African Journal of Pharmacy and Pharmacology Vol. 6(37), pp. 2696-2700, 8 October, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1057
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Promotion of all-trans retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukemia cells by Bufalin

Zhitu Zhu[1]*, Yunpeng Liu[2], Kezuo Hou[2], Enze Li[1], Hongzhi Sun[1], Ying Wang[1], Zhenghua Wang[1], Xiaomei Liu[1] and Qingjun Wang[1]

[1]Department of Oncology, the First Affiliated Hospital of Liaoning Medical University, Jinzhou, Liaoning 121001, China.
[2]Department of Oncology, the First Affiliated Hospital of Chinese Medical University, Shenyang, Liaoning 120000, China.

Accepted 24 September, 2012

All-trans retinoic acid (ATRA) has been shown as a promising agent for the treatment of acute promyelocytic leukemia (APL). However, the clinical application of ATRA is limited by the resistance of APL to ATRA-induced differentiation. Bufalin is a steroid isolated from Chan'su and could induce leukemia cell differentiation. This study is aimed to determine whether bufalin treatment could promote ATRA-induced differentiation of APL cell lines and primary cells. The cell surface differentiation marker CD11b, nitro blue tetrazolium (NBT) reduction and morphological changes in the cells were examined. The results showed that bufalin promoted ATRA-induced differentiation of APL primary cells and NB4 cell line, although low dose of bufalin alone had no direct effects on APL cell differentiation. In addition, bufalin and ATRA had synergistic effects to down-regulate survivin expression in NB4 cells. Taken together, these data suggest that bufalin is a potential regimen to be used in combination with ATRA to promote the differentiation of APL cells, which could achieve better therapy efficacy.

Key words: Bufalin, all-trans retinoic acid, surviving, acute promyelocytic leukemia, differentiation.

INTRODUCTION

Acute promyelocytic leukemia (APL) is a lethal blood disease because hematopoietic progenitors are arrested at the promyelocyte stage and could not be differentiated. APL is characterized by the formation of PML-RARα and RARα-PML fusion genes due to the reciprocal translocation t(15;17)(q22;q21), and the expressed fused proteins are critical for the development of APL (Reiter et al., 2004). All-trans retinoic acid (ATRA) is a ligand for the retinoic acid (RA) nuclear receptor family (RARs, RARα, RARβ and RARγ). ATRA can bind with the PML/RARα fusion protein and promote the differentiation of APL cells (He et al., 1998).

ATRA has been shown as a promising agent for APL treatment. However, the clinical application of ATRA is limited by the resistance of APL to ATRA (Kamimura et al., 2011). Therefore, there is an urgent need to develop new APL therapy strategies that overcome the resistance of APL to ATRA induced differentiation.

Bufalin is a steroid isolated from the traditional Chinese medicine Chan'su and has been shown to induce leukemia cell differentiation under certain experimental conditions (Chen et al., 2009; Yamada et al., 1998). This study is aimed to determine whether bufalin treatment could promote ATRA-induced differentiation of APL cell lines and primary cells. Our results demonstrated that bufalin promoted ATRA-induced differentiation of APL primary cells and NB4 cell line, suggesting the potential application of bufalin in APL chemotherapy.

MATERIALS AND METHODS

Reagents

Bufalin was purchased from Sigma (St. Louis, MO, USA), dissolved in ethyl alcohol as 0.01 mol/L stock solution, and kept at -20°C and

*Corresponding author. E-mail: zhuzhitu@hotmail.com. Tel: +86 416 4197189. Fax: +86 416 4197256.

diluted in phosphate buffer saline (PBS) when used. ATRA was purchase from Sigma, dissolved in ethyl alcohol as 0.01 mol/L stock solution, and sterile-filtered.

Cell culture

Primary APL cells were obtained from bone marrow with informed consent from twelve cases of APL. The diagnosis of APL was established according to clinical presentation and morphological criteria of the French-American-British (FAB) classification, and was confirmed by cytogenetic assay for t(15;17)(q22;q21) and reverse transcription polymerase chain reaction (RT-PCR) analysis for *PML-RAR* transcripts as described previously (Ghavamzadeh et al., 2006). Fresh cells were separated by centrifugation on Ficoll's solution and collected as primary APL cells. NB4 cell line was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), where they were tested and authenticated based on cross species checks, DNA authentication and quarantine. Primary APL cells and NB4 cells were cultured at 37°C with 5% CO_2 in an air atmosphere in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin (Sigma). Cells at the logarithmic growth stage were used in all of the experiments.

