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APOPTOSIS-INDUCING EFFECT OF SPIROAMINOPYRIMIDINE ANALOGUE IN NB4 LEUKEMIA CELLS VIA DOWN-REGULATION OF *BIRC5* EXPRESSION

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Aim: **It has been reported that spiroaminopyrimidine derivatives inhibited the growth and proliferation of various cancer cell lines. In the present study, we evaluated cytotoxic and apoptosis-inducing effects of *2,4-diamino-1,3-diazaspiro[5.5]-9-tert-butyl-2, 4-diene-5-carbonitril* (9-tBAP) on NB4 acute promyelocytic leukemia (APL) cells.** ***Materials and Methods:*** **The cells were treated with 10–100 μM of 9-tBAP. Cytotoxic activity of the compound was measured using the MTT assay. Apoptosis was investigated by Hoechst 33258 staining as well as by Annexin V/PI double staining.** ***Results:*** **The compound under study was found to be highly active cell growth inhibitor with IC_{50} of 30.0 ± 3.5 μM inducing apoptosis in NB4 cells. Cell cycle analysis by flow cytometry showed a time-dependent increase in sub-G_1 cell population. Real-time polymerase chain reaction analysis revealed that the treatment with the compound down-regulated the *BIRC5* expression in a time-dependent manner.** ***Conclusion:*** **9-tBAP displayed potent anti-leukemic activity *in vitro* thus warranting further investigation.**

Key Words: **spiroaminopyrimidine, apoptosis, cell cycle, NB4 cell line.**

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Leukemia is the appearance of the inappropriate expression of hematopoietic progenitor cells due to a block of cell maturation at early stages in the lineages that give rise to the various cell types that constitute normal blood [1]. Acute myeloid leukemia (AML) is a heterogenous group of leukemias that result from malignant transformation of hematopoietic precursors. Acute promyelocytic leukemia (APL) is a subtype of AML characterized by at t(15;17) translocation that yields a PML-RARα fusion oncoprotein, which blocks maturation and apoptosis of neutrophils at the promyelocyte stage [2]. Apoptosis or programmed cell death is a normal process that ensures equilibrium between cell proliferation and cell death, as well as plays a regulatory role in controlling the size of cell populations and tissues homeostasis [3]. It has been demonstrated that excessive or improper regulation of apoptosis plays a critical role in tumor development and invasion. Accordingly, chemotherapeutic agents that target the apoptosis pathways can be selected for the cancer treatment. In fact, many commonly used chemotherapeutic drugs induce apoptosis in the cancer cells [4]. Apoptosis is regulated by multiple pro- and anti-apoptotic factors. Among the most extensively studied are members of inhibitor of apoptotic protein (IAP) family, which inhibit apoptosis via a pathway distinct from Bcl2 family members. Survivin (coding gene *BIRC5*) is a unique member of the IAP family that is expressed in most human tumors, but is barely detected in normal adult tissues [5]. Overexpression of survivin in tumors is generally associated with poor prognosis and drug resistance. Nuclear expression of survivin has been established as a good prognostic marker in several cancers [6]. Down-regulation of survivin induces apoptosis in cancer cells and suppresses tumor growth. Regulation of mitosis by forming chromosomal passenger complex with other proteins and apoptosis suppression by inhibition of protease activity of caspase-3 and -7 are the major function of survivin in cancer cells [5]. In many human malignancies, apoptosis resistance is a cause for concern in developing effective chemotherapeutic drugs. Consequently, down-regulation of survivin, combined with cytotoxic agents, can be an effective route in the treatment of cancer. Aminopyrimidines have been introduced as a new series of potent apoptosis-inducing agents. These compounds possess several critical properties including antitumor, antibacterial, and antifungal function in the cells [7, 8]. Presently, the significant known effects of aminopyrimidines are linked to their anticancer responses via inhibiting the reaction of multiple-kinase and also of PI3-kinase/mTOR pathway [7, 8]. AMN107 as a bioavailable ATP-competitive inhibitor, inhibited proliferation associated with the induction of apoptosis in Philadelphia-positive acute lymphoblastic leukemia cells [9].

In this study, we investigated the growth inhibitory and apoptosis-inducing effects of *2,4-diamino-1,3-diazaspiro [5.5]-9-tert-butyl-2,4-diene-5-carbonitril* (9-tBAP) on human APL NB4 cell line.

