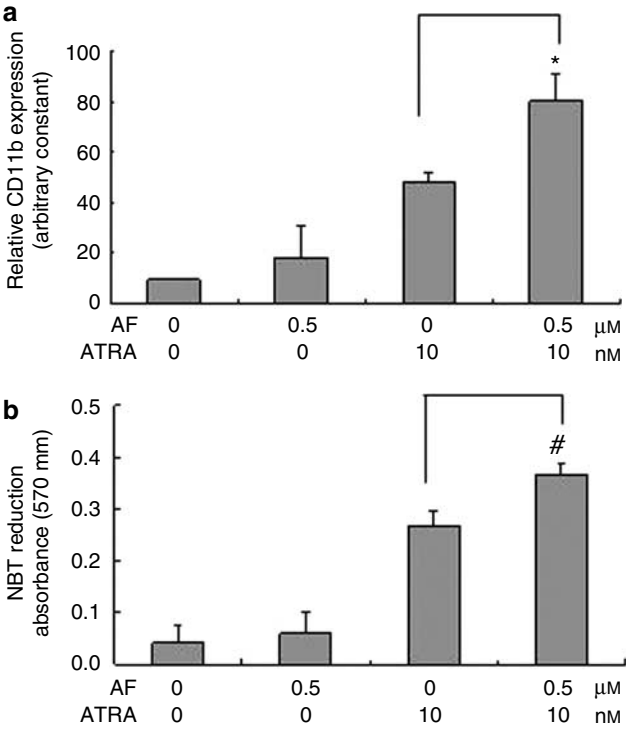

Figure 2 Stimulatory effect of auroanofin (AF) on cell differentiation triggered by all-*trans*-retinoic acid (ATRA) at low dose. The acute promyelocytic leukaemia (APL) cells, isolated from leukaemia patients, were seeded at 1×10^5 cells in 12-well plates and treated with AF (0.5 μM) and ATRA (10 nM) alone or in combination for 5 days. Differentiation of the APL cells was evaluated by flow cytometric analysis using R-phycoerythrin (RPE)-conjugated CD11b antibody (**a**) and an nitroblue tetrazolium (NBT) reduction assay (**b**), as described in Methods. The results represent the means ± s.d. of data from three separate experiments. $^{*,\#}P<0.05$ compared with cells treated with 10 nM ATRA alone.

RPE-conjugated monoclonal mouse antihuman CD11b antibody, RPE-conjugated mouse IgG1 antibody were obtained from Dako Cytomation (Carpinteria, CA, USA) and Cy3-conjugated rabbit antimouse IgG was obtained from Jackson Immunoresearch Lab (West Grove, PA, USA). Antibodies directed against PML (PG-M3), PTEN, p21, cyclin A, Cdk2 and Cdk4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and fluorescein isothiocyanate-conjugated monoclonal mouse antihuman CD14 antibody, p27, hypophosphorylated pRb were from BD Biosciences (San Diego, CA, USA). The ChIP assay kit and acetylated histone H3 antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Results

Combined effects of auroanofin and ATRA on granulocytic differentiation of APL cells isolated from patients
Our previous study showed that auroanofin enhances the differentiation of NB4 cells in the presence of subeffective concentrations of ATRA that alone could not induce significant cell differentiation (Kim *et al*., 2004). Although the NB4 cell line is derived from an APL patient, its

characteristics are not the same as those of primary APL cells. To confirm whether auroanofin also has stimulatory effects on the differentiation of primary APL cells, fresh APL blasts were purified from bone marrows of APL patients (Table 1). The primary APL cells were incubated for 5 days in medium containing auroanofin (0.5 μM) and ATRA (10 nM). The cells were stained with Giemsa solution and the morphological changes were observed in a microscope. Untreated primary APL cells were predominantly promyelocytes with round and regularly shaped nuclei. The cells treated with auroanofin and ATRA together showed features of granulocytic differentiation, such as nuclear lobulation, numerous granules and vacuoles and a lower ratio of nucleus to cytoplasm; in contrast, the cells treated with auroanofin or ATRA alone showed weak differentiation (Figure 1).

To further study the induction of differentiation by auroanofin, quantitative flow cytometric analysis of CD11b surface antigen and the NBT reduction test were performed. The expression of CD11b increased more after combined treatment with auroanofin (0.5 μM) and ATRA (10 nM) than after treatment with auroanofin or ATRA alone (Figure 2a). Consistent with these results, the formation of formazan by NBT reduction was increased when the APL blasts were treated with the combination of auroanofin and ATRA (Figure 2b). These findings suggest that, in primary APL blasts, auroanofin prompts granulocytic differentiation, which is triggered by subeffective dose of ATRA.

