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Effects of berberine on the secretion of cytokines and expression of genes involved in cell cycle regulation in THP-1 monocytic cell line

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ABSTRACT

Objective(s): Current acute myeloid leukemia (AML) therapeutic strategies have irreversible side-effects. Berberine (BBR) is an isoquinoline alkaloid, which has been known as an aryl hydrocarbon receptor (AhR) ligand. AhR is a cytoplasmic receptor, which is involved in the regulation of cellular and immune responses. Here, we investigated the expression profile of genes involved in the cell cycle and different cytokines upon BBR-mediated AhR activation on AML THP-1 cell line.

Materials and Methods: THP-1 cells and normal monocytes were treated with different concentrations of BBR (10 μM, 25 μM, 50 μM, and 100 μM) for 24 and 48 hr. The cell viability was measured by MTT assay. Real-time RT-PCR was conducted to evaluate the expression of AhR, cytochrome P450 1A1 (CYP1A1), interleukin 1 beta (IL1β), p21, p27, cyclin-dependent kinase 2 (CDK2) and p53. Cellular expression of AhR was also assessed using immunofluorescence method. ELISA was used to determine the level of IL-10 and IL-12 cytokines.

Results: BBR inhibits the proliferation of THP-1 cells in a dose- and time-dependent manner with minimal toxicity on normal monocytes. Phorbol 12-myristate 13-acetate (PMA) treatment increased the cellular expression of AhR. The AhR target genes (CYP1A1, IL1β) were overexpressed upon BBR treatment. BBR downregulated Cdk2 and upregulated p21, p27 and p53 genes in THP-1 cells. IL-10 was significantly increased upon BBR treatment, while IL-12 was not significantly changed in all combinations.

Conclusion: BBR could be introduced as an effective chemotherapeutic agent against AML by giving rise to the expression of CDK inhibitors and anti-inflammatory cytokines and downregulation of CDK2.

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Introduction

Acute myeloid leukemia (AML) is a systemic heterogeneous hematologic malignancy, which is the most predominant form of acute leukemia in older people. It accounts for the largest number of morbidity and mortality (1). Current therapeutic strategies mostly rely on aggressive approaches such as chemotherapy and radiotherapy. These options not only seem to be ineffective in achieving long-term survival for AML, but also are accompanied by different side effects (2). Therefore, investigation of alternative treatment possibilities and therapeutic targets with minimal side effects and higher efficiencies should be considered.

Recently, the importance of small molecules from natural sources with anti-cancer and anti-inflammatory properties has been noticed. Berberine (BBR) (PubChem CID: 2353) is an isoquinoline derivative alkaloid, which could be isolated from the stems and roots of several plants, such as *Berberis vulgaris* (3). *Berberis vulgaris* L. from Berberidaceae that have been widely used in Persian traditional medicine are mostly grown in Europe and Asia, especially in Iran (4). In recent years, several medicinal properties of BBR have been reported including anti-inflammatory (5) and anti-cancer effects (6). BBR has been extensively studied for its cytotoxic activities against cancer cells of different origins by inducing cell cycle arrest and apoptosis with minimal toxicity to normal cells (7, 8), indicating that BBR could be an attractive anti-cancer compound. Several molecular mechanisms have been proposed for these anti-proliferative roles of BBR against cancer cells (3, 9, 10). However, BBR as an aryl hydrocarbon receptor (AhR) agonist (11) has not been studied thoroughly (11, 12). AhR is a cytoplasmic receptor and transcription factor, which is known for its role in xenobiotic-induced

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detoxification for environmental and endogenous toxicants in a ligand-dependent manner (13, 14). AhR also regulates cellular responses (15) to immune regulation and inflammation (16). Recently, the role of AhR in carcinogenesis as a potential drug target is also being studied (17). The role of AhR as a cell cycle mediator is associated with multiple aspects in cell proliferation and differentiation, gene regulation and tumor development (15). However, there have been controversies on the role of AhR and interaction of its ligands with cancer cells. Although, 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) as the classic AhR agonist is proposed to act as a tumor promoter (18), there are several evidences reporting the tumor-suppressive role of ligand-dependent activation of AhR against multiple cancer types (19-24).

