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Research Paper

Andrographolide inhibits growth of acute promyelocytic leukaemia cells by inducing retinoic acid receptor-independent cell differentiation and apoptosis

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Abstract

Objectives The growth inhibiting potential of andrographolide was evaluated in three acute promyelocytic leukaemia cell line models (HL-60, NB4 and all-*trans* retinoic acid (ATRA)-resistant NB4-R2).

Methods In elucidating the mechanisms of growth inhibition, a special emphasis was placed on assessing the induction of differentiation and apoptosis by andrographolide in the primary acute promyelocytic leukaemia NB4 cells.

Key findings The compound was 2- and 3-fold more active in inhibiting the growth of HL-60 and NB4-R2 cells compared with NB4 cells, respectively. At IC50 (concentration at which growth of 50% of the cells (compared with medium only treated control cells) is inhibited; 4.5 μM) the compound exhibited strong cell-differentiating activity in NB4 cells, similar to ATRA (IC50 1.5 μM). In the presence of a pure retinoic acid receptor antagonist AGN193109, the growth inhibition of NB4 cells by ATRA was reversed, whereas the activity of andrographolide was not affected. This clearly suggested that andrographolide's cell differentiating activity to induce growth inhibition of NB4 cells most likely occurred via a retinoic acid receptor-independent pathway. At higher concentration ($2 \times$ IC50), andrographolide was an efficient inducer of apoptosis in NB4 cells.

Conclusions Taken together, these results suggest andrographolide and its derivatives, apparently with a novel cell differentiating mechanism and with ability to induce apoptosis, might be beneficial in the treatment of primary and ATRA-resistant acute promyelocytic leukaemia.

Keywords andrographolide; apoptosis; cell differentiation; promyelocyte/retinoic acid receptor alpha

Introduction

Acute promyelocytic leukaemia (APL), being the focus of this study, is associated with favourable prognosis and uniquely characterised by its ability to enter complete remission with current treatment modalities consisting of cell differentiating and cytotoxic agents without the patients undergoing marrow aplasia (Lo Coco *et al*. 1998).[1] Molecular pathogenesis studies of APL reveal that more than 95% of APL cases have the chromosome translocation *t*(15;17) (q22;q21), which resulted in the expression of chimeric fusion protein promyelocyte/retinoic acid receptor alpha (PML-RARα) (Mistry *et al*. 2003).[2] Presently, cell differentiation inducing agent all-*trans* retinoic acid (ATRA), in combination with anthracyline-based chemotherapy, has become standard first-line treatment in the management of APL (Tallman *et al*. 2002).[3] ATRA exerts its effect by inducing differentiation of immature promyeloblasts with infinite proliferative potential into mature granulocytes with a finite life span.[4] The most compelling evidence supporting the induction of differentiation as a viable mode of cancer therapy arises with the treatment of ATRA in a clinical study, in which complete remission was attained in patients with APL.[5] At physiological concentrations, ATRA promotes normal granulopoiesis by binding to RARα–RXR (retinoid X receptor) complex for transducing signalling for the production of mature functional neutrophils.[2] However, RXR on its own is unable to bind ATRA. The ATRA-bound RARα–RXR complex induces signalling by binding retinoic acid response elements (RAREs) located in the promoter regions of RAR-specific target genes to induce terminal

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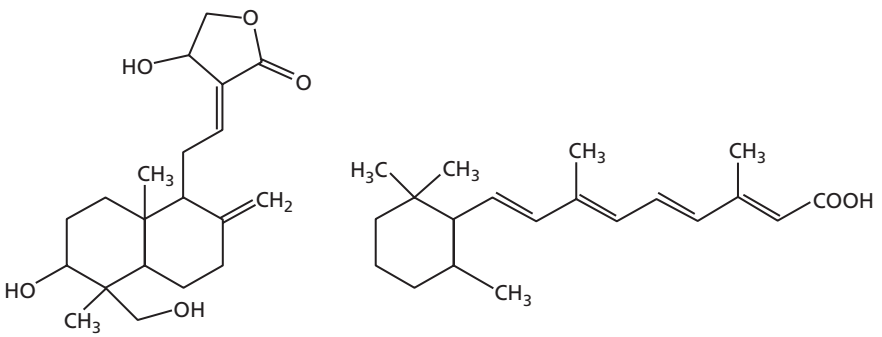
myeloid differentiation. Without the presence of ATRA, the RARα–RXR complex binds co-repressors to repress transcription of RAR target genes by binding RAREs with high affinity, which leads to inhibition of differentiation. In APL, the PML-RARα protein associates with co-repressors histone deacetylase (HDAC) and DNA methytransferases (Dnmt's) to bind target promoters, repressing transcription and activation mediated by native RARα–RXR.[2] High pharmacological doses of ATRA (> 1 μM) release these co-repressors from PML-RARα for positive signalling to occur, in which RAR target genes involved in cell differentiation are expressed.[4] This strategy also leads to the caspase and ubiquitin–proteosome system mediated degradation of PML-RARα protein to overcome the dominant negative effect of this protein.[6]

Although the initial remissions attained with ATRA and cytotoxic drugs were impressive, unfortunately these have been short-lived because the treatment failed to eradicate the malignant clones completely and led to a more devastating situation due to development of resistance to drugs, particularly ATRA.[7–10] Therefore, new agents with cell differentiation inducing activity via non-RAR pathways are desirable to be effective against APL cells that are non-responsive and have acquired resistance to ATRA. Moreover, it has become more apparent in recent times that it is an added advantage to develop agents with growth inhibitory effect mediated not only through cell differentiation but also via other mechanisms, such as apoptosis,[11,12] to be active against APL cells that are inherently resistant to undergoing differentiation.

