Chemosensitivity enhancement toward arsenic trioxide by inhibition of histone deacetylase in NB4 cell line

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Abstract

Objective: To investigate the cytotoxic effects of suberanilohydroxamic acid (vorinostat) in combination with arsenic trioxide (ATO) on the human NB4 cell line *in vitro*.

Methods: The rates of cell proliferation following treatment with vorinostat with or without ATO were measured. Flow cytometry of Annexin-V/propidium iodide double-stained cells was used to measure apoptosis. Acridine Orange and ethidium bromide staining was used to observe morphological changes characteristic of apoptosis. Western blot analysis was used to measure protein levels.

Results: Vorinostat and ATO, alone and in combination, inhibited the proliferation of NB4 cells in a time- and dose-dependent manner and the effect was additive. NB4 cells treated with vorinostat + ATO demonstrated greater levels of apoptosis compared with cells treated with either drug alone. Both vorinostat and ATO alone and in combination resulted in lower levels of promyelocytic leukaemia/retinoic acid receptor alpha fusion protein and increased levels of acetyl-histone H3 and acetyl-histone H4 proteins compared with controls. Vorinostat + ATO resulted in lower levels of Akt protein compared with either drug alone.

Conclusion: The combination of vorinostat and ATO inhibited cell proliferation, induced apoptosis, and enhanced the chemosensitivity of NB4 cells. The mechanism might be associated with increasing histone acetylation levels as well as downregulation of the Akt signalling pathway.

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Introduction

Histone acetylation and deacetylation comprise one of the most common modifications found in epigenetics.[1] Histone acetylation helps transcription factors bind to DNA templates and activates transcription; in contrast, histone deacetylation inhibits transcription.[2–5] In 2006, the second generation hydroxamic acid histone deacetylase inhibitor (HDACi) suberanilohydroxamic acid (vorinostat; also known as SAHA) was approved by the US Food and Drug Administration for the clinical treatment of relapsed or refractory cutaneous T-cell lymphoma (CTCL).[6]

Acute promyelocytic leukaemia (APL) is a form of acute myeloid leukaemia with specific epidemiological, pathogenetic and clinical features.[7] The molecular hallmark of APL is the presence of a balanced reciprocal translocation resulting in the promyelocytic leukaemia (*PML*)/retinoic acid receptor alpha (*RAR-α*) gene fusion, which represents the target of all-*trans* retinoic acid (ATRA) therapy.[8] The introduction of ATRA in conjunction with anthracyclines marked a turning point in the treatment of APL.[9] However, this treatment combination is unable to completely eradicate the malignant APL clone.[10] Moreover, drug resistance has been observed in clinical practice.[11] Given our previous experience with the K562 cell line,[12] we hypothesized that in order to better eradicate the APL clone and overcome drug resistance, vorinostat could be used to augment chemosensitivity by combining it with arsenic trioxide (ATO), another widely used chemotherapeutic agent used to treat haematological malignances. This present study investigated the antileukaemic effect of vorinostat combined with ATO on the NB4 cell line, which is a maturation inducible cell line with a t(15;17) marker that was isolated from a human acute promyelocytic leukaemia.[13]

Materials and methods

Cell culture

The NB4 cell line was a gift from the Tianjin Institute of Haematology, Tianjin, China. The NB4 cell line was preserved and cultured in Fujian Provincial Key Laboratory on Haematology, Department of Haematology, Fujian Institute of Haematology, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, China following a standard protocol as previously described.[14,15] Generally, the NB4 cells were cultured in RPMI-1640 culture medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Tianjin Haoyang Biological Manufacture Company, Tianjin, China) at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents

Dimethyl sulphoxide (DMSO) was diluted to a concentration of 10 mmol/l. ATO (Heilongjiang Harbin Medical University Pharmaceutical Co., Harbin, China) was diluted to a concentration of 2 mmol/l in 10 mM phosphate-buffered saline (PBS; pH 7.4). Vorinostat was provided by Aton Merck Pharmaceutical (Kenilworth, NJ, USA). Vorinostat was dissolved in DMSO. Both vorinostat and ATO were stored at −20°C. Penicillin and streptomycin

were purchased from Hyclone (Burlington, Canada). Protein extraction reagents were purchased from Wuhan Boster Biological Company (Wuhan, Hubei, China).