Cytochrome P450 1A1 (CYP1A1) is one of the xenobiotic metabolizing enzymes (XMEs), which is induced by polycyclic aromatic hydrocarbons (PAHs) through AhR activation (25). Interleukin 1 beta (IL1β) is a member of the interleukin 1 family, which is mostly produced by activated monocytes and/or macrophages. IL1β is an inflammatory cytokine, which is involved in a variety of cellular responses such as cell proliferation, differentiation, and apoptosis (26). CYP1A1 and IL1β are also known as major AhR target genes (11). Interleukine-10 (IL-10) is an anti-inflammatory cytokine with multiple effects in immunoregulation. IL-10 mostly downregulates Th1 cytokines and co-stimulatory molecules on macrophages and can suppress the expression of several pro-inflammatory cytokines such as IL-12 (27). The cyclin-dependent kinase (CDK) inhibitors CDKN1A (p21) and CDKN1B (p27) regulate the progression of cell cycle in the G0-G1 phase, and overexpression of these factors causes a blockade of the G1 to S transition. The expression of p21 and p27 is tightly associated with the tumor suppressor p53 (28). CDK2 as a major cyclin-dependent kinase could represent the effect of CDK inhibitory factors (28).

In the present study, we aimed to investigate the expression profile of genes involved in the regulation of cell cycle and secretion of inflammatory and anti-inflammatory cytokines upon BBR-mediated AhR activation on AML THP-1 cell line.

Materials and Methods

Reagents and materials

Berberine chloride, phorbol 12-myristate 13-acetate (PMA) and MTT solution were purchased from Sigma (Sigma-Aldrich, USA). THP-1 AML cell line was provided by Dr Shokri's Laboratory (Department of Immunology, Tehran University of Medical Sciences). fetal bovine serum (FBS), high glucose RPMI 1640, penicillin/streptomycin antibiotics solution, and dimethyl sulfoxide (DMSO) were purchased from Gibco (Life Technologies, USA). Commercially available IL-10 and IL-12 ELISA kits as well as anti-AhR monoclonal antibody and DyLight 488 conjugated goat anti-rabbit IgG (H+L) secondary antibody were purchased from eBioscience (Thermo Fisher, USA). Biozol total RNA extraction reagent (BioFlux, China) was used for RNA extraction. Real-time PCR (qPCR) SYBR Green Master Mix and cDNA synthesis kit were from Bioron (Bioron, Germany).

Cell culture and treatments

THP-1 AML cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM Glutamax and incubated in a fully humidified atmosphere at 37 °C with 5% CO_2. Cells (2-5x10^5/ml) were seeded in 6 well plates following by resuspension in complete growth media. THP-1 monocyte cells were then treated with 10 ng/ml PMA as a tumor promoter to enhance the expression of AhR (29) and were treated with increasing concentrations of BBR (10 µM, 25 µM, 50 µM, and 100 µM). Control experiments were carried out by adding DMSO to PMA-primed THP-1 cells. Treated and non-treated cells were incubated for 24 and 48 hr at 37 °C with 5% CO_2. The cell culture supernatants were then collected by aspiration and centrifugation at 1000 g for 5 min and stored at -70 °C until cytokine analysis. THP-1 cells were harvested and kept at -70 °C until total RNA extraction. In order to address the effects of BBR on normal monocytes, peripheral blood mononuclear cells (PBMCs) from 6 healthy donors were isolated using Ficoll-Paque gradient centrifugation. Monocytes were enriched using a modified attachment method as previously described (30).

RNA extraction and real time RT-PCR

Total RNA was extracted from collected cells by Biozol according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed to cDNA with random hexamer primers. Real-time RT-PCR was performed with Bioer real-time PCR detection system (Hangzhou high tech, Bioer Technology, China). Gene specific primers for AhR, CYP1A1 and IL1β (as target genes regulated by AhR), 18srRNA (as an internal control), p21, p27, CDK2, and p53 (genes involved in cell cycle regulation) are summarized in Table 1. PCR amplifications were performed using Bioron master mix.

