

Flow Cytometric Analysis of 4-HPR-induced Apoptosis and Cell Cycle Arrest in Acute Myelocytic Leukemia Cell Line (NB-4)

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Abstract

In many acute leukemias, normal differentiation does not occur. However, in many cell lines derived from hematologic malignancies, differentiation or programmed cell death (apoptosis) can be induced by variety of agents including: Vitamin analogs, demethylating agents, cyclic AMP analogs and anti-proliferative agents. To the best of our knowledge there has been not any study specifically to analyze apoptotic and anti-proliferative effects of 4-HPR (a vitamin analog) in NB-4 cell line. To test whether this drug has activity in acute myeloid leukemia (AML), we first analyzed the anti-proliferative effect of 4-HPR in one AML cell line (NB-4) using MTT Assay. Next we tested whether this drug induced apoptotic cell death. The ability of this compound to induce apoptosis of cancer cells was examined by Annexin V-FITC Assay using Flow cytometry. We also analyzed the cell cycle progression by PI staining using flow cytometry. Using MTT assay, NB-4 cells exhibited increased inhibition of proliferation at micromolar concentrations of 4-HPR at 24, 48 and 72 *hrs* post treatment. Flow cytometry analysis indicates that 4-HPR is a potent inducer of *in vitro* apoptotic cell death, and cell cycle analysis revealed an increase in S phase population. In total, the results indicate that 4-HPR is a strong inhibitor of AML cell proliferation and a potent inducer of *in vitro* apoptotic cell death. Further studies are required to evaluate the *in vitro* effects of 4-HPR in AML blasts derived from AML patients.

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Introduction

Acute myelocytic leukemia (AML) is a malignant neoplasm of hematopoietic cells, often associated with abnormal proliferation of myeloid precursor cells, and cessation of cell differentiation at several stages of hematopoietic precursors⁽¹⁾. AML is the most common type of leukemia often occurring in

adults, with about 11,900 cases per year in the United States⁽¹⁾. The incidence of AML increases with age, and the peak of onset is in the sixth decade of the human life⁽¹⁾. AML contains less than 1% of all cancers, and about 34% of all types of leukemia⁽²⁾.

Treatment phase of AML contains two

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general parts including remission induction and post-remission therapy. Cytarabine, Anthracycline, Daunorubicin (DNR), and Thioguanine are commonly used for remission induction ⁽³⁾. And some studies suggest that Idarubicin or Mitoxantrone ⁽⁴⁾, and the high-dose use of Cytarabine ⁽⁵⁾ are effective, too. Whether leukemia patients in the first remission should receive bone marrow transplant immediately or after relapse has been a controversial issue ⁽⁶⁾. Although the successful results of transplant especially in persons less than 20 years of age are notable, the most significant effects of bone marrow transplant has been seen in patients failing chemotherapy ⁽⁶⁾. Despite all progresses in the treatment of AML, patients with this cancer are faced with some complications and second malignancies ⁽⁷⁾.

Since most patients with AML will experience the relapse, and long-term survival remains unresolved, there is a continued need to discover new strategies for treatment of leukemia. Recently, with the increase of laboratory proofs, researchers have begun targeted treatment of AML based on differentiation induction ⁽⁸⁻¹⁰⁾. Researchers have so far evaluated many differentiation inducing agents which affect the proliferation and differentiation of tumor cells or can induce apoptotic cell death in cancer cells ⁽¹¹⁻¹³⁾.

One of the synthetic analogs of ATRA (all-trans-retinoic acid) is N-(4-Hydroxyphenyl)-all-trans retinamide (fenretinide) which is also known as 4-HPR ⁽¹⁴⁾. The substitution of the carboxyl group of ATRA with a 4-hydroxyphenyl group connected to an amide can reduce dominantly the side effects including liver toxicity. Moreover, since fenretinide is not able to induce point mutations and chromosomal aberrations, it is not considered to be a genotoxic agent ⁽¹⁵⁾. Although natural retinoids such as ATRA and 13-cis-retinoic acid often induce differentiation in cancer cells or cause cessation of the cell cycle, fenretinide triggers some specific biological effects ⁽¹⁶⁾. Some of these effects are the generation of Reactive Oxygen Species (ROS)

and lipid second messengers that seems to be involved in the induction of apoptosis in transformed and malignant cells *in vitro* ^(17, 18).