Cell proliferation assay

A cell-counting kit-8 (CCK-8) assay was used to measure NB4 cell line proliferation according to the manufacturer's instructions (Dojindo Molecular Technologies, Rockville, MD, USA). In brief, a total of 5×10^4 cells/ml were seeded into individual wells of a 96-well cell culture plate to a final volume of 100 μl and cultured at 37°C in a humidified atmosphere containing 5% CO_2. Blank control (medium only), negative control (PBS-treated) and drug-treated groups exposed to various concentrations were set up (vorinostat: 0.125, 0.25, 0.5, 1 μmol/l; ATO: 0.5, 1, 2, 4 μmol/l). The assay was completed in triplicate for each group. After 24, 48, and 72 h of treatment, 10 μl of CCK-8 reagent was added into each well and incubated for 1–4 h at 37°C in a humidified atmosphere containing 5% CO_2. The absorbance of the cell culture medium at 450 nm was measured using a microplate reader (ELx808 Absorbance Reader; BioTek, Winooski, VT, USA). The inhibition rate was calculated using the following equation: inhibition rate = (negative control group – experimental group)/ negative control group $\times$ 100%. An inhibition of proliferation curve was plotted based on the drug concentration and the proliferation inhibition rate. The Q value was calculated by combining the inhibitory effects of vorinostat and ATO. Q was calculated using the following equation: Q = EA + B/[(EA + EB)–(EA × EB)], where EA + B, EA and EB represented the inhibition rate of combination treatment, A only and B only, respectively. Then Q ≤ 0.85, 0.85 ≤ Q < 1.15, and Q ≥ 1.15 represented

respectively antagonistic, additive and synergistic effects.[14,15]

Annexin-V and PI staining

Apoptosis was detected using an apoptosis detection kit (Annexin-V-fluorescein iso-thiocyanate [FITC], propidium iodide [PI] double staining; Roche, Shanghai, China) according to the manufacturer's instructions. Approximately 1×10^6 NB4 cells were incubated with the appropriate concentration of the test drugs for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested after a single wash with 10 mM PBS (pH 7.4) at room temperature. Then, binding buffer (100 μl) from the kit was added to each well and the NB4 cells were resuspended. Annexin-V (2 μl) and PI (2 μl) solutions from the kit were added to each well and the cells were incubated at room temperature for 10–15 min in the dark. The NB4 cells were analysed by flow cytometry (BD FACSVerse^{TM} flow cytometer; Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Annexin-V$^-$/PI$^-$ cells were living cells, Annexin-V$^+$/PI$^-$ cells were early apoptotic cells and Annexin-V$^+$/PI$^+$ were late apoptotic cells. This experiment was repeated three times.

AO/EB fluorescence staining for apoptosis

Approximately 1×10^6 NB4 cells were incubated with the appropriate concentration of the test drugs for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Approximately 5×10^6 cells/ml suspended in 95 μl were mixed with Acridine Orange/ ethidium bromide (AO/EB) (5 μl, 100 μg/ml for both reagents; Amresco, Solon, OH, USA). Immediately after mixing, one drop of suspended cells was placed on a clean glass slide and observed using fluorescence microscopy (Eclipse TE2000-U

Inverted Microscope; Nikon, Tokyo, Japan) at an excitation wavelength of 490 nm at ×400 magnification. Cells with green fluorescence in the nucleus and cytoplasm were living cells; cells with yellow-green fluorescence in the nucleus or cytoplasm were apoptotic cells; and cells with red fluorescence in nucleus were necrotic cells.