MATERIALS AND METHODS

Reagents. 9-tBAP was obtained from Saeed Balalaie, Department of Organic Chemistry, Khaje Nasir Toosi University of Technology, Tehran, Iran. The RPMI1640 medium, fetal bovine serum and penicillin-

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Abbreviations used: AML – acute myeloid leukemia; APL – acute promyelocytic leukemia; IAP – inhibitor of apoptotic protein; PS – phosphatidylserine.

streptomycin were obtained from Gibco (BRL, Life Technologies, Paisley, Scotland). The culture plates were purchased from SPL (South Korea). Annexin V FITC Apoptosis kit was bought from Biosciences, BD (USA). Hoechst 33258, propidium iodide (PI), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich company (Germany). 9-tBAP (Fig. 1) was synthesized by Balalaie *et al*. at Department of Organic Chemistry, Khaje Nasir Toosi University of Technology, Tehran, Iran and identified by elemental analysis, spectroscopic methods (UV-Vis, FTIR, ^{1}HNMR, and ^{13}CNMR) and electrospray ionization mass spectrometry [10].

Cell culture conditions. The NB4 cell line was bought from Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 µg/ml penicillin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Determination of cytotoxic activity. Cytotoxicity of the compound was measured using the MTT assay. The NB4 cells ($2 \cdot 10^5$ cells/ml) were cultured in 96-well plates and then exposed to the various concentrations (10–100 µM) of the compound. For IC_{50} values determination, DMSO was added to each well and then incubated 4 h at 37 °C. Cell viability was calculated by measuring the absorbance at 570 nm by a multiwell plate reader (Quant Biotek Instruments, USA) [11].

Morphologic assessment of apoptosis. For morphological studies, the cells were seeded in 12-well plates at a concentration of $2 \cdot 10^5$ cells/well, in 2 ml of the growth medium. When the cells reached 90% confluence, 9-tBAP was added to the cells at indicated concentration (IC_{50} value). After 24–72 h of incubation, the cell morphology was assessed by an inverted microscope (Zeiss, Germany). DNA was also stained with Hoechst 33258 (1 mg/ml) in PBS for 5 min, and then washed twice with PBS for 5 min each, then examined using a fluorescence microscope (Zeiss Axoscope 2 Plus) [4].

Cell cycle analysis by flow cytometry. Briefly, the NB4 cells were cultured in 96-well plates for various times (24–72 h). The cells ($2 \cdot 10^5$ cells/well) were treated with IC_{50} of the compound. The cells were harvested and washed twice with cold PBS, fixed with 70% (v/v) cold ethanol and stored at –20 °C for several weeks until analysis. Afterward, the control (untreated) and treated cells were incubated with 50 µg/ml PI containing 20 µg/ml RNase A in the darkroom at 37 °C for 2 h. The stained cells were analyzed by flow cytometry (BD FACSCalibur™, BD Biosciences, CA, USA) [12].

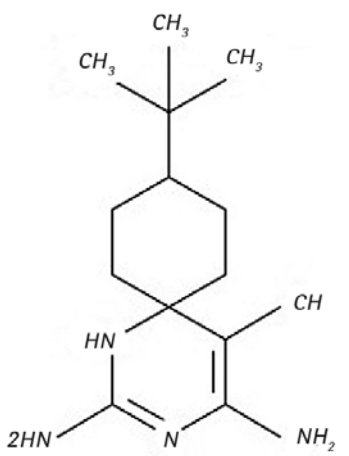

Fig. 1. Chemical structure of 9-tBAP

Flow cytometric assessment of apoptosis. To confirm the outcomes of former analysis, apoptotic cells were quantified with FITC-AnnexinV and PI double staining method with analyzing of the presence of phosphatidyl serine on the outer surface of the apoptotic cell membrane [13, 14]. The NB4 cells were seeded in 96-well cell culture microplates for 24 h prior to treatment and treated with 9-tBAP for 24, 48 and 72 h. After double washing of treated and control cells with PBS, $12 \cdot 10^5$ cells were resuspended in 100 µl binding buffer (1×) and then 5 µl of FITC-conjugated Annexin V and 10 µl PI were added. Afterward, cells were incubated for 15 min in the dark at room temperature and analyzed by flow cytometry (BD FACSCalibur™, BD Biosciences, CA, USA) [13, 14].