There is little information available about the functions of 4-HPR in myeloid malignancies. Therefore, in the present study, the effects of 4-HPR in acute myelocytic leukemia cell line (NB-4) were evaluated. Results have shown great effects of 4-HPR in inhibiting the growth and proliferation of AML cells (NB-4). Furthermore, 4-HPR was found to be a strong inducer of apoptosis in these cells.

Materials and Methods

Cell lines

The acute myeloid leukemia cell line (NB-4) was purchased from Iran cell bank. Human NB-4 cell line was grown in RPMI 1640 culture medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY).

MTT assay

Cell proliferation was evaluated by the MTT [3, (4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4-sulfophenyl)-2H-tetrazolium] (SIGMA-ALDRICH®, Steinheim, Germany) assay. NB-4 cells were washed twice in RPMI 1640 by centrifugation at 300×g for 5 min. Cell concentration and viability were determined by hemocytometer counts of cells with 0.1% trypan blue dye. 5×10^4 cells were plated in a total volume of 100 μ l in 96-well plates (FALCON, USA). Then, cells were suspended in medium with 4-HPR (SIGMA-ALDRICH®, Steinheim, Germany) at concentrations of 1, 2.5, 5, 7.5, and 10 μ M. The cells were incubated for 24-72 hrs at 37 °C in a humidified 5% CO₂ atmosphere. Following the incubation, 0.01ml of MTT solution (at a final concentration 0.5 mg/ml) was added per well. The plates were then incubated for additional 3 hr at 37 °C in a humidified 5% CO₂ atmosphere. After incubation, cells were deposited by centrifugation at 300 ×g, and the medium was removed. 100 μ l of stop solution (isopropanol containing 0.04% HCl) was added per well. Immediately after solving the blue formazon

crystals, the absorbance of samples was read using a 96-well plate reader (ELISA Reader, Antus 2020) at a wave length of 570 *nM*. Each data point was determined eight times prior to analysis. Statistical analysis of the cell proliferation assay was performed with the Microsoft Excel program version 2007.

Microscopy

According to differences that exist between apoptotic and necrotic cells, two fluorescent dyes (AnnexinV-FITC and Propidium Iodide (SIGMA-ALDRICH®, Steinem, Germany)) were used in this study to analyze the apoptotic and necrotic cells, respectively. NB-4 cells were treated by 4-HPR at concentration 7.5 μM . After 6 *hr*, cells were washed and stained by two fluorescent dyes. Then, they were analyzed by fluorescence microscope.

Apoptosis assay

This procedure is based on evaluating apoptotic cells by detecting the phosphatidylserines. Annexin V-FITC is a fluorescent probe binding to phosphatidylserine.

In this study, 1×10^6 *cells/ml* suspension of NB-4 cells was induced for apoptosis by addition of several concentration of 4-HPR (1, 2.5, 5, 7.5 μM). 1×10^6 *cells/ml* suspension of non-induced NB-4 cells was established as a negative control. Both control and test samples of NB-4 cell cultures were incubated for 24 *hr* in a 37 °C, 5% CO₂ incubator. Following the incubation, the cells were washed twice with DPBS (Dulbecco's Phosphate Buffered Saline). After that, the cells at concentration of about 1×10^6 *cells/ml* were resuspended in 1X binding buffer. 500 λ of the apoptotic cell suspension was added to a plastic 12 $\times$ 75 *mm* test tube, and 500 λ of the non-induced cell suspension was added to a second plastic tube.

Next, 5 λ of AnnexinV-FITC (SIGMA-ALDRICH®, Steinem, Germany) and 10 λ of propidium iodide (SIGMA-ALDRICH®, Steinem, Germany) were added to each cell suspension. Then the tubes were incubated at room temperature for exactly 10 *min* and protected from light. Finally, the fluorescence

of the cells was immediately determined by a flow cytometer (Partec FloMax, version 2.3).