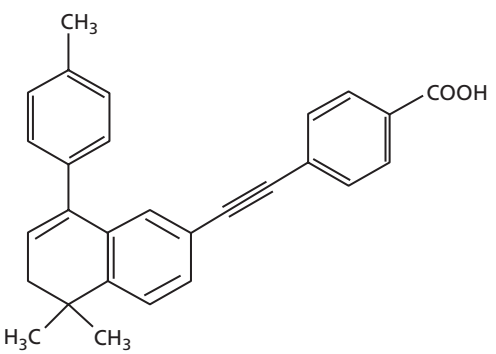
For this purpose we studied andrographolide (AGP, Figure 1), a naturally occurring diterpenoid lactone for cell differentiating and apoptosis inducing properties in APL cell models. A previous study revealed the mouse myeloid cell differentiating activity of AGP and its natural derivatives found in the famous Asian herb *Andrographis paniculata*.[13] However, to date the cell-differentiating effect of AGP on human myeloid leukaemia cells, with the purpose of developing new agents for the treatment of APL, has yet to be reported. AGP has various pharmacological actions.[14–19] Therefore, this study attempts to shed some light on the in-vitro anti-leukaemia effect of AGP on APL cells by determining the effect of AGP on the proliferation of ATRA-sensitive NB4,[20] ATRA-resistant NB4 (NB4-R2)[21] and HL-60 (late M2 stage) cells[22] by assessing the compound's ability to induce growth inhibition, differentiation and apoptosis. The HL-60 and NB4 cells are common APL cell line models used to study myeloid cell differentiation and are sensitive to retinoic acid.[23,24] The NB4 cells have the classical APL chromosomal translocation *t*(15;17), whereas HL-60 cells do not display this type of chromosomal abnormality.[20,22] The NB4-R2 cells have the mutated form of PML-RARα gene and are resistant to ATRA with no apparent signalling of differentiation.[21]

In this study, we found AGP to be effective against the NB4 cells by inducing differentiation and apoptosis, and to inhibit the proliferation of ATRA-resistant NB4-R2 cells,



AGP

ATRA



AGN193109

Figure 1 Chemical structures of andrographolide (AGP), all-*trans* retinoic acid (ATRA) and AGN193109.

which strongly suggested the potential of AGP, and possibly its derivatives, in the treatment of primary and ATRA-resistant APL cases.

Materials and Methods

Compounds and chemicals

AGP was isolated and purified from *A. paniculata* by Mr Sreenivasa Rao Sagineedu (Universiti Putra Malaysia) according to the method of Matsuda *et al*. (1994).[13] The purity of AGP was > 98%, as tested using HPLC. ATRA was obtained from Hoffmann-La Roche Ltd (Basel, Switzerland). AGN193109 (retinoic acid receptor antagonist) was kindly provided by Dr Richard Beard (Allergan Pharmaceuticals, Irvine, CA, USA). Etoposide was obtained from Pharmacia & Upjohn (Netherlands). Test compounds were dissolved in dimethyl sulfoxide (DMSO) to make up stock solution of 10 mM.

Phosphate-buffered saline (PBS) tablets, ethylene-diamine-tetraacetic-acid (EDTA), tris-HCl, RNase, trypan blue, nitroblue tetrazolium (NBT) dye, acridine orange (AO), propidium iodide (PI), proteinase K, Triton X-100, phenol–chloroform–isoamyl alcohol (25 : 24 : 1), molecular biology grade agarose gel, ethidium bromide and DMSO were obtained from Sigma Chemicals (St Louis, MO, USA). Absolute ethanol, glutaraldehyde and sodium acetate anhydrous were purchased from the BDH Laboratory (UK). The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was supplied by Phytotechnology Laboratories (Kansas, USA).

Cell culture

The APL cell lines HL-60, NB4 and NB4-R2 were used in this study. HL-60 cells were obtained from the American Type Culture Collection whereas the NB4 and NB4-R2 cells were generously provided by Dr M. Lannotte (Hôpital Saint-Louis, Paris, France). All cell lines were maintained in RPMI 1640 medium containing 2 mM L-glutamine (GIBCO BRL, USA), supplemented with 20% heat-inactivated foetal calf serum (PAA Laboratories, Linz, Austria), 100 IU/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories, Linz, Austria). Cells were cultured in 25 cm^2 culture flasks with 0.2 μm polysulfone filter (Nalge Nunc International, Denmark) at 37°C in 5% CO_2 humidified incubator. The cells were routinely viewed under an inverted phase contrast microscope (Olympus CK 40) to assess their growth characteristics. The number of viable cells in the culture was determined by the trypan blue exclusion method. Cell suspension with more than 90% viability was used in all assays.