Reorganization of nuclear PML body and degradation of PML–RARα

PML protein is normally localized in specific subnuclear domains characterized by PML bodies. However, in APL cells, PML and PML–RARα proteins are colocalized throughout the nucleoplasm in a micropunctate pattern (Daniel *et al*., 1993). Treatment of the APL cells with ATRA at pharmacologically high concentration induces a degradation of PML–RARα protein and reorganizes the PML bodies (Dyck *et al*., 1994; Yoshida *et al*., 1996). We examined whether auroanofin acts in the processes of degradation of the PML–RARα fusion protein and reconstitution of the PML bodies. When the localization of PML and PML–RARα was observed by confocal

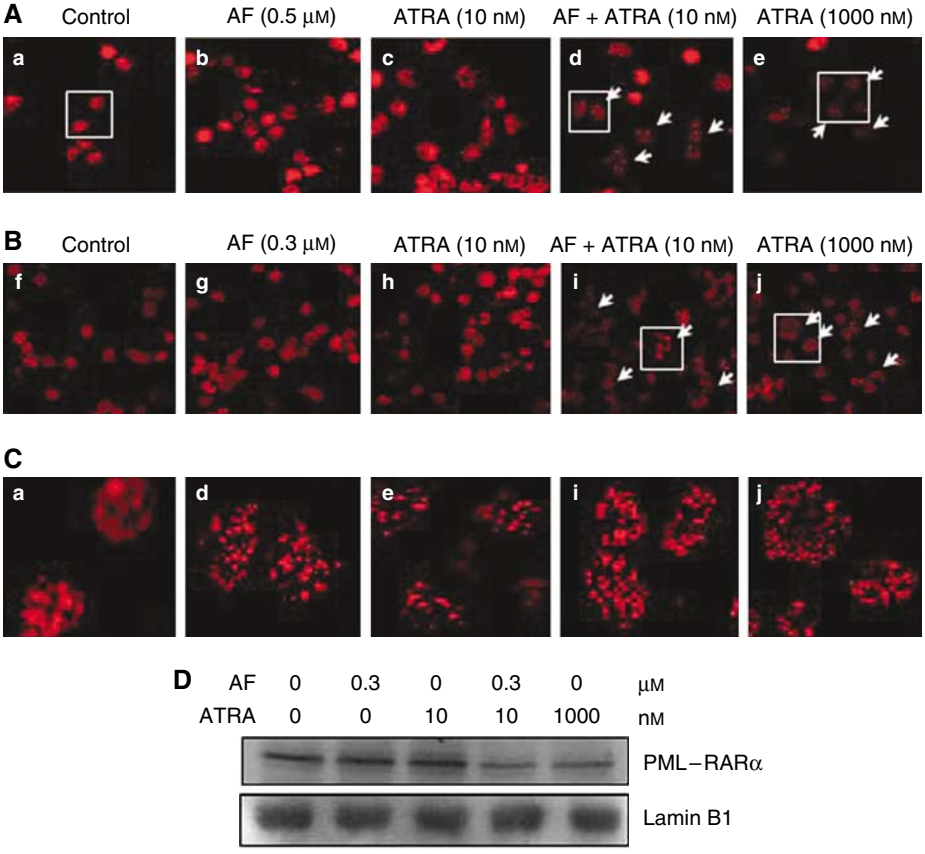

Figure 3 Immunofluorescence analysis using antipromyelocytic leukaemia (PML) antibody. Acute promyelocytic leukaemia (APL) cells from patients (**A**) and NB4 cells (**B**) were incubated in media containing auroanofin (AF) or all-*trans*-retinoic acid (ATRA) or both. After 5(**A**) or 4 (**B**) days, the cells were spun onto glass slides, fixed and stained with a mouse antihuman PML antibody and Cy3-conjugated rabbit antimouse IgG. The stained patterns were observed using a confocal microscope. (**C**) Represent enlarged photographs for squared portions of **a**, **d**, **e**, **i** and **j**. The controls are ethanol (vehicle)-treated cells. Note that the apparent speckles (arrows) show in the cells treated with AF and ATRA in combination, which are similar to the pattern observed in positive control cells treated with a high dose of ATRA (1000 nM). (**D**) Degradation of PML–RARα by cotreatment with AF and ATRA. Nuclear extracts from NB4 cells were analysed by western blot with PML antibody. Lamin B1 was used as a specific marker for nuclear proteins to ensure equal protein loading.