In order to adjust the flow cytometer for evaluating the apoptotic effects of anticancer drug in NB-4 cell line, a positive and a negative control sample was used (Figure 1A). As a positive control, apoptosis was induced in a 1×10^6 *cells/ml* suspension of AML cells by addition of 1 $\mu\text{g/ml}$ Staurosporine (Figure 1B). Flow cytometric analysis was performed using Partec FloMax software.

Cell cycle analysis

The DNA content during cell cycle steps were evaluated with flow cytometry. In brief, 5×10^6 cells were treated with 4-HPR at concentration 5 μM . After 24 *hr*, the cell pellets were washed and resuspended in 2 *ml* of 1% paraformaldehyde in PBS and incubated for 15 *min* at 4 °C. Then, cells were centrifuged and 1 *ml* of cold perm buffer III (BD. Co, USA) solution was added; cells were incubated for 30 *min* at 4 °C and then were washed twice in PBS. Next, 500 λ of propidium iodide (SIGMA-ALDRICH®, Steinem, Germany) staining buffer (50 $\mu\text{g/ml}$ PI, 10 $\mu\text{g/ml}$ RNase in PBS) was added and incubated for 1 *hr* at room temperature in the dark. After staining of DNA by PI, samples were evaluated by a flow cytometer. Flow cytometric analysis was performed using Partec FloMax software.

Results

Anti-proliferative and inhibitory effects

First, the anti-proliferative effects of 4-HPR were analyzed using the MTT assay. To assess cell proliferation, Microsoft Excel 2007 software was used to graph all data from MTT assay. Eight samples were tested for each drug concentration and each experiment was repeated three times prior to MTT analysis. Results of one representative experiment are shown in Figure 2. 4-HPR inhibits the growth of NB-4 cells in a concentration dependent manner. The effective dose that inhibits 50% growth (ED₅₀) of these cells was calculated; 4-HPR inhibits the growth of NB-4 cells with an ED₅₀ of approximately 5