MTT cell viability assay

The MTT assay used for the in-vitro assessment of drug effects on the growth of cells was carried out according to a previously described method.[25] Briefly, cells growing in 25 cm^2 flasks containing RPMI 1640 medium in log phase were counted and plated at 10 000 cells in 180 μl of medium per well in a 96-well flat-bottomed microplate (Nalge Nunc, Rockilde, Denmark). Wells containing 200 μl of medium alone served as blank. AGP and ATRA stocks were diluted in medium to achieve concentrations in the range of 1–1000 μM. Volumes of 20 μl drug dilution at various concentrations were dispensed ($n = 8$) to yield a final concentration range of 0.1–100 μM. Control wells received 20 μl of medium. The microplate was incubated at 37°C with 5% CO_2 in a humidified incubator. At the 96-h time point, 50 μl of MTT (2 mg/ml) was added into each well and the microplate was incubated for 4 h, followed by centrifugation at 450*g* for 10 min to obtain a cell pellet. After discarding the MTT supernatant, 100 μl DMSO was added to dissolve the formazan crystals formed in viable cells. The optical density of the formazan solution, as a measure of cell viability, was read using a microplate reader (Digiscan, Asys Hitech, Austria) at 550 nm. Dose–response cell viability curves were plotted and the 50% inhibitory concentration (IC50) was determined. IC50 is the concentration at which growth of 50% of the cells (compared with medium only treated control cells) was inhibited.

Morphological study

Morphological changes of NB4 cells were observed using an inverted phase contrast microscope (Olympus CK 40). Images of cells treated with 0.1–100 μM of AGP in a microplate were captured using a Hyper HAD camera (Digital Sony) at 24-, 48-, 72- and 96-h time points to observe morphological changes to identify cell differentiation and apoptosis processes.

Quantitation of cells undergoing differentiation

A suspension of control or test agent treated cells (100 μl) was mixed with 100 μl of 1 mg/ml NBT dye and 100 μl of 1% DMSO (as stimulant) in a sterile centrifuge tube and incubated at 37°C for 15 min. This was followed by further incubation at room temperature for 15 min. After centrifugation at 200*g* for 10 min, the cell pellet was fixed in 2.5% glutaraldehyde for 15 min at room temperature. Differentiation of NB4 cells was assessed by the ability of the cells to produce superoxide, as measured by the reduction/degradation of NBT. A minimum of 200 cells were scored microscopically and at least two scores were performed per experimental condition. Percentages of NBT positive cells were determined in both control and treated cells. Each treatment was carried out in triplicate and the experiment was repeated three times.

Analysis of retinoic acid receptor antagonist action

The growth-inhibiting effects of AGP and ATRA on NB4 cells were evaluated in the presence of AGN193109 (a pure RAR antagonist).[26] AGN193109 concentrations of 1 and 10 μM were used. The number of viable cells was determined using MTT assay as described earlier.

Assessment of cell death

NB4 cell death induced by AGP was assessed by phase contrast microscopy and confirmed by fluorescence microscopy of cells stained with fluorescence dyes, AO and PI. Cells treated with 9 μM of AGP for 48 h were collected and washed three times with PBS and centrifuged (200*g*, 10 min). The resulting cell pellet was resuspended in 20 μl of AO/PI

dyes (2.5 μg/ml each) and incubated on ice for 10 min. Viable cells were determined by the uptake of AO (green fluorescence) and exclusion of PI (red fluorescence) stain. Apoptotic cells were identified by their nuclear condensation, fragmentation, apoptotic bodies and cytoplasmic blebbing whereas the necrotic cells were identified by uniform labelling of the cells with PI.

DNA fragmentation analysis

DNA fragmentation occurring during apoptosis was determined by agarose gel electrophoresis after a selective extraction of degraded DNA from apoptotic cells was carried out according to the method of Subashini *et al.* (2005).[27] A total of 1×10^6 cells were washed three times with PBS and centrifuged (200*g*, 10 min). The resulting cell pellet was lysed in 100 μl of lysis buffer (10 mM Tris-HCl, 0.5% Triton X-100, 20 mM EDTA, at pH 7.5) containing 500 μg/ml proteinase K and 100 μg/ml RNAse at 50°C for 30 min. The lysate was centrifuged at 2600*g* at 4°C for 30 min. Supernatant was collected and DNA was extracted with phenol–chloroform–isoamyl alcohol (25 : 24 : 1). The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to determine the purity and concentration of DNA by measuring the absorbance at 260 and 280 nm. The DNA content was measured by assuming that 1 unit of absorbance at 260 nm is equal to 50 μg/ml of double-stranded DNA. DNA purity (A_{260}/A_{280}) in the range of 1.6–1.9 was used. Approximately 16 μg DNA in 20 μl TE buffer was separated in a 2% agarose gel containing 0.1 μg/ml ethidium bromide at 5 V/cm in TBE buffer. Agarose gel containing the separated DNA was photographed under UV transillumination. Untreated cells were used as a negative control, whereas cells treated with etoposide (42.5 μM, 6 h) were used as a positive control for induction of apoptosis.