Western blot analysis

Approximately 1×10^6 NB4 cells were incubated with the appropriate concentration of the test drugs for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. After the cells were lysed using a Protein Extraction Kit (Wuhan Boster Biological Company, Hubei, China) following the manufacturer's instructions, the supernatant was collected and quantified. Total protein (40 µg) was transferred to a polyvinylidene diuoride membrane after sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After blocking with 5% nonfat milk (Lulong Biotech, Xiamen, China) in $1 \times$ Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.4) at room temperature for 2 h, the rabbit antihuman polyclonal antibody against PML-RAR-α (1:500 dilution; Abcam®, Cambridge, MA, USA), and the antihuman monoclonal antibodies against Bcl-2 (1:500 dilution), p21 (1:500 dilution), acetyl-histone H3 (1:1000 dilution), acetyl-histone H4 (1:1000 dilution), pAkt (1:1000 dilution) and Akt (1:1000 dilution) (all monoclonal antibodies from Cell Signaling Technology®, Danvers, MA, USA) were added and incubated at 4°C overnight. After washing three times with TBST for 10 min at room temperature, the secondary antibody (horseradish peroxidase-conjugated mouse antirabbit immunoglobulin G, 1:5000 dilution; Cell Signaling Technology®) was added and incubated for 2 h at room temperature. Membranes were washed three times with TBST for 10 min at room temperature. The chemiluminescence reaction was performed and immunoreactive signals were visualized using an Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad, Hercules, CA, USA). β-actin was used as the internal loading control and immunostained with rabbit antihuman antibodies (1:2000 dilution; Origene, Beijing, China) as described for the target proteins.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 11.5 (SPSS Inc., Chicago, IL, USA) for Windows®. The mean ± SD values were compared using Dunnett's *t*-test. A *P*-value < 0.05 was considered statistically significant.

Results

When NB4 cells were exposed to various concentrations of vorinostat or ATO, both drugs inhibited cell proliferation as measured by the CCK-8 assay compared with the blank control group in a time- and dose-dependent manner (Figure 1) (highest concentration versus the blank control group for both drugs, $P < 0.05$). When vorinostat was used in combination with ATO it demonstrated an additive effect on the inhibition of NB4 cell proliferation (Table 1).

Annexin-V-FITC/PI double staining flow cytometry can distinguish between living cells, early apoptotic and late apoptotic cells. In the flow cytometry scatter plots, the lower left quadrant contains living cells; the lower right quadrant contains early apoptotic cells; the upper right quadrant contains late apoptotic cells; and the upper left quadrant contains necrotic cells. The results showed that 95% of the cells in the control group were living cells (Figure 2A). The rate of apoptosis in NB4 cells increased in a dose-dependent manner after 48 h of exposure to vorinostat and ATO used
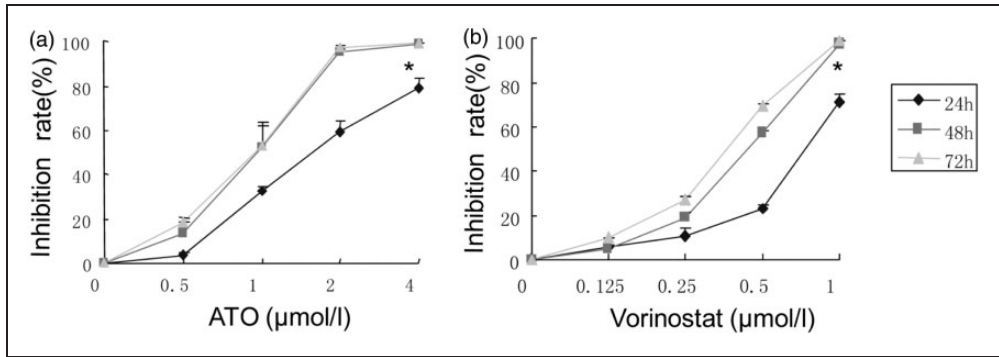
Figure 1. Inhibition of NB4 cell proliferation during treatment with vorinostat or arsenic trioxide (ATO) at various concentrations and over three time periods (24 h, 48 h, and 72 h). (a) The inhibition of proliferation curve of NB4 cells treated with ATO alone. (b) The inhibition of proliferation curve of NB4 cells treated with vorinostat alone. Data presented as mean $\pm$ SD ($n = 3$).
*$P < 0.05$ versus the blank control group; Dunnett's *t*-test.