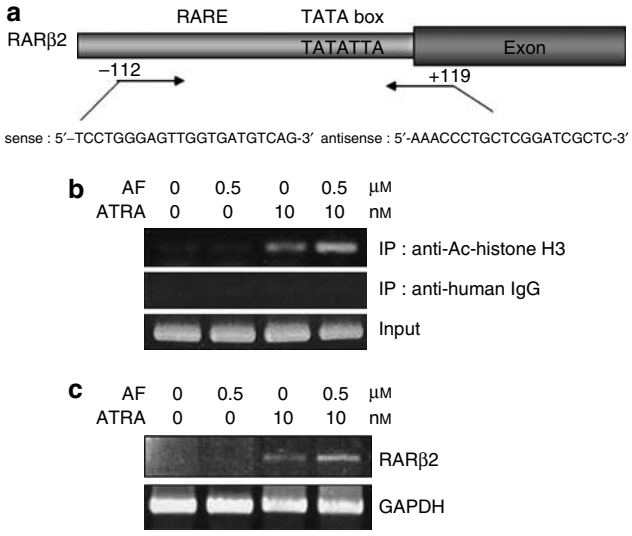

Figure 4 Auroanofin (AF) enhances acetylation of histone H3 at the promoter region of the *RARβ2* gene. (**a**) Schematic representation of about 230 bp containing the two retinoic acid response element (RARE) sites and the 5′ portion of exon 1 of the *RARβ2* gene. (**b**) The promoter DNA immunoprecipitated with antiacetylated histone H3 antibody was amplified by PCR using the specific primers described in panel a. PCR products were analysed by electrophoresis on a 1.5% agarose gel. Mouse antihuman IgG antibody was used as a negative control. (**c**) The relative mRNA level of RARβ2 in the cells treated with AF or all-*trans*-retinoic acid (ATRA) or both was measured by reverse transcription PCR (RT-PCR).

microscopy, immunofluorescence-staining patterns of PML showed diffusely throughout the nucleoplasm in primary APL cells from patients (Figure 3A, a) and the NB4 cells (Figure 3B, f). In contrast, the cells treated with both auroanofin and ATRA showed an apparent speckled pattern (Figures 3A, d and 3B, i) similar to the pattern of positive controls of cells treated with a high dose of ATRA (1000 nM) (Figures 3A, e and 3B, j), suggesting that PML protein relocalizes to the nuclear PML body. The cotreatment with auroanofin and ATRA also decreased PML–RARα protein (110 kDa) level (Figure 3D). These findings suggest that auroanofin and ATRA cooperate in degradation of PML–RARα and reorganization of PML body in APL cells.

Effect of auroanofin on histone acetylation at promoter of the RARβ2 gene

The PML–RARα fusion protein, bound on RARE sites of the ATRA-target promoters, recruits histone deacetylase complex and blocks the transcriptions of the target genes (Di Croce, 2005). Because auroanofin was involved in PML–RARα degradation, we examined whether auroanofin could restore the histone acetylation around the promoter of the *RARβ2* gene, which is an ATRA target and plays a crucial role in APL cell differentiation (Lin *et al*., 1998; Fazi *et al*., 2005). The ChIP assay using an antiacetylated histone H3 antibody showed that the histone acetylation at the *RARβ2* gene promoter increased significantly in APL cells differentiated by cotreatment with auroanofin and ATRA in low doses (Figure 4b). Consistent with this, the transcription of RARβ2 also increased (Figure 4c). These results suggest that auroanofin acts synergistically with a suboptimal dose of ATRA to restore histone acetylation and to upregulate the expression of differentiation-associated genes, leading to APL cell differentiation.

Enhancement of monocytic differentiation by combined treatment with auroanofin and 1,25(OH)$_2$ vit D$_3$

The HL-60 cell line has been used widely as a model system of APL differentiation, because high doses of ATRA or 1,25(OH)$_2$ vit D$_3$ induce terminal differentiation toward granulocytes or monocytes, respectively (Breitman *et al*., 1980; McCarthy *et al*., 1983). To investigate whether auroanofin also stimulated 1,25(OH)$_2$ vit D$_3$-mediated monocytic differentiation, HL-60 cells were treated for 3 days with auroanofin (0.5 μM) or 1,25(OH)$_2$ vit D$_3$ (3 nM) or both. The NBT reduction assay indicated that the combined treatment induced differentiation to the same extent as treatment with 1,25(OH)$_2$ vit D$_3$ (100 nM, positive control) (Figure 5a).