Statistical analysis

The results are expressed as means ± SD. Statistical analyses of data were performed using one-way analysis of variance and Mann–Whitney U non-parametric test. Upon analysis of variance, individual differences between groups were evaluated using Bonferroni post-hoc test and those at $P < 0.05$ were considered statistically significant. Statistical analyses were performed with SPSS version 14 for Windows.

Results

Growth inhibitory effects of andrographolide and all-*trans* retinoic acid on acute promyelocytic leukaemia cells

Dose–response growth curves were generated using the percentage of cell survival converted from the absorbance readings at 550 nm (MTT end point), from which the IC50 values were determined (Figure 2). AGP inhibited the growth of all three cell lines, HL-60, NB4-R2 and NB4 cells. Interestingly, AGP had lower IC50 values in NB4-R2 (1.5 ± 0.3 μM) and HL-60 (2.4 ± 0.5 μM) compared with NB4 cells (4.5 ± 0.5 μM). Therefore, AGP was 2- and 3-fold more efficient in inhibiting the growth of HL-60 and NB4-R2

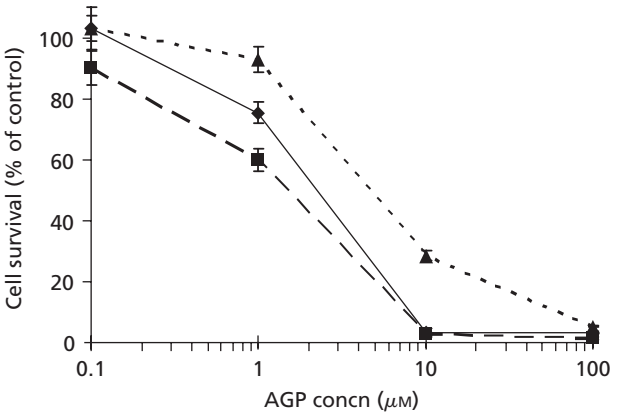


Figure 2 Representative graph of a dose–response growth inhibiting effect of andrographolide (AGP) on acute promyelocytic leukaemia cells obtained at 96-h time point. Cell viability after AGP treatment was determined by MTT assay. ♦, HL-60; ■, NB4-R2; ▲, NB4. Data are presented as means ± SD, $n = 6$.

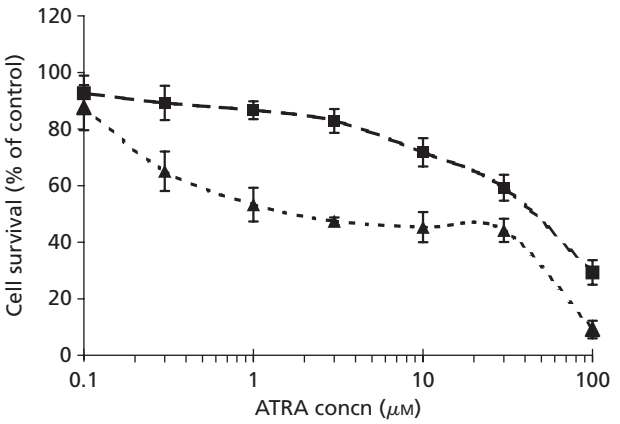


Figure 3 Representative graph of dose–response growth inhibitory effects of all-*trans* retinoic acid on NB4 cells. The absorbance at 550 nm determined by MTT assay is proportional to the number of living cells. ▲, NB4; ■, NB4-R2. Data are presented as means ± SD, $n = 6$.

as compared with NB4 cells ($P < 0.001$). The IC50 of ATRA in NB4 cells was 1.5 μM compared with 48.3 μM in NB4-R2 cells (Figure 3). This confirmed that NB4-R2 cells were approximately 32-fold more resistant to ATRA when compared with NB4 cells ($P < 0.001$).

Induction of differentiation

Routine light microscopy observation of NB4 cells exposed to IC50 concentration of AGP (4.5 μM) for 96 h revealed that the majority of cells underwent morphological differentiation. As a result, the cell-differentiating potential of AGP was quantified and compared with that of ATRA. Both AGP and ATRA induced similar time-dependent cell-differentiating activity. Between 24 and 72 h, ATRA was more active than AGP but comparable efficacy to ATRA was shown by AGP at 96 h, when AGP induced 72% cell differentiation compared with 79% by ATRA (Figure 4). Throughout the experimental period, the control cells did not