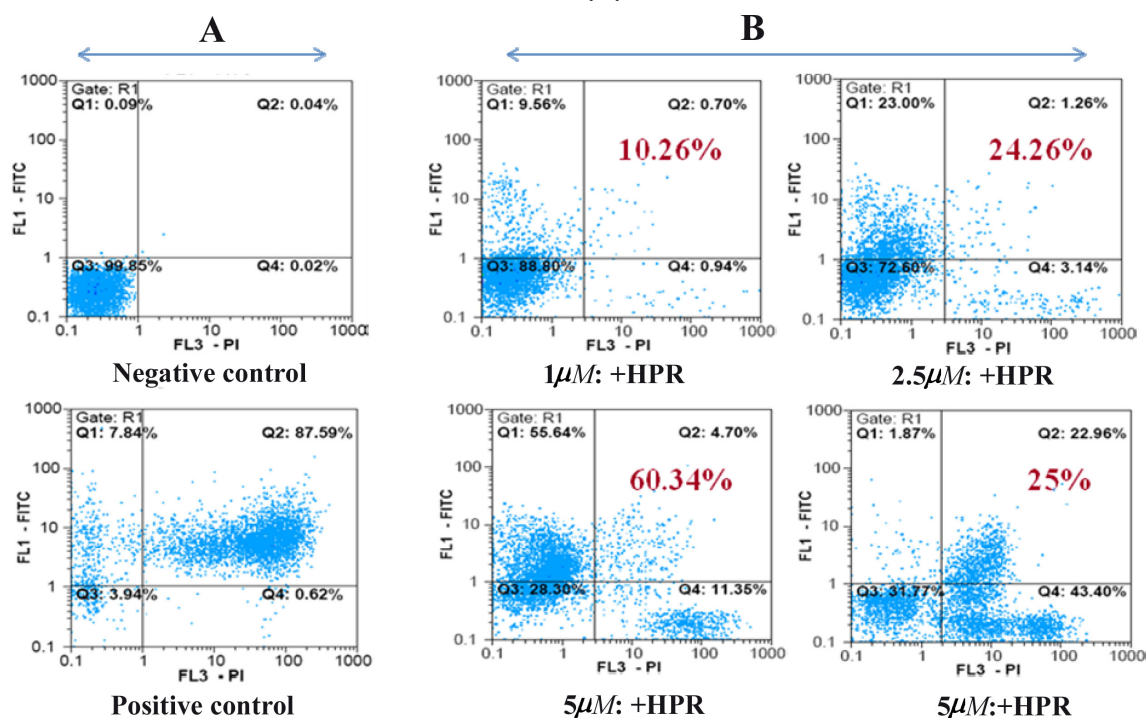


Figure 1. (A) Negative and positive control, (B) Apoptotic effect of 4-HPR in NB-4 cells at concentration 1, 2.5, 5 and 7.5 μM . Lower left quadrants, viable cells. Lower right quadrants, necrotic cells. Upper left quadrants, early apoptotic cells. Upper right quadrants, nonviable late apoptotic cells.

μM after 24 *hr* treatment (Figure 2). The most significant inhibitory effects of 4-HPR in NB-4 cells were around 80% at 10 μM and 7.5 μM after 24 *hr* and 48 *hr* treatments, respectively.

Morphological study of the apoptotic and necrotic cells

Figure 3 (Panel F) shows NB-4 cells stained with fluorescent dyes [AnnexinV-FITC (A) and Propidium Iodide (B)] after treatment with 4-HPR, respectively. As the pictures of inverted phase microscope show (Figure 3A, Panel P), apoptotic cells have a

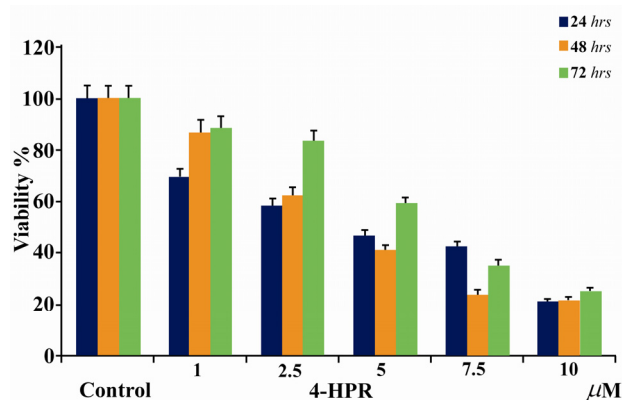


Figure 2. Anti-proliferation effects of 4-HPR in NB-4 cells after 24, 48, and 72 *hrs*. Eight samples were tested for each drug concentration and each experiment was repeated three times prior to MTT analysis

dense chromatin and still maintaining their cell membrane during apoptosis. However, in necrotic cells, the cell membrane is completely destroyed and their cell content were thrown out (Figure 3B, Panel P).

Apoptosis

To determine whether the growth inhibition by 4-HPR in NB-4 cells was associated with the induction of apoptotic cell death the apoptosis index was evaluated using the AnnexinV-FITC staining method (Figure 1). According to this method, NB-4 cells were evaluated by flow cytometry 24 *hr* post treatment with the anticancer drug. Staurosporine was used to induce apoptosis in NB-4 cells as a positive control. It is also known as a strong inducer of apoptosis in several cell lines, and it is a potent inhibitor of phospholipid/ Ca^{2+} dependent protein kinase (protein kinase C) ⁽¹⁹⁾. Many studies have shown the apoptotic effects of Staurosporine in several mammalian cell types ⁽²⁰⁾. All data were analyzed by specific software Partec FloMax. Although the percentages which are shown in Figure 1 demonstrate the dose-dependent increase in the apoptotic fraction at first,