The expression of CD14 surface antigen, a marker of monocytes/macrophages, was also detected. As shown in Figure 5b, combined treatment with auroanofin and 1,25(OH)$_2$ vit D$_3$ markedly increased antigen expression (81%), in contrast to the low expression induced by auroanofin (0.6%) or 1,25(OH)$_2$ vit D$_3$ (15%) alone. These results suggest that auroanofin also contributes to the monocytic differentiation of HL-60 cells.

Analysis of the cell cycle and cell cycle regulators

Cell differentiation is associated with G$_0$/G$_1$ arrest (Furukawa, 2002). To determine the effect of auroanofin on cell cycle progression, flow cytometric analysis of propidium iodide-stained nuclei was carried out. As shown in Figure 6, the accumulation of cells in the G$_0$/G$_1$ phase increased more after treatment with the combination of auroanofin and 1,25(OH)$_2$ vit D$_3$ than with each compound alone. The number of cells in the S phase decreased concomitantly. Because the cell cycle is controlled by cyclins and Cdks, the expression of these regulators was measured. Figure 7a indicates that the expression levels of p21, p27 and PTEN increased and the levels of cyclin A, Cdk2 and Cdk4 decreased in cotreated cells. The pRb protein is also an inhibitor of cell cycle progression and, in G$_0$/G$_1$-arrested cells, pRb mainly exists in hypophosphorylated form, whereas pRb is hyperphosphorylated by G$_1$/S-Cdks in actively proliferating conditions (Hollingsworth *et al*., 1993). We also examined the phosphorylation states of pRb in untreated and auroanofin/1,25(OH)$_2$ vit D$_3$-treated HL-60 cells. The cells treated without and with auroanofin (0.5 μM) or 1,25(OH)$_2$ vit D$_3$ (3 nM) showed little hypophosphorylated pRb. However, the hypophosphorylated pRb was markedly increased in combined treatment with auroanofin and 1,25(OH)$_2$ vit D$_3$, to a similar extent as a positive control (100 nM 1,25(OH)$_2$ vit D$_3$) (Figure 7b). These findings suggest that auroanofin and 1,25(OH)$_2$ vit D$_3$ acts synergistically on cell cycle arrest at G$_0$/G$_1$ phase.

Effect of auroanofin on acetylation of histone around the promoter of the p21 gene

We examined the histone acetylation around the p21 gene promoter, a modulator of the cell cycle (Hovhannisyan *et al*., 2003; Sakajiri *et al*., 2005). Induction of monocytic differentiation by incubating HL-60 cells in medium containing auroanofin and 1,25$(OH)_2$ vit D_3 increased the acetylation of histone H3 at the p21 promoter (Figure 8b). Consistent with this, the transcriptional level of p21 also increased (Figure 8c). These observations suggest that the stimulatory effect of combined treatment with auroanofin and 1,25$(OH)_2$ vit D_3 on monocytic differentiation is related to the cooperative actions of the two compounds on chromatin remodelling through histone acetylation of the target genes.

Discussion

In a previous study, we found that auroanofin enhanced granulocytic differentiation of the NB4 cell line when combined with a subeffective dose of ATRA (Kim *et al*., 2004). To identify whether auroanofin also stimulates the differentiation of primary APL cells, we purified fresh APL cells from the bone marrow of APL patients. We found that auroanofin reinforces the granulocytic differentiation of primary APL cells induced incompletely by a suboptimal dose of ATRA (10 nM) (Figures 1 and 2). When we obtained APL cells from the bone marrow of a patient undergoing ATRA treatment in the clinic, about 75% of the cells had become positive for CD11b expression after treatment with auroanofin (0.5 μM) alone, whereas 22% of cells were positive