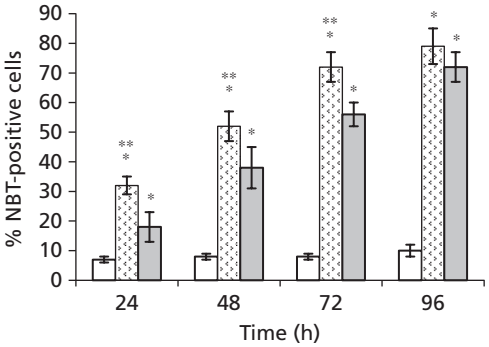


Figure 4 Representative graph of induction of differentiation by 1.5 μM all-*trans* retinoic acid (ATRA) and 4.5 μM andrographolide (AGP), determined by quantifying percentage NBT positive cells. Both all-*trans* retinoic acid (ATRA) and andrographolide (AGP) significantly ($P < 0.05$) induced increased cell differentiation in a time-dependent fashion, except for ATRA from 72 h to 96 h. AGP induced comparable differentiation to ATRA at 96 h. Open columns, control; stippled columns, ATRA; shaded columns, AGP. *$P < 0.05$, compared with control; **$P < 0.05$, compared with AGP (analysis of variance followed by Bonferroni post-hoc test). Data are presented as means ± SD, $n = 3$.

show significant changes in the number of cells undergoing differentiation, which was ≤ 10%.

Effect of pure retinoic acid receptor antagonist AGN193109 on the growth inhibitory effects induced by andrographolide and all-*trans* retinoic acid

It has been well established that differentiation of NB4 cells by ATRA is mediated through the RARα nuclear receptor pathway.[24] To determine whether the growth inhibitory effect of test compounds is mediated via the RAR pathway, a pure RAR antagonist, AGN193109, was used to block RAR in both ATRA- and AGP-treated cells. NB4 cells were treated with AGP and ATRA in the absence and presence of the antagonist at concentrations of 1 μM and 10 μM for 96 h. The IC50 of ATRA in NB4 cells in the absence of RAR antagonist was 1.5 ± 0.5 μM, but in the presence of 1 μM and 10 μM antagonist the IC50 values increased to 4.6 ± 0.3 μM and > 100 μM, respectively ($P < 0.001$) (Table 1). Therefore, AGN193109 at 1 μM and 10 μM decreased the growth inhibitory action of ATRA by 3-fold and > 67-fold, respectively. However, no change in the growth inhibitory effect of AGP was observed in the presence of the antagonist. It should be noted that AGN193109 alone at 1 and 10 μM did not affect the growth NB4 cells (data not shown), which validates its application as a pure antagonist of RAR, without partial agonist activity, as reported previously.[26]

Table 1 Effect of AGN193109 on the growth inhibitory effect induced by all-*trans* retinoic acid (ATRA) and andrographolide (AGP) on NB4 cells

AGN193109 (μM)	IC50 (μM)	
	ATRA	AGP
0	1.5 ± 0.5	4.5 ± 0.5
1	4.6 ± 0.3*	4.5 ± 0.3
10	> 100 ± 0*	4.5 ± 0.6

Values are means ± SD obtained from three independent experiments. *$P < 0.05$ compared with ATRA treatment without the addition of AGN193109.

Types of cell death induced by andrographolide in NB4 cells

Using an inverted phase contrast microscope, morphological features of nuclear condensation, fragmentation and apoptotic bodies indicative of apoptotic cell death were observed in NB4 cells treated with 9 μM AGP (Figure 5). The intensity of apoptosis occurred in a time-dependent manner and almost all cells underwent apoptosis at the 96-h time point. A small fraction of cells undergoing differentiation was observed in control cells. Fluorescence microscopy using AO and PI, two DNA binding dyes, further supported the presence of apoptotic cells when treated with 9 μM AGP for 48 h (Figure 6).

DNA fragmentation analysis

Mode of cell death by apoptosis was further confirmed by the presence of 200 bp internucleosomal DNA fragmentation on agarose gel electrophoresis in cells treated with 9 μM AGP (Figure 7). The DNA fragmentation was prominent in NB4 cells treated for 48 h with subsequent thickening of DNA bands at 72 h and 96 h. Etoposide, a clinically used cytotoxic anticancer agent, was included as a positive control to validate this process. The 200 bp DNA ladder was absent in the untreated control cells. Although the 24-h treatment showed some cells with morphological features of apoptosis (Figure 5), this effect could not be corroborated with DNA fragmentation. It is believed that this might have been due to low amounts of cells undergoing the 200 bp DNA fragmentation.

Discussion

The current treatment regime for APL is a combination of ATRA with anthracyline-based chemotherapy, which has produced excellent remissions and survival in patients.[3] However, even with this improved treatment strategy some patients do relapse after ATRA treatment and, unfortunately, most of these patients are resistant to further treatment with ATRA.[7–10] To overcome this form of resistance, presently, arsenic trioxide (ATO) and gemtuzumab are being employed in the clinic with good success rate.[28,29] Owing to the fact that new agents with different mechanisms of growth inhibition might offer therapeutic advantage over present drugs for the management of APL, we investigated AGP for its efficacy against APL cell line models, represented by ATRA-sensitive NB4 and HL-60, and ATRA-resistant NB4-R2 cells. AGP was previously shown to have mouse myeloid cell-differentiating activity,[13] and in the present study AGP's cell-differentiating and apoptosis-inducing potential were examined in NB4 cells to elucidate the mechanism of growth inhibition by AGP in this cell line, representing primary APL.