Table 1. The effects of treatment with vorinostat with or without arsenic trioxide (ATO) for 48 h on the inhibition of proliferation of the NB4 cell line.

Concentration, μmol/l		Inhibition, %	Q value	Additive effect
Vorinostat	ATO			
Vorinostat	ATO			
0	0	0		
0.125	0	5.00		
0.25	0	19.00		
0.5	0	57.61		
1	0	97.80		
0	0.5	13.47		
0	1	52.29		
0	2	95.13		
0	4	98.53		
0.125	0.5	19.49	1.094	+
0.125	1	46.50	0.850	+
0.125	2	84.35	0.884	+
0.25	0.5	33.78	1.128	+
0.25	1	52.83	0.861	+
0.25	2	86.23	0.898	+
0.5	0.5	69.68	1.100	+
0.5	1	86.70	1.087	+
0.5	2	98.84	1.009	+

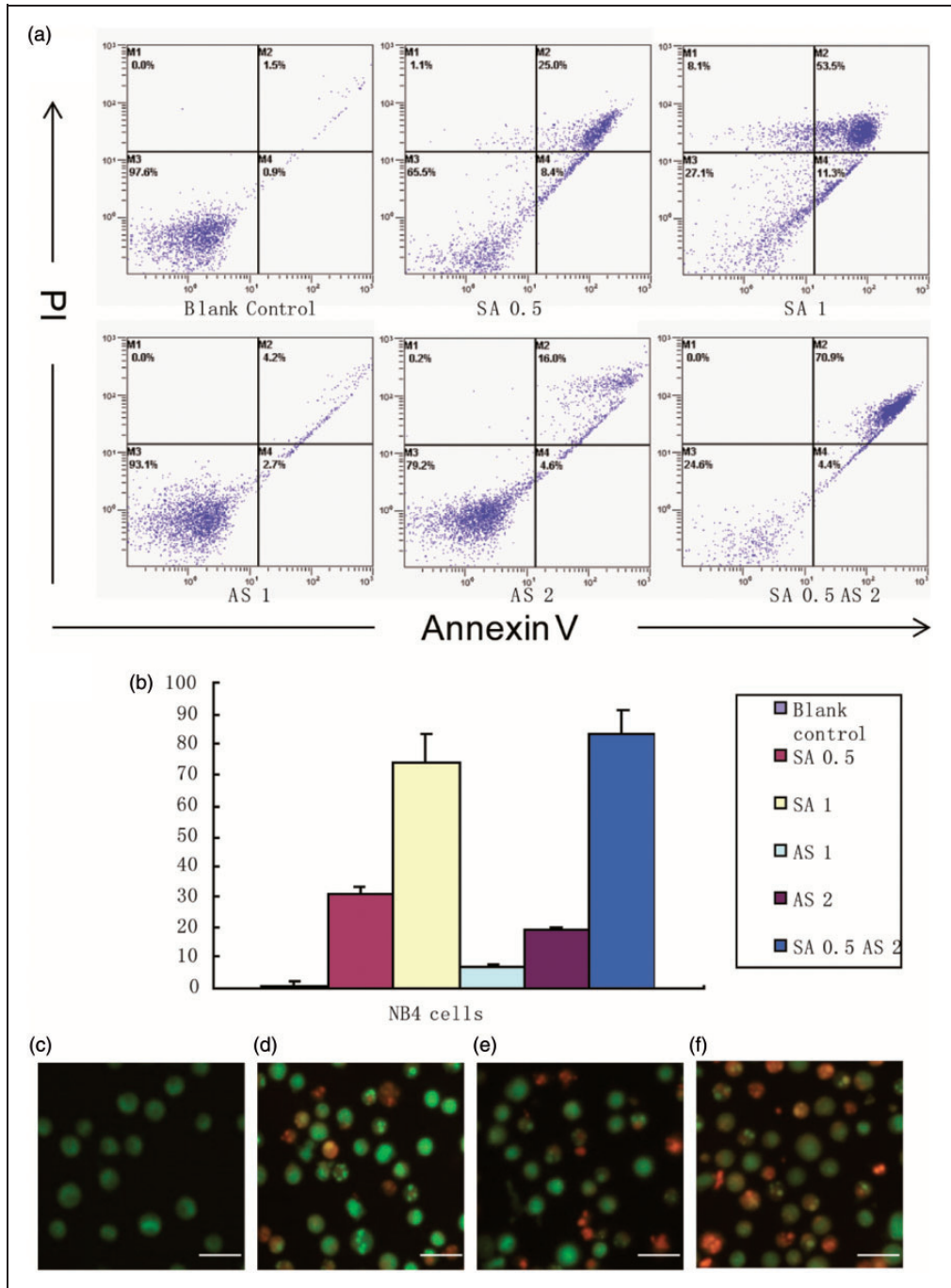Data presented as mean ($n = 3$).