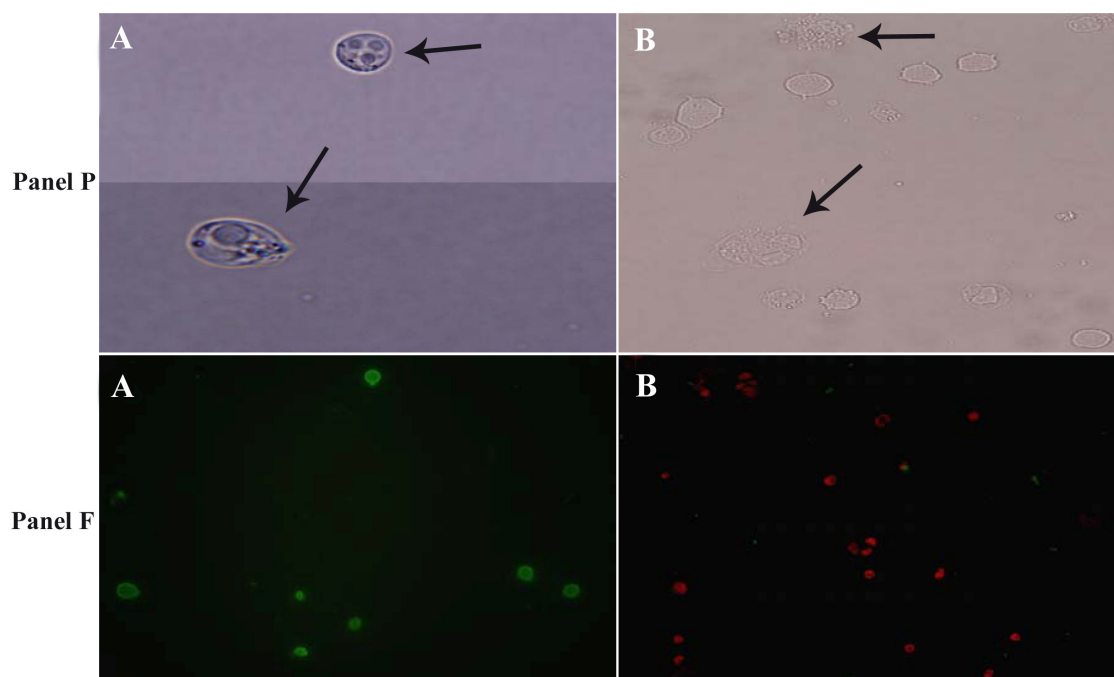


Figure 3. Morphological study of the apoptotic (A) and necrotic (B) cells using inverted phase microscope (Panel P) and fluorescence microscope (Panel F)

however these percentages were significantly decreased at the highest concentration used in this assay ($7.5 \mu\text{M}$). The population of cells that underwent necrosis was increased with higher 4-HPR concentration. The most apoptotic fraction of NB-4 cells measured by flow cytometry was more than 60% at concentration $5 \mu\text{M}$ of 4-HPR.

Cell cycle analysis

In this study, the DNA content of NB-4 cells during cell cycle was measured in order to obtain information about the cell cycle progression. Since it has been previously shown that 4-HPR causes the cessation of cell cycle in the G1 to S phase⁽²¹⁾, the effect of 4-HPR on progression of NB-4 cell cycle was tested in this study.

Figure 4 shows the distribution of DNA content during cell cycle in NB-4 cells. In control samples (untreated NB-4 cells), 51.59% of all cells accumulated in G0/ G1 phase, 8.66% in S phase, and 39.74% in G2 M phase. Treatment of NB-4 cells with 4-HPR at concentration of $5 \mu\text{M}$ causes a decrease (about 11%) in the proportion of cells in G0/ G1 phase (Figure 4). Cells were observed to significantly accumulate in S

phase (27.74% in treated cells versus 8.66% in control samples).

Discussion

Over the recent years, it has been demonstrated that retinoids regulate biological processes including cell proliferation, differentiation, and apoptosis⁽²²⁾. Retinoids also modulate cellular functions at many levels which are deregulated during carcinogenesis.