Flow cytometry analysis

The expression of cell surface differentiation antigen CD11b was detected as described previously (Drayson et al., 2001). After treatment with ATRA or/and bufalin, primary APL cells or NB4 cells were collected and incubated with FITC labeled CD11b antibody (Coulter Immunology). Samples were analyzed using a FACS Calibur flow cytometer within 30 min after the staining.

Nitro blue tetrazolium (NBT) assay

NBT assay was performed to assess the differentiation of APL cells as described previously (Idres et al., 2001). After treatment with ATRA and/or bufalin, primary APL cells or NB4 cells were collected and incubated with RPMI-1640 medium containing 0.1% NBT (Sigma,St. Louis, MO, USA) and 1 μg/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) for 30 min at 37°C in the dark. For each sample, total 200 cells were counted under microscope and the cells stained blue were judged as positive cells for NBT assay.

RT-PCR

Total RNA was extracted from NB4 cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. cDNA was synthesized by reverse transcription using RT kit (Promega, Madsion, WI, USA) following the manufacturer's protocol. PCR amplification of survivin and β-actin was performed using Taq Master Mix (Promega, Madison, WI, USA). The primers used are as follows: PML/RAR 5'-AGCTGCTGGAGGCTGTGGACGCGCGGTACC-3' (forward) and 5'-AGTGTACGCCTTCTCCATCA-3' (reverse), product 214 bp; survivin 5'-CTTTCTCAAGGCCACCGCATC-3'(forward) and 5'-CAATCCATGGCAGCCAGCTGC-3' (reverse), product 393 bp; β-actin 5'-CGCTGCGCTGGTCGTCGACA-3' (forward) and 5'-GTCACGCACGATTTCCCGCT-3' (reverse), product 619 bp. Amplification conditions are as follows: 5 min at 95°C (one cycle); 30 s at 94°C; 1 min at 55°C and 1 min at 72°C (35 cycles); and 72°C for 5 min (one cycle). The PCR products were analyzed by 1.5% agarose electrophoresis and ethidium bromide staining.

Western blot analysis

After treatment with ATRA and/or bufalin, NB4 cells were collected and lysed in RIPA buffer for 30 min on the ice. Lysates were collected after centrifuging at 12,000 rpm for 20 min at 4°C. Protein levels in the lysates were quantified using Lowry method. Equivalent amounts of protein (50 μg/lane) were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluouride (PVDF) membranes. The membranes were blocked in PBS containing 5% non-fat dry milk (w/v) for 1 h, and then incubated with mouse antibodies for survivin and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 30 min, and developed using enhanced chemiluminescence reagent and exposed to X-ray film.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) from experiments performed in triplicate. T-test was used to identify statistically significant differences between the experimental and control groups. The statistical analyses were performed using the SPSS software 13.0 (SPSS Inc., Chicago, IL, USA). P-value < 0.05 was considered statistical significance.

RESULTS

Promotion of ATRA-induced differentiation of primary APL cells by Bufalin

To examine the effects of bufalin on the differentiation of primary APL cells, we treated primary APL cells with bufalin and ATRA alone or in combination. Flow cytometry analysis showed that the expression of CD11b was significantly increased in primary APL cells treated by both bufalin and ATRT, compared to those treated by either bufalin or ATRT alone (Figure 1A to D). In addition, NBT assay showed that ATRA alone promoted the differentiation of primary APL cells in a dose and time dependent manner. Notably, bufalin at the dose of 5 nM exhibited synergistic effects to enhance ATRT-induced cell differentiation (Figure 1E). Taken together, these results suggest that bufalin promotes ATRA-induced differentiation of primary APL cells.