Assessment of BIRC5 expression by real-time polymerase chain reaction (RT-PCR). After treatment of the cells with 9-tBAP (at IC_{50}), total RNAs were extracted from treated and untreated cells using RNX-plus reagent as described by the manufacturer (Cinagen, Tehran, Iran). 1 µg of total RNA was reverse transcribed into cDNA using Reverta-L kit (Amplisens, Russia) [15]. Specific primers were used for the:

Beta-actin Forward:
5′-TGCCCATCTACGAGGGGTATG-3′;
5′-CTCCTTAATGTCACGCACGATTTC-3′.
BIRC5 Forward:
5′-CAGATTTGAATCGCGGGACCC-3′.
BIRC5 Reverse:
5′-CCAAGTCTGGCTCGTTCTCAG-3′.

The reaction for survivin and β-actin were run for 30 and 35 cycles, respectively. The PCR products were electrophoresed on a 2% agarose gel and visualized under UV light by ethidium bromide staining [15, 16]. β-actin gene was used as an internal control.

Statistical analysis. All experiments were performed in triplicate and the data are presented as mean ± SD. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by a specific post hoc test to analyze the difference.

RESULTS

Cell viability assay. The cells were exposed to different concentrations (10–100 µM) of the compound for 24, 48 and 72 h. As shown in Fig. 2, at 10–50 µM, 9-tBAP reduced cell viability by 70% after 72 h. Following the treatment with different doses of the compound for 24–72 h, the cell viability decreased in a time-dependent manner (Fig. 2). The IC_{50} value was calculated as 30 ± 3.5 µM after 24 h exposure to the compound.

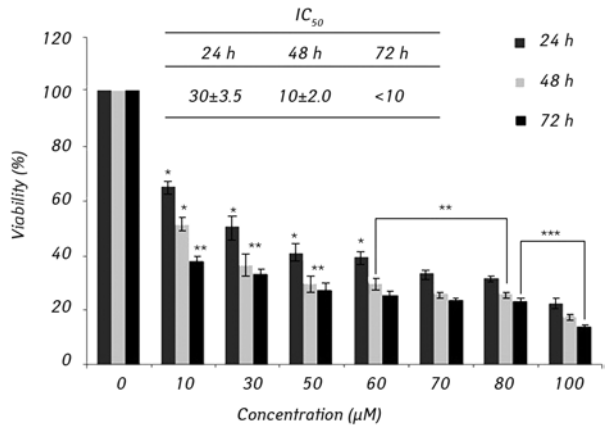

Fig. 2. Effect of 9-tBAP on the viability of the NB4 cells. The NB4 cells were treated with various concentrations (10–100 μM) of the compound for 24–72 h and then the viability of the cells was investigated by MTT assay. Data are shown as mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Morphological assay of the apoptotic cells. The morphological changes of the treated cells with 9-tBAP (at IC_{50} value) were visualized by invert and fluorescence microscopy after 24–72 h (Fig. 3). We notified that the cells cultured in control condition were in round shape, while some of the cells exposed to the test compound became wrinkle and condensed (Fig. 3, *a*). Moreover, using fluorescence microscopy we observed that the viable and control cells were in normal blue color, while the apoptotic cells were in blue color with bright dots in their nuclei due to condensation of the nuclei and fragmented chromatins (Fig. 3, *b*).

Cell cycle analysis. Flow cytometric analysis of cellular DNA content was performed to identify the effects of 9-tBAP on the cell cycle distribution. It indicated that treatment of NB4 cells with 30 μM 9-tBAP notably enhanced the number of cells in the sub-G_1 phase (apoptotic cells). The results showed a time-dependent increase in sub-G_1 peak. Findings showed that the percentage of NB4 cells in sub-G_1 phase was almost 3.64% at control (untreated cells), 24.8%, 32.3% and 37.9% at 24, 48 and 72 h, respectively (Fig. 4). Therefore, obviously, the peak accumulation of the cells at sub-G_1 phase confirmed the apoptotic function of 9-tBAP in NB4 cells.