We showed that AGP had growth inhibitory potential in all the three APL cell line models. The NB4 cell line is a relevant model in this study as it exhibits the reciprocal

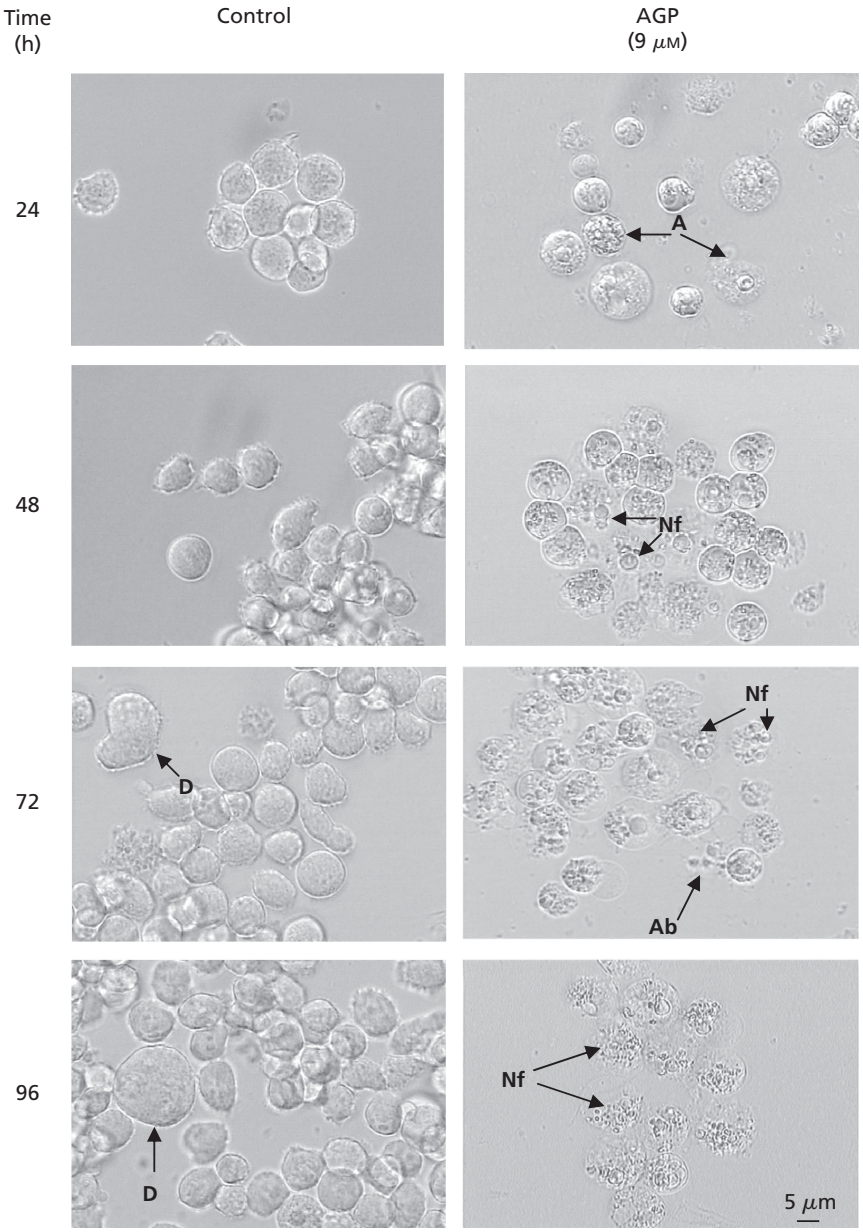


Figure 5 Phase contrast photomicrographs of NB4 cells treated with 9 μM andrographolide (AGP) at various time points. A, apoptosis; Nf, nuclear fragmentation; Ab, apoptotic bodies. Control cells show some spontaneously differentiating cells (D).

chromosomal translocation *t*(15;17) specific for APL, with the resultant production of the chimeric protein PML-RARα.[20] This abnormal fusion protein blocks differentiation of promyelocytes into granulocytes, resulting in the accumulation of promyeloblasts with infinite proliferative capacity coupled with resistance to undergo apoptosis. ATRA exploits this underlying molecular abnormality by binding the RARα region of the PML-RARα complex to overcome maturation defect by promoting promyeloblasts to differentiate and ultimately undergo apoptosis, a paradigm of a disease sensitive to differentiation therapy.[24] As reported previously,[4] we managed to show in this study that, when treated at a pharmacological concentration of 1.5 μM, ATRA induced differentiation of NB4 cells as indicated by morphological features of maturation (not shown) and increased NBT-positive cells (Figure 4), in a time-dependent manner.