Bufalin promotes ATRA-induced differentiation of NB4 cells

Next, we used NB4 cells as the model to examine the effects of bufalin on the differentiation of APL cells. Flow cytometry analysis showed that the expression of CD11b was significantly increased in NB4 cells treated by both bufalin and ATRT, compared to those treated by either bufalin or ATRT alone (Figure 2A to D). In addition, NBT assay showed that bufalin exhibited synergistic effects to enhance ATRT-induced NB4 cell differentiation in a dose-dependent manner from 1 to 5 nM, but the synergistic

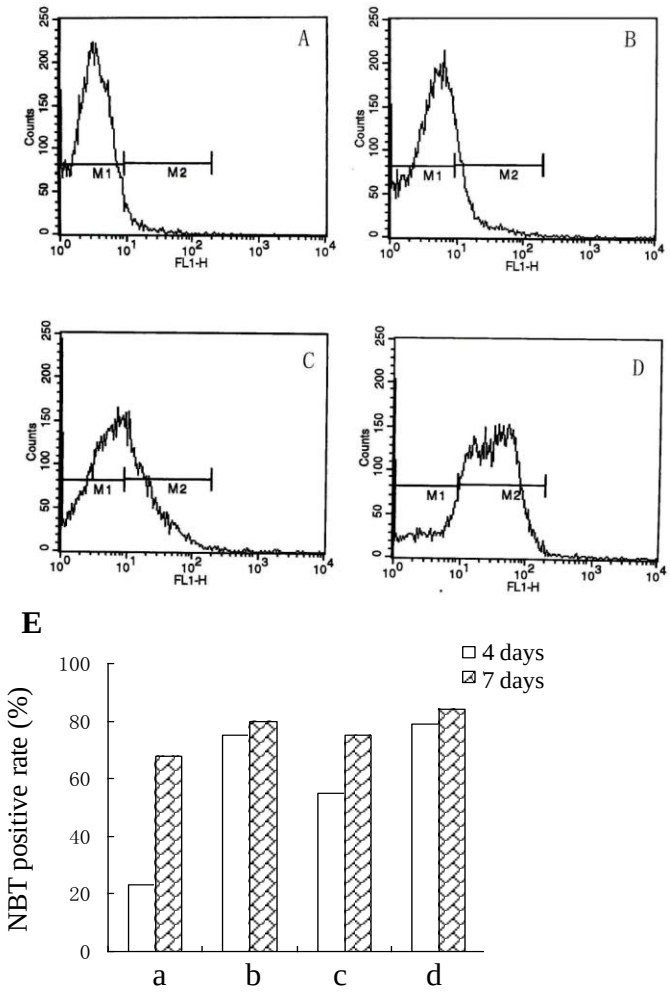

Figure 1. Bufalin promotes ATRA-induced differentiation of APL primary cells. A to D: The expression of cell surface marker CD11c in APL primary cells. Cells were treated as indicated for 4 days and then incubated with CD11c antibody. 10^4 cells were analyzed by FACScan flow cytometer. A, PBS treated control; B, 5 nM bufalin; C, 30 nM ATRA; D, 5 nM bufalin plus 30 nM ATRA; E, NBT assay. Cells were treated as indicated for 4 and 7 days and the reduction of NBT by the cells was determined. a, PBS treated control; b, 5 nM bufalin; c, 30 nM ATRA; d, 5 nM bufalin plus 30 nM ATRA.