Apoptosis assay by flow cytometry. Exposure of phosphatidylserine (PS) on the surface of the plasma membrane is an obvious and immediately-synthesized apoptotic marker. The results of Annexin V/PI assay demonstrated that the cells in the lower left quarter ($Annx^-/PI^-$) were viable cells, and the cells in the lower right quarter ($Annx^+/PI^-$) were in early apoptotic state. The upper left quarter ($Annx^-/PI^-$) showed necrotic cells and in the upper right quarter ($Annx^+/PI^+$) cells were in the late apoptotic situation. The rate of early apoptosis ($Annx^+/PI^-$) and late apoptosis ($Annx^+/PI^+$) in the cells treated with 30 μM of 9-tBAP was calculated as 47.58%, 47.67% and 49.05% and 0%, 8.54%, 17.23% at 24, 48 and 72 h, respectively (Fig. 5). As shown in Fig.5, when the NB4 cells were exposed to the compound, the percent of early and late apoptotic cells increased in a time dependent manner.

Evaluation of BIRC5 expression in NB4 cells. We used RT-PCR technique for examination of *BIRC5* expression in NB4 cells before and after treatment with 30 μM of 9-tBAP. According to Fig. 6, *a, b*, exposure of NB4 cells to 9-tBAP for 48–72 h led to significant reduction of *BIRC5* expression. However, dramatic down-regulation of this gene was observed after 24 h. Because of cell resistance to the 9-tBAP, *BIRC5* expression level did not change during 24 h of exposure. Nonetheless, longer exposure decreased expression of this gene to very low levels (Fig. 6).

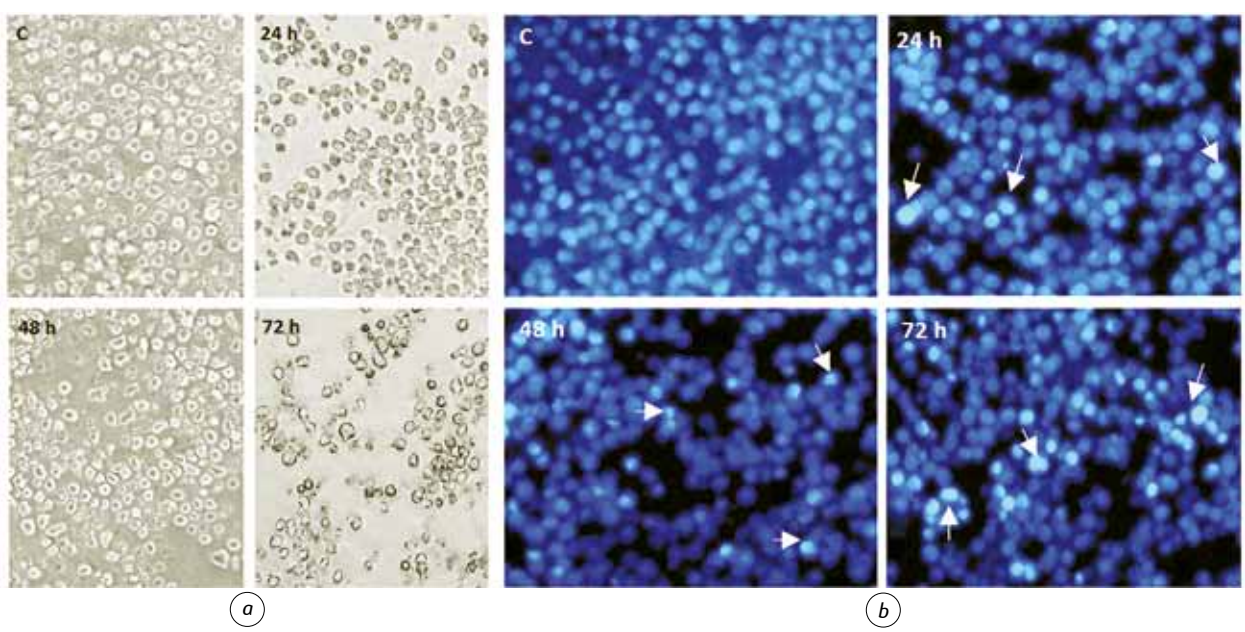

Fig. 3. Morphological study of the NB4 cells treated with 30 μM of the 9-tBAP in 24, 48 and 72 h. (*a*) The morphological changes of the NB4 cells treated with the compound as compared to the control cells, including the deformation of the cells and increasing number of cells with membrane shrinkage. (*b*) Fluorescence microscopy of the control and 9-tBAP-treated NB4 cells after 24–72 h. 9-tBAP induced condensation and fragmentation of the nuclei (arrows)