Figure 2. Results of experiments into the effects of vorinostat and arsenic trioxide (ATO) on the rates of apoptosis of NB4 cells. (a) Representative flow cytometric scatter plots using Annexin-V and propidium iodide (PI) staining to identify apoptotic NB4 cells after treatment for 48 h with vorinostat with or without ATO;

individually (Figure 2B). The mean $\pm$ SD rates of apoptosis of the NB4 cells were $31.25 \pm 2.15\%$, $19.25 \pm 1.35\%$, and $83.05 \pm 7.75\%$ at 48 h after treatment with $0.5\,\mu mol/l$ vorinostat, $2\,\mu mol/l$ ATO and $2\,\mu mol/l$ ATO $+ 0.5\,\mu mol/l$ vorinostat, respectively. Vorinostat $(0.5\,\mu mol/l)$ in combination with ATO $(2\,\mu mol/l)$ showed a significant increase in the level of apoptosis of NB4 cells compared with the equivalent drug doses used alone ($P < 0.01$ for both comparisons; Figure 2B).

The apoptosis results from the flow cytometry were confirmed by AO/EB staining. In the blank control group, NB4 cells showed uniform cell size and morphology and homogeneous green fluorescence in the nucleus and cytoplasm (Figure 2C). At 48 h after vorinostat $(0.5\,\mu mol/l)$ or ATO $(2\,\mu mol/l)$ treatment alone, differences in cell size and morphology were observed (Figure 2D & 2E), which were characterized by dense nuclei that showed yellow-green fluorescent debris. Vorinostat $(0.5\,\mu mol/l)$ in combination with ATO $(2\,\mu mol/l)$ showed more apoptotic characteristics than the single drugs alone (Figure 2F).

Western blot analysis showed that treatment of NB4 cells for 48 h with vorinostat $(0.5\,\mu mol/l)$ and ATO $(2\,\mu mol/l)$ alone and in combination resulted in lower levels of PML-RAR-α protein and Bcl-2 compared with the blank control cells (Figure 3). Treatment with vorinostat $(0.5\,\mu mol/l)$ and the combination group resulted in increased levels of acetyl-histone H3 and acetyl-histone H4 proteins compared with the untreated control cells and the cells treated with ATO $(2\,\mu mol/l)$ alone. Treatment with vorinostat $(0.5\,\mu mol/l)$ alone resulted in increased levels of p21, while ATO $(2\,\mu mol/l)$ alone and in the combination group showed no changes in p21 protein levels. The vorinostat $(0.5\,\mu mol/l)$ and ATO $(2\,\mu mol/l)$ combination group showed lower levels of both Akt and pAkt protein compared with either drug alone.