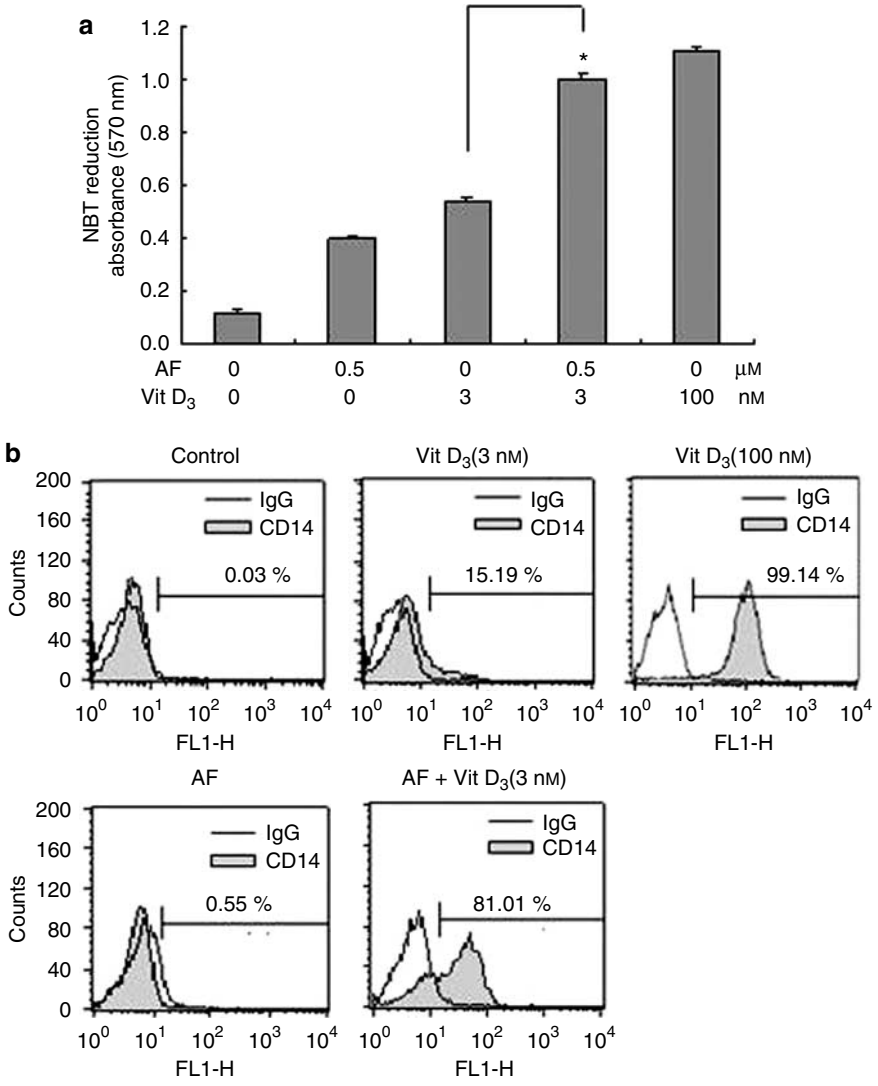

Figure 5 Synergistic effect of auroanofin (AF) and 1,25$(OH)_2$ vit D_3 on monocytic differentiation. HL-60 cells were seeded at 2×10^5 cells in 12-well plates and treated with AF (0.5 μM) and 1,25$(OH)_2$ vit D_3 (3 nM) for 3 days. The cells treated with a high concentration of 1,25$(OH)_2$ vit D_3 (100 nM) were used as a positive control for monocytic differentiation. (**a**) The treated cells were incubated in medium containing 1 mg mL^{-1} nitroblue tetrazolium (NBT) and 5 μg mL^{-1} 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 30 min and then lysed with isopropanol containing 0.04 M HCl. Dissolved formazan was detected by absorbance at 570 nm. The results represent the mean ± s.d. of data from experiments performed in triplicate. *$P<0.005$ compared with cells treated with 1,25$(OH)_2$ vit D_3 (3 nM) alone. The result shown here is representative of three independent experiments. (**b**) Expression of the cell surface marker CD14 was identified by flow cytometric analysis using fluorescein isothiocyanate-conjugated antibody. The result is representative of three independent experiments.

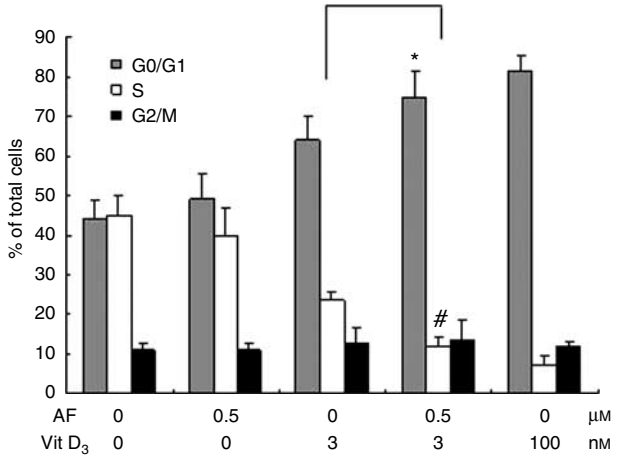

Figure 6 Cell cycle analysis during differentiation. HL-60 cells were incubated with auroanofin (AF) or 1,25(OH)$_2$ vit D$_3$ or both for 3 days. After incubation, the cell cycle was analysed by flow cytometry. The data represent the mean ± s.d. of three independent experiments. $^{*,\#}P<0.05$ compared with cells treated with 1,25(OH)$_2$ vit D$_3$ (3 nM) alone.