Cell viability (MTT) assay

THP-1 and enriched normal monocytes were seeded at 5x10^4 cells/well in 24-well plate with different concentrations of BBR chloride and observed for 24 hr and 48 hr followed by MTT assay (31). Briefly, cells were incubated in triplicates with different concentrations of BBR (10 µM, 25 µM, 50 µM and 100 µM) in a final volume of 200 µl of phenol red free RPMI 1640 for 20 hr at 37 °C with 5% CO_2. 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 hr at 37 °C with 5% CO_2. 24-

well plate was then centrifuged at 400 g for 5 min and media was removed. 300 µl DMSO was then added to each well as a cell lysis solution. Percentage of cell viability was assessed by spectrophotometry at 570 nm using ELx800 Absorbance Reader (Biotek, USA).

Table1. Gene specific primers used for real time RT-PCR

Primer *(Accession)*	Sequence (5'>3')	T_m	Amplicon size
CYP1A1 *(NM_000499)*	F: TCCTGGAGCCTCATGTATT	61	200 bp
	R: TCTCTTGTTGTGCTGTGG		
IL1β *(NM_000576)*	F: GGCTTATTACAGTGGCAATG	60	135 bp
	R: TAGTGGTGGTCGGAGATT		
P27 *(NM_004064)*	F:CAGGAGAGCCAGGATGTC	59	178 bp
	R:GAGTAGAAGAATCGTCGGTT		
P21 *(NM_001798)*	F: GAGGCCGGGATGAGTTGGGAGGAG	61	221 bp
	R: CAGCCGGCGTTTGGAGTGGTAGAA		
CDK2 *(NM_001220778)*	F: GCTAGCAGACTTTGGACTAGCCAG	60	85 bp
	R: AGCTCGGTACCACAGGGTCA		
P53 *(NM_001126114)*	F: CCCCTCCTGGCCCCTGTCATCTTC	61	265 bp
	R: GCAGCGCCTCACAACCTCCGTCAT		
AHR (NM-001621)	F: ACATCACCTACGCCAGTCGC	60	101 bp
	R: TCTATGCCGCTTGGAAGGAT		
18srRNA *(M10098)*	F: CAGCCACCCGAGATTGAGCA	61	252 bp
	R: TAGTAGCGACGGGCGGTGTG		

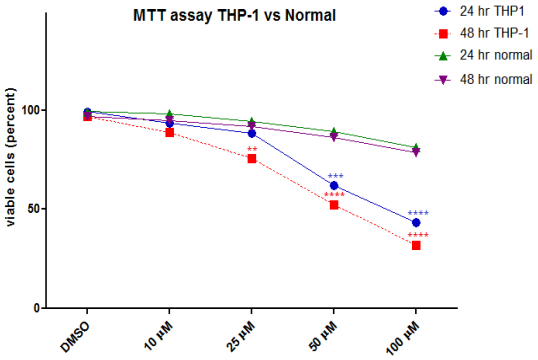

Figure 1. Effect of berberine (BBR) on the viability of THP-1 cells compared to normal monocytes. THP-1 cells and normal monocytes were treated with dimethyl sulfoxide (DMSO) (as zero dose negative control) and increasing concentrations of BBR (10, 25, 50 or 100 µM) for 24 hr and 48 hr. All treatments were subjected to a MTT assay for cell viability. Data represent the mean±SE from three independent experiments. Significant differences are evaluated using independent samples t-test. *P*-values lower than 0.05 are considered statistically significant. ***P*<0.01, ****P*<0.001, *****P*<0.0001