4-HPR is one of the synthetic derivatives of vitamin A⁽¹⁷⁾. In many investigations, 4-HPR has shown cytotoxic activity against several cancer cell lines such as neuroblastoma, breast cancer, prostate cancer, colon cancer, ovarian cancer, bladder cancer, and human head and neck squamous carcinoma^(23 - 25). Furthermore, it is proved that 4-HPR affects the hematopoietic neoplasms; for example, this drug inhibits the growth of ATRA-resistant myeloid leukemia cell lines, T lymphoma cells, and T lymphoblastic leukemia cells^(26, 27). 13-cis-Retinoic acid increases the event-free survival of children with high-risk neuroblastoma, and ATRA (all-trans-retinoic acid) has recently become an important part of Acute Promyelocytic Leukemia (APL) treatment⁽²⁸⁾.

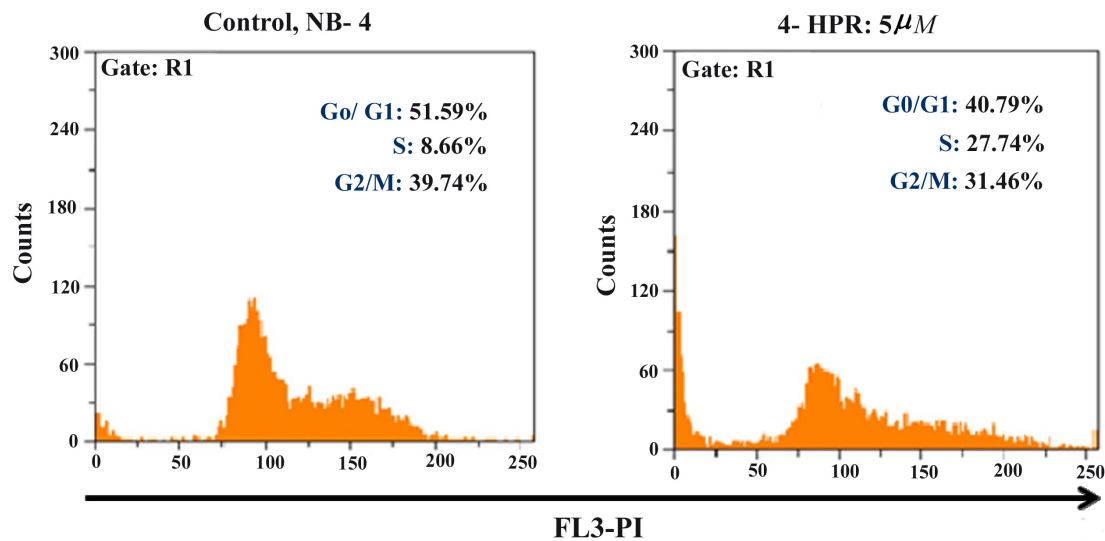


Figure 4. Effects of 4-HPR in NB-4 cell cycle progression after 24 hr, stain with PI

In animal models, it is proved that fenretinide (4-HPR) is an effective agent to prevent adverse side effects of chemotherapy against several cancers including breast cancer ⁽²⁹⁾, prostate and pancreas cancer ⁽³⁰⁾, and skin tumors ^(31, 32). Moreover, clinical trials have shown that fenretinide reduce the progress rate of prostate cancer in men who are diagnosed in early stages of disease ⁽³³⁾. This drug can also prevent the development of ovarian cancer and second breast malignancies in women who are being treated against breast cancer in early stages ⁽³⁴⁾. However, very few is known about the effects of 4-HPR in acute myelocytic leukemia. Therefore, the mechanisms by which 4-HPR acts in these cell systems are not totally understood.

This study evaluated the effects of 4-HPR in acute myelocytic leukemia cell line (NB-4) which have not been reported previously. We have demonstrated that 4-HPR strongly inhibits the growth of NB-4 cells. As we have shown in Figure 2, inhibition of proliferation of NB-4 cells was about 80% at 4-HPR concentration of 7.5 and 10 μ M. In this study, the inhibitory effects of 4-HPR in NB-4 cells after 72 hr treatment was shown to be less than the effects after 24 hr and 48 hr treatment. This effect was not an artifact, since the anti-proliferative effect of 4-HPR in NB-4 cells was evaluated three times and the results

were approximately the same each time. This effect might have happened due to reduced presence of 4-HPR in the test culture medium. Such reduction may have allowed the viable cells to proliferate and grow in culture medium.