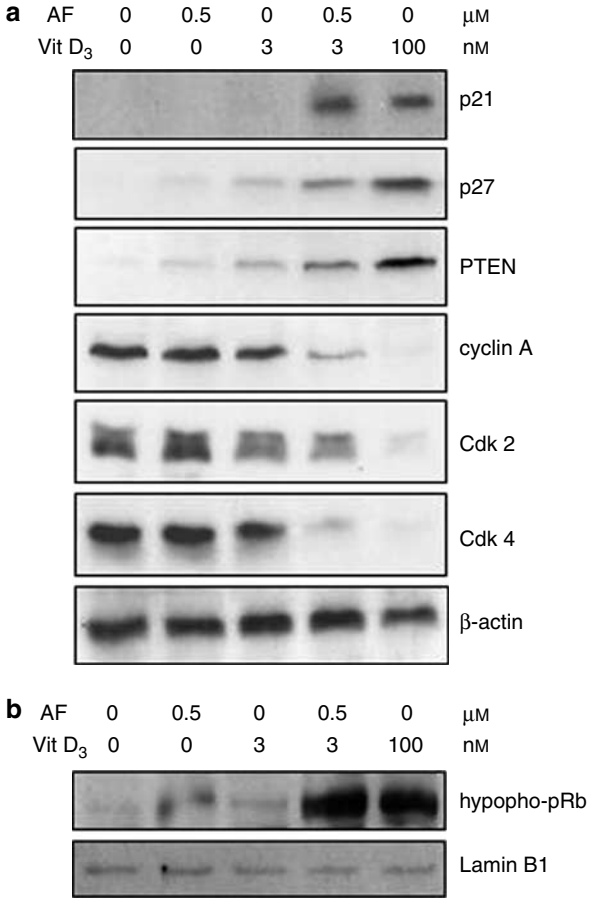

Figure 7 Regulation of cell cycle-related gene expression during differentiation. The HL-60 cells (6×10^5 cells in 6-well plate) were treated with auroanofin (AF) or 1,25(OH)$_2$ vit D$_3$ or both for 3 days. (**a**) Equal amounts of the cell lysates were loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and detected with the indicated antibodies. β-actin was used as an internal marker to ensure equal protein loading. (**b**) Hypophosphorylation of hypophosphorylated retinoblastoma protein (pRb) in differentiated HL-60 cells. Nuclear extracts obtained from HL-60 cells were run on 8% SDS-polyacrylamide gels and analysed with the antihypophosphorylated pRb antibody.

in untreated cells (data not shown). These findings suggest that auroanofin can prompt the differentiation of APL cells triggered by ATRA.

The PML–RARα fusion protein is one target of APL therapy, because the fusion protein is characteristic of APL cells and plays a crucial role in the pathogenesis of APL (Kakizuka *et al.*, 1991; Grignani *et al.*, 1993; Dyck *et al.*, 1994). In fact, ATRA-induced APL differentiation is associated with degradation of the PML–RARα protein and subsequent reorganization of PML bodies. We examined whether auroanofin participated in the degradation of the PML–RARα protein and the reformation of PML bodies. In immunocytochemical results (Figure 3), the cells treated with both auroanofin and a suboptimal concentration of ATRA (10 nM) clearly showed speckled nuclear structures, which represented the formation of PML bodies. In addition, the combined treatment with auroanofin and ATRA induced degradation of PML–RARα. However, the degradation of PML–RARα was not sufficient to explain the mechanism underlying auroanofin-stimulated APL differentiation, because cotreatment with auroanofin and ATRA also induced the differentiation of HL-60 cells, which have no PML–RARα fusion protein (data not shown).

When HL-60 cells were cotreated with auroanofin and subeffective concentrations of 1,25(OH)$_2$ vit D$_3$ (3 nM), the stimulatory effects of auroanofin on monocytic differentiation were similar those of auroanofin-stimulated granulocytic differentiation in NB4 cells triggered by subeffective concentrations of ATRA. These results suggest that auroanofin acts on a common pathway of differentiation induced by ATRA and 1,25(OH)$_2$ vit D$_3$. Both ATRA and 1,25(OH)$_2$ vit D$_3$ transduce signals through their ligand-inducible nuclear receptors and upregulate gene expression associated with differentiation by induction of histone acetylation at the promoter regions of the target genes (Kliewer *et al.*, 1992; Guidez *et al.*, 1998). The ChIP assay and RT-PCR demonstrated that auroanofin promoted histone acetylations and gene transcriptions mediated by ATRA or 1,25(OH)$_2$ vit D$_3$ (Figures 4 and 8). Therefore, it is likely that auroanofin induced differentiation of APL cells by stimulating chromatin remodelling, which caused expression of genes involved in cell differentiation.