Discussion

There are a variety of HDACis undergoing clinical trials for the treatment of solid tumours and haematological malignancies, especially for the treatment of CTCL, peripheral T-cell lymphoma, and Hodgkin's lymphoma, which have shown promising results.[16,17] As a novel HDACi, vorinostat was approved by the US Food and Drug Administration for the treatment of progressive clinical recurrence of CTCL.

Arsenic trioxide is an important medicine for treating leukaemia, particularly APL.[18] In addition, chemotherapy regimens based on ATO are the focus of considerable research.[19] In recent years, the mechanism of action and the clinical applications of ATO have been reported.[20] ATRA and arsenic compound-based combination therapy was reported to be effective in reinducing morphological remission in relapsed

(b) Bar chart showing the rates of apoptosis of NB4 cells as determined by the flow cytometric analysis: Blank control: untreated cells (medium only); SA 0.5: 0.5 $\mu mol/l$ vorinostat; SA 1: 1 $\mu mol/l$ vorinostat; AS 1: 1 $\mu mol/l$ ATO; AS 2: 2 $\mu mol/l$ ATO; SA 0.5 + AS 2: 0.5 $\mu mol/l$ vorinostat + 2 $\mu mol/l$ ATO. Data presented as mean $\pm$ SD ($n = 3$); (C–F) Representative fluorescence microscopy images (original magnification $\times 400$) showing NB4 cells after treatment for 48 h with vorinostat with or without ATO stained with Acridine Orange/ethidium bromide: (c) blank control cells; (d) 0.5 $\mu mol/l$ vorinostat; (e) 2 $\mu mol/l$ ATO; (f) 0.5 $\mu mol/l$ vorinostat + 2 $\mu mol/l$ ATO. Cells with green fluorescence in the nucleus and cytoplasm were living cells; cells with yellow-green fluorescence in the nucleus or cytoplasm were apoptotic cells; and cells with red fluorescence in the nucleus were necrotic cells. Scale bar 25 μm. The colour version of this figure is available at: http://imr.sagepub.com.
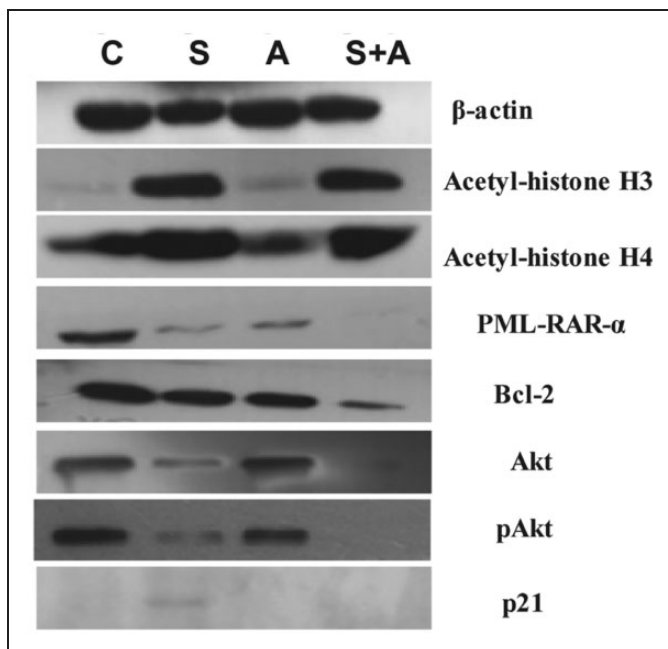
Figure 3. Western blot analysis of protein levels in NB4 cells treated with vorinostat with or without arsenic trioxide (ATO) for 48 h. The proteins detected were as follows: promyelocytic leukaemia (PML)/ retinoic acid receptor alpha (RAR-α) fusion protein, Bcl-2, p21, acetyl-histone H3, acetyl-histone H4, Akt and pAkt. β-actin was used as an internal loading control. C: blank control; S: 0.5 μmol/l vorinostat; A: 2 μmol/l ATO; S + A: 0.5 μmol/l vorinostat + 2 μmol/l ATO.

patients with APL who had previous exposure to ATRA and arsenic compounds, but molecular remission rates remained low and there was a high risk of secondary relapse.[21] This present study demonstrated enhanced chemosensitivity of the NB4 cell line when vorinostat was combined with ATO, which suggests that a combination of vorinostat and ATO therapy could have clinical applications in the treatment of leukaemia.