We were interested to test the ability of 4-HPR to induce apoptosis in this cell line; therefore flow cytometry was employed. The flow cytometric results show that 4-HPR is a potent inducer of programmed cell death (apoptosis) in NB-4 cells (Figure 1). These data show that 4-HPR at 5 μ M could induce the maximum amount of apoptosis (60.34%) in these cells. However, flow cytometric analysis indicated a continuous increase in the fraction of necrotic cells with increasing concentrations of 4-HPR. At concentration of 7.5 μ M, the percentage of apoptotic cells decreased, and more than 40% of cells died through necrosis. This effect may be due to cytotoxic effects of 4-HPR in concentrations more than 5 μ M.

Initially we demonstrated that 4-HPR is a potent inhibitor of cell proliferation and a strong inducer of apoptosis in NB-4 cells. Next, we evaluated the effects of 4-HPR on the progression of NB-4 cell cycle. This can give useful information about the specific action of cytotoxic drugs. A variety of methods have been used for measuring the

DNA content of cells. All methods use dyes which bind specifically to nucleic acids, and upon binding their fluorescence is enhanced.

As previous studies have shown, one of the essential steps to initiate terminal differentiation is cell cycle arrest⁽³⁵⁾. Even though G1 arrest has been known as a central step in differentiation, other studies report the involvement of G2 and S phase arrest in this phenomenon^(36, 37).

In this study, the flow cytometric investigation revealed that 4-HPR causes the accumulation of cells in S phase (8.66% in the control versus 27.74% in the 4-HPR treated NB-4 cells after 24 hr) (Figure 4). Hence, the decrease in proliferation was associated with S-phase arrest in these cells. Indeed, the increase in the percentage of S-phase cells was associated with a decrease in G0/ G1 and/or G2/M cells up to 24 hr. This result is in agreement with other previous investigations. For example in 2003 Faderl et al, used cell cycle analysis and reported that 4-HPR at concentration of 5 μ M could block the Z-119 (ALL) cells in S phase, and it prevents cell cycle progression⁽¹⁷⁾. The cell cycle is regulated in response to the environment and basic check points are located at the end of G1 and G2 phases⁽³⁸⁾.

Over the past few years, studies have shown that 4-HPR is a strong inducer of apoptosis in some different cell systems. Chan et al have demonstrated that 4-HPR is a potent and selective inducer of apoptosis in murine T lymphoma and T lymphoblastoid cells⁽³⁹⁾.

4-HPR was 50 to over 150 times more effective to inhibit the growth and induce apoptosis in cell lines when compared to the other retinoids⁽³⁹⁾. Moreover, 4-HPR can affect steroid-resistant cell lines as well as steroid-sensitive counterparts⁽⁴⁰⁾. In an investigation Delia et al have demonstrated that 4-HPR inhibits the growth and proliferation of lymphoid and myeloid malignant cell lines in a dose-dependent manner⁽²⁷⁾. Taken all results together, 4-HPR has been shown to have more potent inhibitory effects in several

cancer cell lines than those of other retinoids at an equimolar concentration⁽¹⁴⁾.

In conclusion, this study has demonstrated that 4-HPR is a strong inhibitor of the growth and proliferation of acute myeloid leukemia cell line (NB-4). Furthermore, we have shown that 4-HPR induces programmed cell death and causes cell cycle arrest in S phase in these cells. It seems that 4-HPR acts through some specific and unique pathways to induce apoptosis in malignant cells that significantly distinguish this drug from other kind of retinoids like ATRA⁽¹⁷⁾. To further characterize the signaling pathways involved in AML cells differentiation, and in order to get a better understanding of the anti-proliferative activity of 4-HPR, more *in vitro* studies on AML blasts from leukemia patients are required.

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