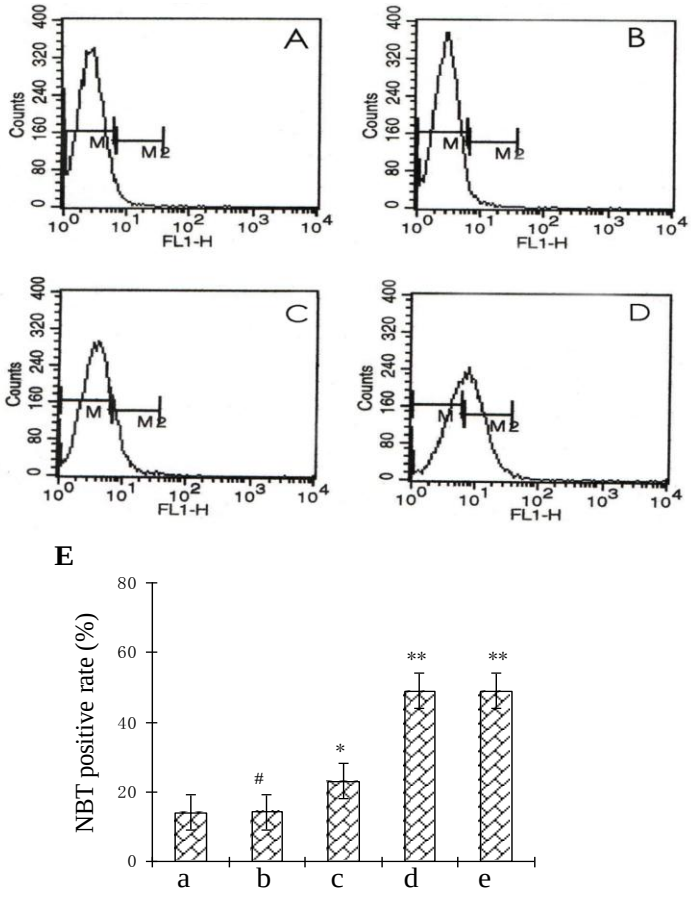

Figure 2. Bufalin promotes ATRA-induced differentiation of NB4 cells. A to D: The expression of cell surface marker CD11c in NB4 cells. Cells were treated as indicated for 7 days and then incubated with CD11c antibody. 10^4 cells were analyzed by FACScan flow cytometer. A, PBS treated control; B, 5 nM bufalin; C, 30 nM ATRA; D, 5 nM bufalin plus 30 nM ATRA; E, NBT assay. Cells were treated as indicated for 7 days and the reduction of NBT by the cells was determined. a, 30 nM ATRA alone; b,: 30 nM ATRA plus 1 nM bufalin; c, 30 nM ATRA plus 3 nM bufalin; d, 30 nM ATRA plus 5 nM; e, 30 nM ATRA plus 7 nM. #P > 0.05 versus a; *P < 0.05 versus a; **P < 0.01 versus a.

effects could not be further promoted when the dose of bufalin increased to 7 nM (Figure 2E).

Furthermore, we observed morphological differentiation of NB4 cells exposed to ATRA and/or bufalin. The results showed that NB4 cells treated by the combination of 30 nM ATRA and 5 nM bufalin demonstrated typical granulocytic maturation patterns: polylobular nuclei, decreased nuclear:cytoplasmic ratio, and decreased cytoplasm staining, while NB4 cells treated by ATRA or bufalin alone demonstrated less patterns of granulocytic maturation (Figure 3A to 3D). These results of cell morphology observation, NBT assay, and CD11c expression analysis proved that bufalin promotes ATRA-induced differentiation of NB4 cells.

Bufalin synergizes with ATRA to down-regulate survivin expression in NB4 cells

To explore the molecular mechanism by which bufalin promotes ATRA-induced differentiation of NB4 cells, first we examined the modulation of PML/RARa expression by bufalin. NB4 cells were treated with 5 nM bufalin and 30 nM ATRA alone or in combination. RT-PCR analysis showed that there were no significant differences in PML/RARa expression in NB4 cells treated differently (data not shown). Next, we detected survivin expression in these differently NB4 cells. RT-PCR analysis showed that both ATRA and bufalin could inhibit survivin mRNA expression to different extent with stronger inhibitory effect observed for ATRA. Furthermore, ATRA and bufalin showed synergistic effect to inhibit survivin expression in