Taken together, this study demonstrates that auroanofin in the concentration range of 0.3–0.5 μM cooperatively induces the differentiation of APL cells along the granulocytic lineage when given with suboptimal concentrations of ATRA (10 nM) or along the monocytic lineage when given with a suboptimal concentrations of 1,25(OH)$_2$ vit D$_3$ (3 nM). Auroanofin exerts its differentiation-promoting activity through mechanisms that stimulate the reformation of PML bodies (possibly through degradation of PML–RARα fusion protein), histone acetylation around the promoter regions of genes involved in differentiation and cell cycle arrest. Pharmacological high doses of ATRA or 1,25(OH)$_2$ vit

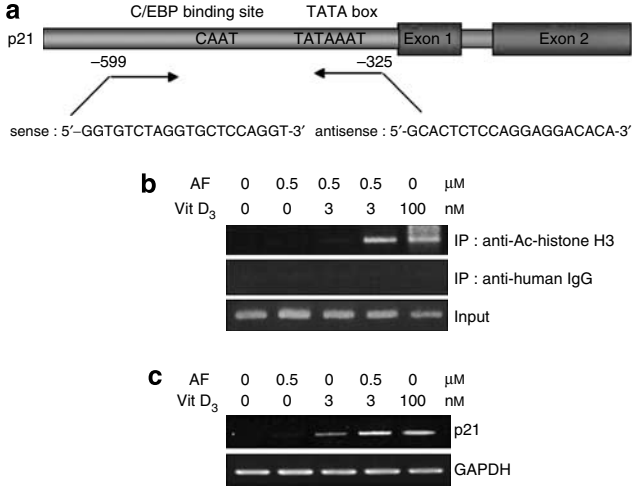

Figure 8 Auroanofin (AF) synergistically induces accumulation of acetylated histone H3 during $1,25(OH)_2$ vit D_3-induced HL-60 cell differentiation. (**a**) Schematic representation of the promoter region of the human p21 gene. The indicated primer sets are for amplification of the 274 bp promoter region containing CCAAT/enhancer-binding protein (C/EBP)-binding site and TATA box. (**b**) Chromatin was immunoprecipitated with an antihuman acetylated histone H3 antibody or antihuman IgG antibody as the negative control, and then amplified by PCR using the specific primers described in panel a. (**c**) p21 mRNA level was measured by reverse transcription PCR (RT-PCR) analysis.

D_3 present problems when applied clinically, such as drug resistance or hypercalcemia (Warrell *et al*., 1993; Hisatake *et al*., 1999; Douer, 2002). Our findings suggest that a combination of auroanofin with a low dose of ATRA or $1,25(OH)_2$ vit D_3 may have therapeutic benefit in treating PML, without the harmful side effects caused by high doses of the drugs.

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Conflict of interest

The authors state no conflict of interest.

References

Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T *et al*. (1981). Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. *Proc Natl Acad Sci USA* **78**: 4990–4994.

Blodgett Jr RC, Heuer MA, Pietrusko RG (1984). Auranofin: a unique oral chrysotherapeutic agent. *Semin Arthritis Rheum* **13**: 255–273.

Borg G, Allander E, Lund B, Berg E, Brodin U, Pettersson H *et al*. (1988). Auranofin improves outcome in early rheumatoid arthritis. Results from a 2-year, double blind placebo controlled study. *J Rheumatol* **15**: 1747–1754.

Breitman TR, Selonick SE, Collins SJ (1980). Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci USA* **77**: 2936–2940.

Daniel MT, Koken M, Romagné O, Barbey S, Bazarbachi A, Stadler M *et al*. (1993). PML protein expression in hematopoietic and acute promyelocytic leukemia cells. *Blood* **82**: 1858–1867.

Degos L, Chomienne C, Daniel MT, Berger R, Dombret H, Fenaux P *et al*. (1990). Treatment of first relapse in acute promyelocytic leukaemia with all-trans retinoic acid. *Lancet* **336**: 1440–1441.

Di Croce L (2005). Chromatin modifying activity of leukaemia associated fusion proteins. *Hum Mol Genet* **14**: R77–R84.

Douer D (2002). Advances in the treatment of relapsed acute promyelocytic leukemia. *Acta Haematol* **107**: 1–17.

Dyck JA, Maul GG, Miller Jr WH, Chen JD, Kakizuka A, Evans RM (1994). A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* **76**: 333–343.

Evans RM (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889–895.