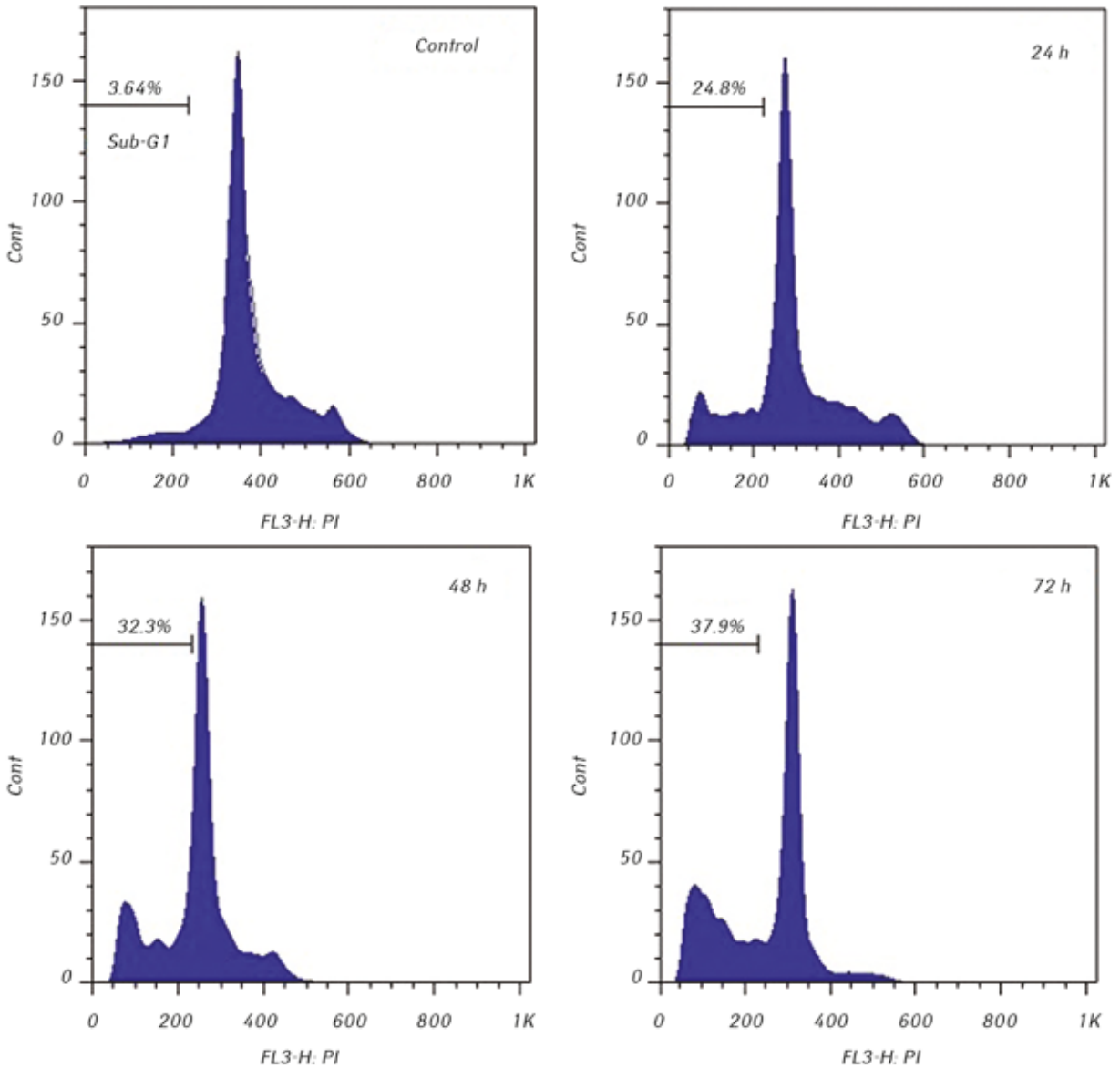

Fig. 4. Cell cycle distribution in NB4 cells after incubation with 9-tBAP (at IC_{50}). The cells were analyzed by flow cytometry and then the percentage of cells was determined in each phase of the cell cycle. The percent of NB4 cells population in sub-G_1 phase in untreated (control) and 9-tBAP-treated (24, 48, 72 h) cells are presented