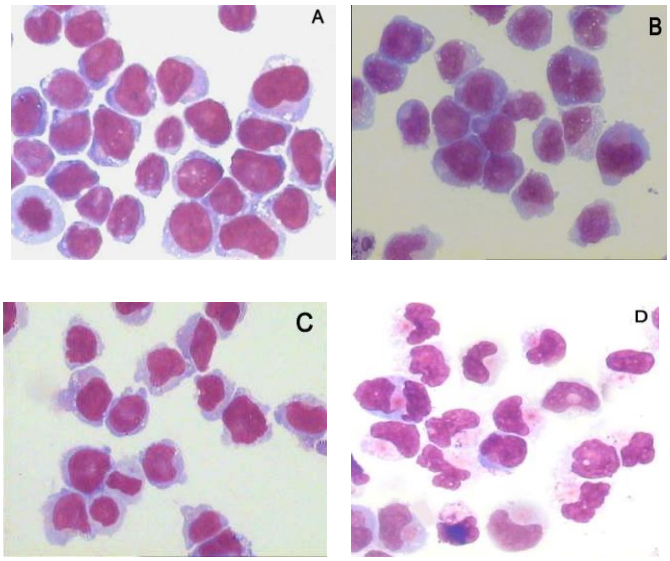

Figure 3. Bufalin promotes morphological differentiation of NB4 cells. A to D: Giemsa staining of NB4 cells. NB4 cells were treated as indicated for 7 days and then subjected to Giemsa staining. A, PBS treated control; B, 5 nM bufalin; C, 30 nM ATRA; D, 5 nM bufalin plus 30 nM ATRA. Magnification: × 200.

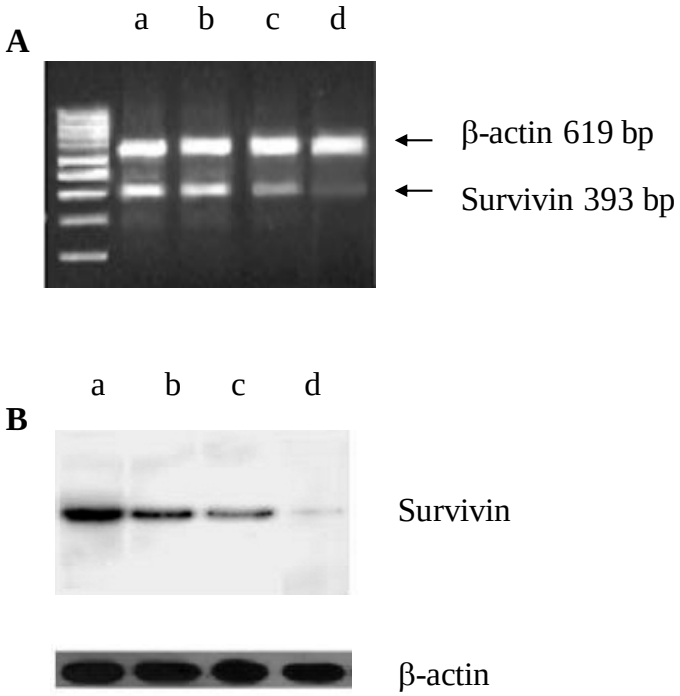

Figure 4. Bufalin synergizes with ATRA to down-regulate survivin expression in NB4 cells. (A), RT-PCR analysis of survivin mRNA level in NB4 cells treated as indicated for 7 days. β-actin served as internal control. (B), western blot analysis of survivin protein level in NB4 cells treated as indicated for 7 days. β-actin served as loading control. Representative images are shown from three independent experiments with similar results. a, PBS treated control; b, 5 nM bufalin; c, 30 nM ATRA; d, 5 nM bufalin plus 30 nM ATRA.

NB4 cells (Figure 4A). Western blot analysis showed similar patterns of survivin expression at protein level in differently treated NB4 cells (Figure 4B). Collectively, these data suggest that bufalin synergizes with ATRA to down-regulate survivin expression in NB4 cells but it has no effect on PML/RARa expression at low dose.

DISCUSSION

In this study, for the first time, we investigated the potential of bufalin in the modulation of ATRA-induced differentiation of APL cells. Based on the analysis of cell surface differentiation marker, NBT reduction and morphological changes, we demonstrated that bufalin promoted ATRA-induced differentiation of APL primary cells and NB4 cell line, although bufalin alone at low dose had no direct effects on APL cell differentiation.

It is known that a variety of molecules participate in the regulation of cell differentiation. Notably, a recent study reported that knockdown of survivin could induce granulocytic differentiation of leukemia cell lines, suggesting that survivin is a negative regulator of granulocytic differentiation of leukemia cells (Balkhi et al., 2008). To provide clues on the mechanism by which bufalin promotes ATRA-induced differentiation of APL cells, we examined the modulation of survivin expression by bufalin. RT-PCR and western blot analysis showed that bufalin synergized with ATRA to down-regulate the expression of survivin in NB4 cells but had no significant effect on PML/RARa expression either alone or in combination with ATRA. Therefore, we propose that bufalin promotes ATRA-induced differentiation of APL cells by inhibiting the expression of survivin, a negative regulator of leukemia cell differentiation. However, further study is necessary to investigate the molecular mechanism by which survivin negatively regulates the differentiation of APL cells.