Comparison of growth inhibition by ATRA in NB4 and NB4-R2 cells confirmed that NB4-R2 cells were 32-fold more resistant towards ATRA. Intriguingly, AGP had an opposite effect, whereby it was more active by 3-fold in the ATRA-resistant cell line compared with the parental cell line, which elegantly supports AGP's potential in the treatment of ATRA-resistant APL. This property of AGP has particular importance in the clinical setting, as some APL patients treated with ATRA develop relapse and acquire resistance to it.[7–10] Although optional treatments utilising ATO and gemtuzumab are presently being employed in such patients with good success rate, identification of new agents

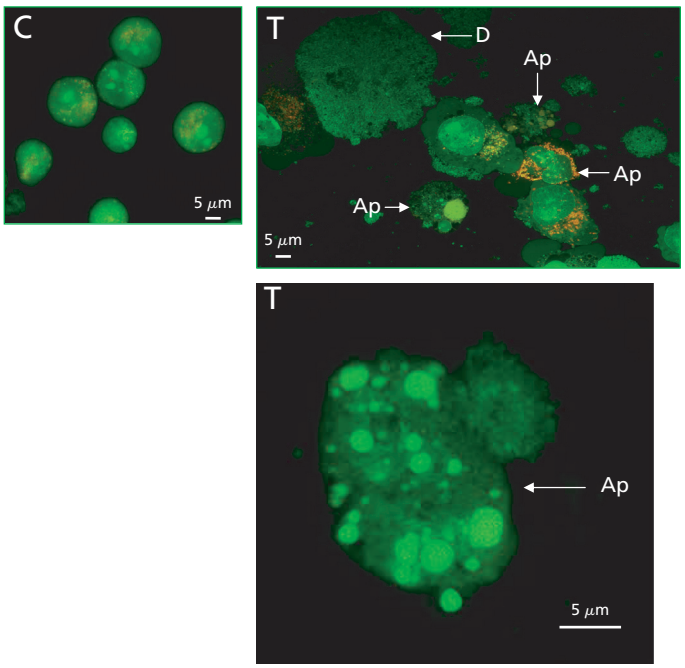


Figure 6 Fluorescence photomicrographs of 48-h control (C) and 9 μM andrographolide treated (T) NB4 cells. Treated cells showed morphological features of apoptosis (membrane blebbing, nuclear condensation and fragmentation, Ap) and differentiation (D). Most of the treated cells underwent apoptosis. The untreated control cells displayed a normal uniform morphology.

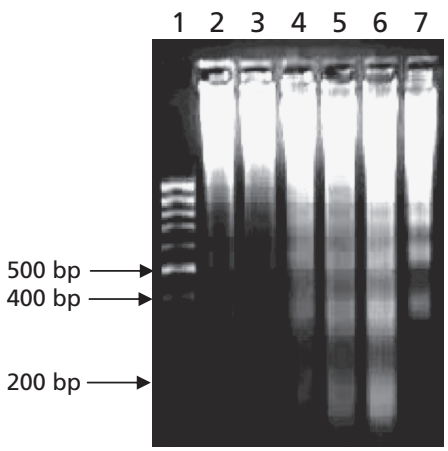


Figure 7 200 bp internucleosomal DNA ladder induced in NB4 cells treated with 9 μM andrographolide (AGP). Lane 1, 100 bp molecular weight markers; lane 2, DNA of vehicle treated control cells; lanes 3–6, DNA from 24-, 48-, 72- and 96-h AGP treated cells, respectively; lane 7, DNA of etoposide treated cells (42.5 μM, 6 h) as positive control. NB4 cells treated with AGP underwent a time-dependent internucleosomal DNA fragmentation, suggesting apoptosis as the main mode of cell death induced by this compound. The control cells treated with 0.009% DMSO as vehicle at various time points did not show DNA fragmentation, represented by cells harvested at 96 h as shown in this figure.

that are able to overcome ATRA resistance might offer alternative agents to be included in the armamentarium of drugs for APL that has acquired resistance to ATRA.

AGP was also shown to be active with regard to differentiation induction in the NB4 cells as evaluated by several criteria, including morphological changes and reduction of NBT. AGP and ATRA at their IC50 concentrations induced maturation of NB4 cells in a time-dependent fashion and reached near equal efficacy at 96 h. The involvement of RAR-mediated cell differentiation by AGP was examined by using a pharmacological antagonist of RAR (AGN193109). The antagonist failed to reverse the growth inhibitory effect of AGP on NB4 cells, suggesting AGP-mediated cell-differentiating activity is not via binding to RAR, unlike ATRA. As anticipated, the growth inhibitory effect of ATRA was blocked by the antagonist (Table 1). The effect of AGN193109 was concentration dependent and this agent at 10 μM caused almost complete reversal of growth inhibition by ATRA. The mechanism of action of ATRA is by binding of the RARα of PML-RARα complex to release co-repressors of myeloid differentiation such as HDAC and Dnmt's, to allow promyelocytes to differentiate into terminal mature neutrophils that ultimately undergo cell death via the apoptotic pathway.[2] ATRA treatment also causes degradation of the PML-RARα chimeric protein.[30] It is important to note that AGN193109 is a pure antagonist of RAR with no intrinsic agonist activity.[26,31] Based on these findings we are tempted to speculate the possibility of AGP acting through the other retinoid receptor pathway mediated by RXR or even being a ligand for coactivator/corepressor of myeloid differentiation, to induce maturation of NB4 cells. The RXR pathway also plays an important role in the induction of differentiation[32,33] and antagonists of this receptor have been shown to induce apoptosis in leukaemia cells.[34] However, the hypothesis on the mechanism of action of AGP needs to be confirmed experimentally to pave the way for the development of new cell-differentiating agents for the treatment of APL. We believe

that upon AGP-induced differentiation of NB4 cells, the mature cells very likely undergo apoptotic cell death. Similar findings, in which cell differentiation induced apoptotic cell death, have been reported for clinical agents like ATRA and ATO.[4,35] As discussed above, the cell-differentiating activity of AGP apparently was not mediated by RAR. The RAR-independent mechanism by AGP is reinforced by the observation of improved activity of AGP against ATRA-resistant NB4-R2 cells as compared with the wild-type NB4 cells.