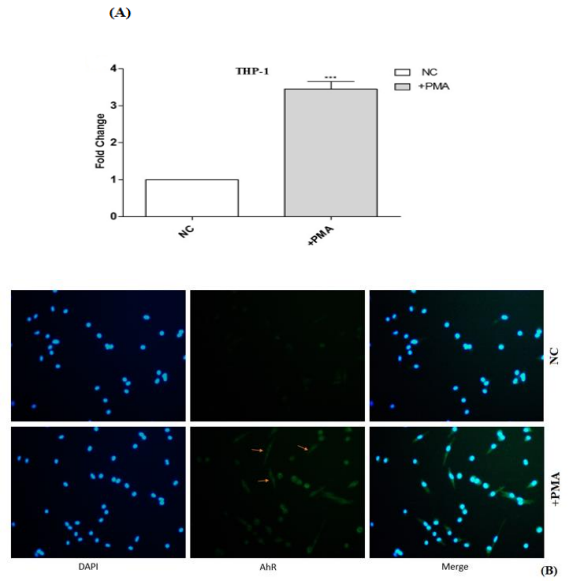

Figure 2. The expression of aryl hydrocarbon receptor (AHR) is increased upon phorbol 12-myristate 13-acetate (PMA induction). **(A)** Quantitative real-time RT-PCR on AhR gene was conducted to confirm the overexpression of AhR upon PMA treatment**.** Data are expressed relatively to mRNA levels in control group, dimethyl sulfoxide (DMSO), arbitrarily set at the value of 1. Data represent the mean ± SE from three independent experiments. Significant differences are evaluated using one-way ANOVA followed by bonferroni *post hoc* test. *P*-values lower than 0.05 are considered statistically significant. ****P*<0.001. **(B)** Immunocytochemical characterization of human THP-1 monocytic cells. The cytoplasmic expression of AhR is increased among PMA-treated (10 ng/ml) cells. Blue 4', 6-diamidino-2-phenylindole (DAPI) stained nucleus of THP-1 cells were merged with green colored AhR-expressing cells as shown by arrows (40X magnification)

ELISA cytokine assay

Cell culture supernatants of treated and non-treated THP-1 cells were centrifuged at 1000 g for 5 min to remove any cellular remnants. The levels of anti-inflammatory IL-10 and inflammatory IL-12

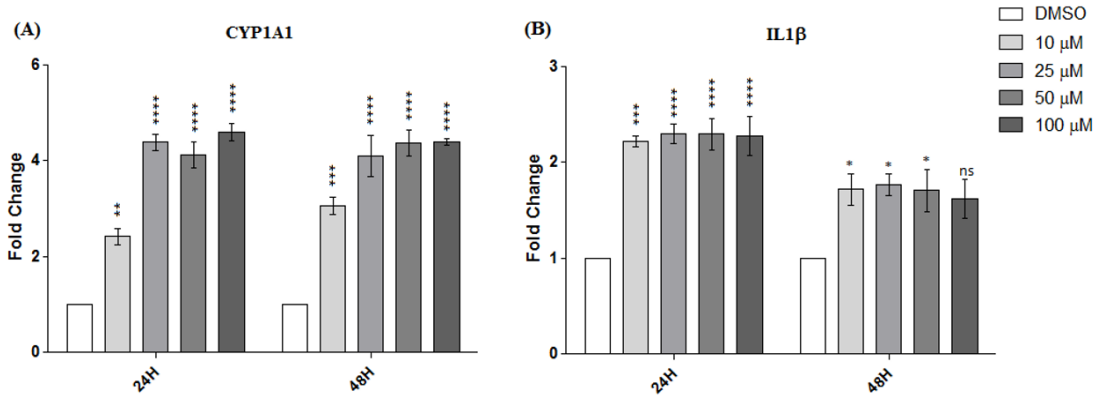

Figure 3. The expression of AhR target genes. Quantitative real-time RT-PCR on CYP1A1 (A) and IL1β (B), as specific target genes, was conducted to confirm the activation of AhR upon BBR treatment**s.** Data are expressed relatively to mRNA levels in control group (DMSO), arbitrarily set at the value of 1. Data represent the mean ± SE from three independent experiments. Significant differences are evaluated using one-way ANOVA followed by bonferroni *post hoc* test. *P*-values lower than 0.05 are considered statistically significant. Ns: not statistically significant, **P*<0.05, ****P*<0.001, *****P*<0.0001

cytokines were determined using commercially available ELISA kits (eBioscience, USA) according to the manufacturer's instructions. According to our previous study, the most crucial element of the detection strategy was considered for this ELISA assay (32). All samples were assayed in triplicates and the results were reported as picograms per ml.

Immunofluorescence

In order to evaluate the expression of AhR in THP-1 monocytic cells upon PMA treatment, an immunofluorescence method was used (25). The cytoplasmic expression of AhR was detected in THP-1 cells using anti-AhR monoclonal antibody (1/100) and visualized using DyLight 488 conjugated goat anti-rabbit IgG (H+L) secondary antibody (1/200). In order to address possible nonspecific antibody bindings, cells were incubated with secondary antibody alone as a negative control. Nucleus was visualized by 4', 6-diamidino-2-phenylindole (DAPI) staining. Cells were washed and immediately visualized by fluorescence microscopy.