Fazi F, Travaglini L, Carotti D, Palitti F, Diverio D, Alcalay M *et al*. (2005). Retinoic acid targets DNA-methyltransferases and histone deacetylases during APL blast differentiation *in vitro* and *in vivo*. *Oncogene* **24**: 1820–1830.

Furukawa Y (2002). Cell cycle control genes and hematopoietic cell differentiation. *Leuk Lymphoma* **43**: 225–231.

Grignani F, Fagioli M, Alcalay M, Longo L, Pandolfi PP, Donti E *et al*. (1994). Acute promyelocytic leukemia: from genetics to treatment. *Blood* **83**: 10–25.

Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M *et al*. (1993). The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* **74**: 423–431.

Guidez F, Ivins S, Zhu J, Soderstrom M, Waxman S, Zelent A (1998). Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RARalpha underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. *Blood* **91**: 2634–2642.

Hisatake J, Kubota T, Hisatake Y, Uskokovic M, Tomoyasu S, Koeffler HP (1999). 5,6-trans-16-ene-vitamin D3: a new class of potent inhibitors of proliferation of prostate, breast, and myeloid leukemic cells. *Cancer Res* **59**: 4023–4029.

Hollingsworth Jr RE, Chen PL, Lee WH (1993). Integration of cell cycle control with transcriptional regulation by the retinoblastoma protein. *Curr Opin Cell Biol* **5**: 194–200.

Hovhannisyan H, Cho B, Mitra P, Montecino M, Stein GS, Van Wijnen AJ *et al*. (2003). Maintenance of open chromatin and selective genomic occupancy at the cell cycle-regulated histone H4 promoter during differentiation of HL-60 promyelocytic leukemia cells. *Mol Cell Biol* **23**: 1460–1469.

Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhoa L *et al*. (1988). Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* **72**: 567–572.

Jeon KI, Jeong JY, Jue DM (2000). Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase. *J Immunol* **164**: 5981–5989.

Kakizuka A, Miller Jr WH, Umesono K, Warrell Jr RP, Frankel SR, Murty VV *et al*. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* **66**: 663–674.

Kato S (2000). The function of vitamin D receptor in vitamin D action. *J Biochem (Tokyo)* **127**: 717–722.

Kim IS, Jin JY, Lee IH, Park SJ (2004). Auranofin induces apoptosis and when combined with retinoic acid enhances differentiation of acute promyelocytic leukaemia cells *in vitro*. *Brit J Pharmacol* **142**: 749–755.

Kim NH, Lee MY, Park SJ, Choi JS, Oh MK, Kim IS (2007). Auranofin blocks interleukin-6 signalling by inhibiting phosphorylation of JAK1 and STAT3. *Immunology* **122**: 607–614.

Kliewer SA, Umesono K, Mangelsdorf DJ, Evans RM (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**: 446–449.

Lavau C, Dejean A (1994). The t(15;17) translocation in acute promyelocytic leukemia. *Leukemia* **8**: 1615–1621.

Lin RJ, Nagy L, Inoue S, Shao W, Miller Jr WH, Evans RM (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* **39**: 811–814.

McCarthy DM, San Miguel JF, Freake HC, Green PM, Zola H, Catovsky D *et al*. (1983). 1,25-dihydroxyvitamin D3 inhibits proliferation of human promyelocytic leukaemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. *Leuk Res* **7**: 51–55.

Mu ZM, Chin KV, Liu JH, Lozano G, Chang KS (1994). PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol Cell Biol* **14**: 6858–6867.

Park SJ, Kim IS (2005). The role of p38 MAPK activation in auranofin-induced apoptosis of human promyelocytic leukaemia HL-60 cells. *Brit J Pharmacol* **146**: 506–513.

Piazza F, Gurrieri C, Pandolfi PP (2001). The theory of APL. *Oncogene* **20**: 7216–7222.

Sakajiri S, Kumagai T, Kawamata N, Saitoh T, Said JW, Koeffler HP (2005). Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. *Exp Hematol* **33**: 53–61.

Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Feusner JH, Ogden A *et al*. (1997). All-trans-retinoic acid in acute promyelocytic leukemia. *N Engl J Med* **337**: 1021–1028.

Warrell Jr RP, de The H, Wang ZY, Degos L (1993). Acute promyelocytic leukemia. *N Engl J Med* **329**: 177–189.

Yoshida H, Kitamura K, Tanaka K, Omura S, Miyazaki T, Hachiya T *et al*. (1996). Accelerated degradation of PML-retinoic acid receptor alpha (PML-RARA) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. *Cancer Res* **56**: 2945–2948.