DISCUSSION

Although it has been considered that most of the patients with APL be completely cured by typical chemotherapeutic agents, there are still elusive factors of drug resistance in leukemia cells [17]. Therefore, new therapeutic strategies such as use the synthetic chemical compounds against leukemia are of intense interest. Aminopyrimidine derivatives have been introduced as a new series of heterocyclic compounds with potent apoptosis-inducing activity. In the present study, we demonstrated that 9-tBAP from spiroaminopyrimidines inhibited proliferation and induced apoptosis in NB4 cells via down regulation of *BIRC5* expression [8, 18, 19]. It has been previously suggested that this compound can inhibit growth and induce apoptosis in chronic myeloid leukemia K562 cells. This compound caused a significant decrease in viability of treated NB4 cells with low concentrations (10–30 μM). Previous data showed that replacement of cyclohexane ring without a substituent by cyclohexane ring with *tert-butyl* at the 9-position caused a reduction in the K562 cell viability [20]. According to previous studies and also the obtained result of MTT assay in the presented study, it can be inferred that 9-tBAP has an anti-proliferative effect, as well as an apoptosis-inducing activity in a dose and time dependent manner. Morphological studies and Annexin V/PI double staining assay indicated the induction of apoptosis in 9-tBAP-treated NB4 cells during 24–72 h. In addition, due to close relevancy of apoptosis to cell cycle, different phases of the cell cycle were analyzed in NB4 cells before and after treatment. The results revealed that there is a reciprocal association between the decrease in proliferation and sub-G_1 arrest. The result of RT-PCR showed a significant reduction in *BIRC5* expression in the 9-tBAP-treated cells. The sub-G_1 cell cycle arrest is concomitant with