Survivin has been implicated in the development of acute leukemia because it is aberrantly expressed in most cancers and hematopoietic malignancies (Wrzesień-Kuś et al., 2004; Liang et al., 2009). As an important inhibitor of apoptosis, survivin could protect leukemia cells from chemotherapy induced apoptosis (Tyner et al., 2012). Given our observation that bufalin inhibited survivin expression in NB4 cells, it would be interesting in our future studies to investigate whether bufalin could inhibit the survival of APL cells by inducing cell apoptosis.

In summary, the present study showed that bufalin promoted ATRA-induced differentiation of APL cells, and this was associated with the down regulation of survivin expression by bufalin and ATRA. These findings provide new insight into the pharmaceutical characteristics of bufalin and suggest that bufalin is a potential regimen to be used in combination with ATRA to improve the efficacy of APL therapy.

ACKNOWLEDGEMENTS

This study was supported by Fund from Department of Science and Technology of Liaoning Province (No. 2010225034 and 2011225015) and Fund for PhD of Liaoning Province (No. 20091051).

REFERENCES

Balkhi MY, Christopeit M, Chen Y, Geletu M, Behre G (2008). AML1/ETO-induced survivin expression inhibits transcriptional regulation of myeloid differentiation. Exp. Hematol. 36:1449-1460.

Chen A, Yu J, Zhang L, Sun Y, Zhang Y, Guo H, Zhou Y, Mitchelson K, Cheng J (2009). Microarray and biochemical analysis of bufalin-induced apoptosis of HL-60 Cells. Biotechnol. Lett. 31: 487-494.

Drayson MT, Michell RH, Durham J, Brown G (2001). Cell proliferation and CD11b expression are controlled independently during HL60 cell differentiation initiated by 1,25 alpha-dihydroxyvitamin D(3) or all-trans-retinoic acid. Exp. Cell Res. 266:126-134.

Ghavamzadeh A, Alimoghaddam K, Ghaffari SH (2006). Treatment of acute promyelocytic leukemia with arsenic trioxide without ATRA and/or chemotherapy. Ann. Oncol. 17, 131-134.

He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A, Pandolfi PP (1998). Distinct interactions of PML/RARalpha and PLZF/RARalpha with co-repressors determine differential responses to RA in APL. Nat Genet. 18:126-135.

Idres N, Benoit G, Flexor MA, Lanotte M, Chabot GG (2001). Granulocytic differentiation of human NB4 promyelocytic leukemia cells induced by all-trans retinoic acid metabolites. Cancer Res. 61: 700-705.

Kamimura T, Miyamoto T, Harada M, Akashi K (2011). Advances in therapies for acute promyelocytic leukemia. Cancer Sci. 102:1929-1937.

Liang QL, Wang BR, Li GH (2009). DcR3 and survivin are highly expressed in colorectal carcinoma and closely correlated to its clinicopathologic parameters. J. Zhejiang. Univ. Sci. B. 10:675-682.

Reiter A, Lengfelder E, Grimwade D (2004). Pathogenesis, diagnosis and monitoring of residual disease in acute promyelocytic leukaemia. Acta Haematol.112:55-67.

Tyner JW, Jemal AM, Thayer M, Druker BJ, Chang BH (2012). Targeting survivin and p53 in pediatric acute lymphoblastic leukemia. Leukemia. 26:623-632.

Wrzesień-Kuś A, Smolewski P, Sobczak-Pluta A, Wierzbowska A, Robak T (2004). The inhibitor of apoptosis protein family and its antagonists in acute leukemias. Apoptosis 9:705-715.

Yamada K, Hino K, Tomoyasu S, Honma Y, Tsuruoka N (1998). Enhancement by bufalin of retinoic acid-induced differentiation of acute promyelocytic leukemia cells in primary culture. Leuk Res. 22:589-595.