Apart from the cell-differentiation inducing potential, AGP also promoted apoptotic cell death in NB4 cells when treated with twice the IC50 (9 μM). This was evaluated by cell morphology (inverted light and fluorescence microscopy) and the presence of 200 bp internucleosomal DNA fragmentation in treated cells. Morphological features of apoptosis induced by AGP include nuclear condensation, fragmentation and presence of apoptotic bodies (Figure 5). The induction of internucleosomal DNA fragmentation shown by the 200 bp ladder confirmed the apoptosis mechanism, as this too occurred in a time-dependent manner similar to morphological features of apoptosis observed under light microscopy. The electrophoretic detection of DNA fragmentation has been accepted as a hallmark of apoptosis.[36] The apoptosis induced by AGP at a concentration of 9 μM most likely occurred due to direct cytotoxic effect of this compound, as only cells undergoing apoptosis and not differentiation were observed under the inverted light microscopy (Figure 5) throughout the experimental period of 96 h. At a lower concentration (4.5 μM), however, this compound seemed to have induced predominantly cell differentiation (Figure 4). Although various researchers[17–19,37,38] have described the mechanisms of action of AGP in inducing apoptosis, we are not certain if similar pathways were involved in NB4 cell apoptosis.

Based on the findings of this study, we would like to propose that the growth inhibition of NB4 cells by AGP could have occurred predominantly via induction of differentiation for granulocyte maturation and ultimately apoptosis at lower concentrations as observed with the IC50 concentration (4.5 μM), and by direct cytotoxic effect to induce mainly apoptosis at the higher concentration of twice the IC50 value (9 μM). This notion was further strengthened by studies reporting the induction of apoptosis by AGP in cancer cells as a mechanism of cytotoxicity.[15–19] Therefore, a combination of cell-differentiating and cytotoxic pathways is thought to be involved in AGP-mediated anti-leukaemic effect *in vitro*, which is indeed desirable to overcome APL cases that have relapsed after ATRA treatment or are refractory to ATRA. Drug-like compounds with cell differentiation and apoptosis-inducing properties are being considered as potential therapeutic agents with better treatment outcome for acute myeloid leukaemia.[11] Further supportive evidence of the potential of AGP in the management of APL comes from the finding that ATO has shown overwhelming clinical efficacy against ATRA-relapsed and -refractory APL,[39,40] and promotes growth inhibition of APL cells by inducing cell differentiation via PML-RARα at low concentrations and apoptosis at higher concentrations,[35] similar characteristics to AGP. Moreover, the importance of discovery of new drugs for ATRA-relapse APL cases has recently been highlighted with the intention of identifying single-chemical entities to overcome resistance to ATRA via cell differentiation and apoptosis pathways.[41,42]

A pharmacokinetic study has shown that an in-vivo plasma concentration in the range of 3–30 μM is achievable in mice treated with a single dose of 150 mg/kg of AGP (intraperitoneally) without inducing a significant change in the body weight.[15] Since this concentration is within the range that induces NB4 cell differentiation and apoptosis *in vitro*, as discussed above, AGP is expected to have in-vivo activity against APL. To establish this proof-of-concept, the investigation of the in-vivo anti-APL activity of AGP is warranted.

Overall, evidence to date indicates that AGP is well tolerated in humans, when used appropriately at doses of 5–10 mg/kg per day as reported by Calabrese *et al.*[43] in a human study to assess the efficacy of AGP against HIV infection. Therefore, AGP and its derivatives may form safe and effective drugs for the treatment of primary and ATRA-resistant APL. Recently, we revealed the synthesis of new derivatives of AGP with cytotoxic property[44,45] but, inferring from the present study, these compounds may also possess myeloid cell-differentiating activity that could be exploited for the development of effective chemotherapeutic agents for the treatment of APL.

Conclusions

In summary, we showed that AGP inhibited cell growth by inducing differentiation and apoptosis in NB4 cells. Additionally, AGP also promoted inhibition of proliferation of NB4-R2 and HL-60 cells, suggesting that its mechanism of action is different from that of ATRA. Therefore, owing to the different mechanisms of action of ATRA and AGP, these compounds, if combined, may produce a synergistic effect in APL. Using a pharmacological antagonist of RAR we concluded the mechanism of growth inhibition of APL cells by AGP is not mediated via RAR. Therefore, it is highly possible that AGP mediates its effect through a novel route and identification of this pathway would lead the way in the discovery of new agents with improved efficacy and potency as cell-differentiating and apoptosis-inducing agents for the treatment of APL.

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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