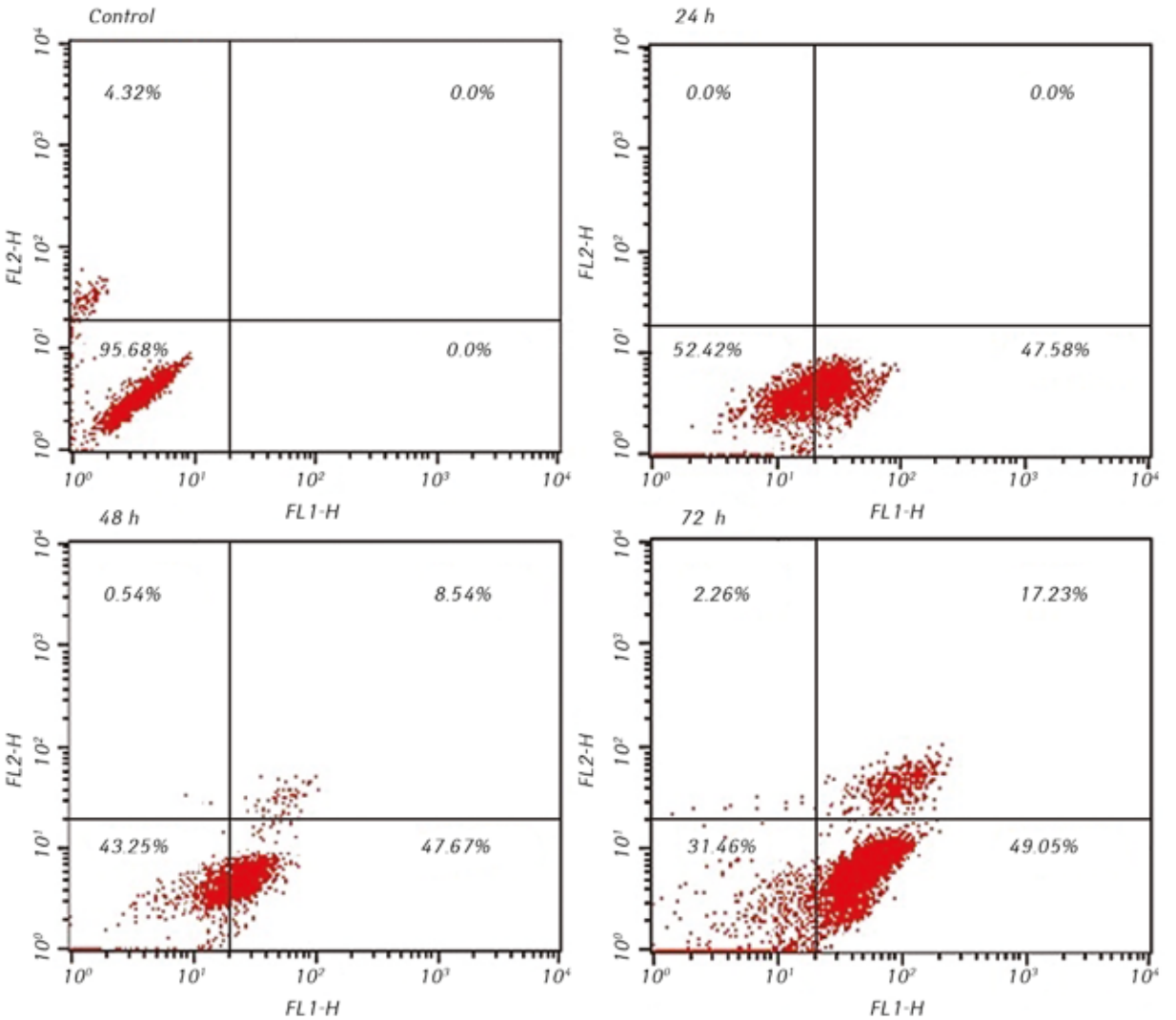

Fig. 5. Quantitatively analysis of apoptosis in the NB4 cells treated with 9-tBAP by annexin-V/PI double staining assay. Flow cytometric analysis showed that 9-tBAP induced apoptosis in NB4 cells in a time-dependent manner

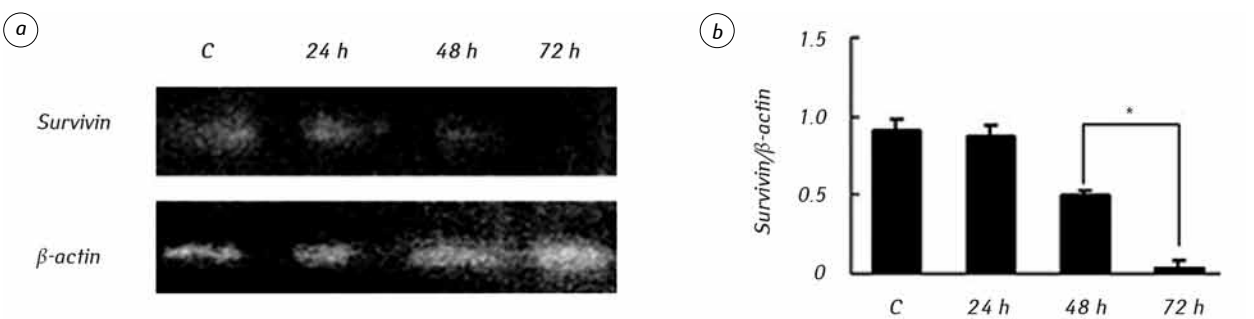

Fig. 6. Evaluation of survivin genes expression by RT-PCR. (*a*) Survivin gene expression level during 24–72 h treatment with 30 μM of 9-tBAP. (*b*) The survivin gene levels in control and treated cells were quantified by ImageJ software and normalized to β-actin band intensity. Data shown are representative of three experiments as mean ± SD (*$p < 0.05$, *vs* control)

down-regulation of survivin gene confirming that cell death indeed occurs by apoptosis in the NB4 cells. The growth-inhibitory and apoptotic effects of aminopyrimidine derivatives on various cancer cells has been reported [8]. Compound 6a from Spiro-cyclic 2-oxindole of Pyrimido[4,5-b]quinoline-4,6-dione derivatives is an attractive target for breast cancer. This compound inhibits proliferation, cell cycle progression and induces apoptosis via up-regulation of pro-apoptotic *TP53*, *BAX* and *CASP3* genes and down-regulation of anti-apoptotic *BCL2* gene in various cancer cells [18]. Thienopyrimidin-2-yl-aminopyrimidines as potent and selective dual pan-PI3-kinase/mTOR inhibitors induced apoptosis through suppression of PI3K/Akt/mTOR pathway in human prostate (PC3) and breast (MCF7.1) cancer cells [8].

In this study, it was found that the 9-tBAP from spiroaminopyrimidines has cytotoxic effect and induces

apoptosis in NB4 cells. This compound causes the classic features of apoptotic cells such as cell shrinkage, nuclear morphological changes, sub-G_1 cell cycle arrest and exposure of PS at the outer plasma membrane. Based on these findings, this compound can be considered for further pharmaceutical investigations for the treatment of APL.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

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