The present study demonstrated that treatment of NB4 cells with vorinostat or ATO inhibited cell proliferation in a time- and dose-dependent manner. The combination treatment showed an additive effect in inhibiting cell proliferation. The present findings also showed that the vorinostat and ATO combination resulted in a greater level of NB4 cell apoptosis than the single drugs used alone. These preliminary *in vitro*

results suggest that this combination should be investigated further as it might offer beneficial effects such as improved chemotherapeutic effects and minimized side-effects as seen with other combinations.[22]

The present study used Western blot analysis to demonstrate that treatment of NB4 cells with vorinostat alone and in combination with ATO resulted in increased levels of acetyl-histone H3 and acetyl-histone H4 protein compared with treatment with ATO alone and the blank control cells. This outcome suggests that vorinostat can increase histone acetylation levels and activate transcription, thereby inducing haematological tumour cell apoptosis.[4,5] p21 is an important cell cycle regulatory protein involved in cell growth, differentiation, aging and death.[23] p21 plays a negative role in the regulation of the cell cycle by

acting on cyclin-dependent kinase inhibitor.[24] For example, the combination of celecoxib and fluvastatin also increased levels of p21, and decreased levels of p-Akt, myeloid cell leukaemia-1 and survivin protein in tumours.[22] In this present study, p21 levels were upregulated after treatment with vorinostat compared with ATO and the blank control cells, thereby blocking the cell cycle and inducing apoptosis. Interestingly, combination therapy did not increase the levels of p21 protein in the Western blot analysis. The mechanism of action of vorinostat requires further research. Akt is a serine/threonine protein kinase that plays a major role in the antiapoptotic pathway in apoptosis signaling.[25] This pathway could be a new target for anticancer therapies, which could have implications for clinical trial design.[26] The present study showed that vorinostat alone and in combination with ATO downregulated the levels of Akt protein compared with ATO alone and the blank control cells. Compared with the single drug treatment groups, the vorinostat + ATO combination group considerably downregulated Akt protein levels, suggesting that the mechanism of apoptosis induction might be associated with the downregulation of the Akt signalling pathway.[27]

This present study had several limitations. Although the study demonstrated that PML-RAR-α fusion protein levels were decreased by vorinostat treatment, this must be further confirmed by measuring the mRNA and protein levels of PML-RAR-α fusion protein in not only NB4 cells but also in primary cells from APL patients. In support of the current findings, similar effects have been seen in a chronic myeloid leukaemia cell line.[12] Similar experiments were also performed in multiple myeloma cell lines (data not shown) and the findings suggested that using ATO combined with an HDACi might be more efficacious for the treatment of those haematological tumours than either treatment alone. Further research is required to confirm the efficacy of the vorinostat + ATO combination therapy and to elucidate its mechanism of action more fully.

In summary, both vorinostat and ATO used alone can inhibit the proliferation of NB4 cells and induce apoptosis. Combined treatment can enhance the inhibition of cell proliferation and the apoptosis-inducing effects. These current findings suggest that the mechanism of action might be associated with increased histone acetylation and the downregulation of the Akt signalling pathway, but this requires further research. These preliminary *in vitro* results provide support for further research into this new ATO-based combination regimen for the treatment of APL.

Statement of author contributions

X.F.L. and N.N.L. contributed to the study conception and design. X.F.L., N.N.L., X.Y.G. and F.L. contributed to the collection and assembly of data. X.F.L., N.N.L., X.Y.G., F.L. and Y.Z.C. contributed to data analysis and interpretation. All authors wrote the manuscript and approved the manuscript for submission.

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Declaration of conflicting interest

The authors declare that there are no conflicts of interests.

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