Statistical analysis

All of the experiments were repeated in triplicates and data were demonstrated as means±SE (Standard Error). Statistical software SPSS 22.0 and Graphpad Prism 5.04 were used for data analysis. Two-way ANOVA with Bonferroni *posttest* were used for comparing means of multiple samples. The independent samples t-test was used to compare differences between two independent groups. *P*-values lower than 0.05 were considered as statistically significant.

Results

BBR inhibits the proliferation of THP-1 cells in a dose-dependent manner over time

The anti-proliferative effects of various concentrations of BBR were first determined on THP-1 leukemic cells and normal monocytes. The percentages of cell viability were measured by MTT assay. BBR treatment resulted in significant reduction in cell proliferation of THP-1 cells in a dose-dependent manner over time, ranging from 30% to 75% (*P*-value<0.05–0.0001). The highest toxicity was observed for 100 µM concentration of BBR after 48 hr. The cell viability of normal monocytes treated with all concentrations of BBR was not changed significantly compared to the control group (normal monocytes treated with DMSO alone), which represents the minimal toxicity of BBR on normal cells (Figure 1).

The cellular AhR is overexpressed among PMA-treated THP-1 cells

Quantitative real-time RT-PCR was conducted to address the alterations in the mRNA expression level of AhR upon PMA treatment. The mRNA expression of AhR is increased upon PMA induction among THP-1 cells (Figure 2A). Moreover, the cellular expression of AhR was assessed using immunofluorescence method, which confirmed the overexpression (Figure 2B).

The expression of AhR target genes is altered upon BBR treatment

In order to confirm the activation of AhR transcription factor upon BBR treatment, the mRNA expression level of CYP1A1 (as the main AhR target gene) and IL1β (as the major regulated inflammatory mediator gene) were evaluated using quantitative real-time RT-PCR. CYP1A1 was overexpressed significantly among all combinations over time (Figure 3A). Although, the mRNA expression level of IL1β was increased upon BBR treatment in THP-1 cells after 24 hr, it was downregulated gradually and reached a level near to control group after 72 hr (Figure 3B).

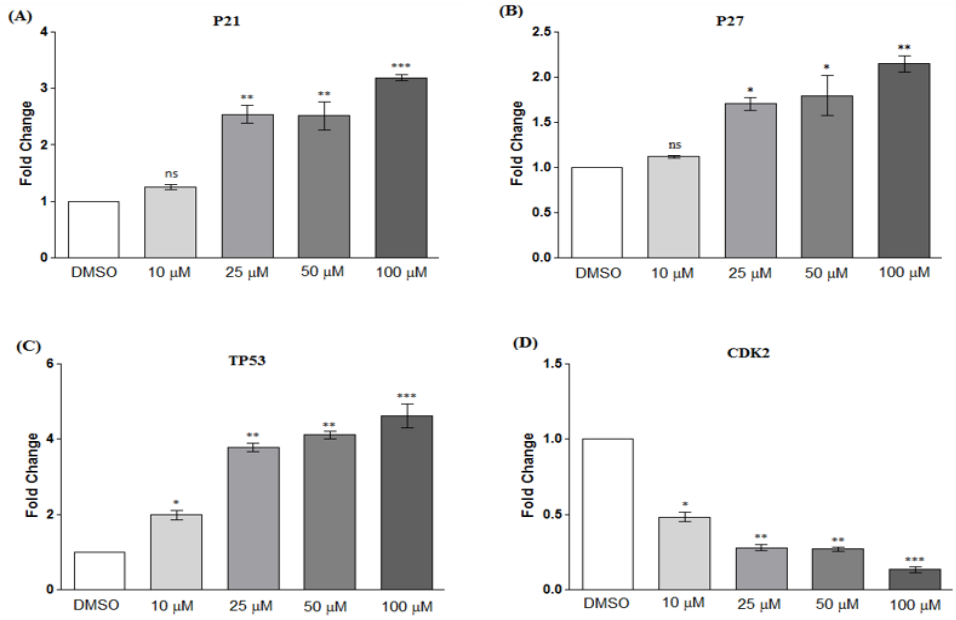

Figure 4. The expression of genes involved in the cell cycle regulation. Quantitative real-time RT-PCR on p21 (A) and p27 (B), p53 (C) and CDK2 (D), was conducted to address the changes in the expression of genes involved in cell cycle upon BBR treatment. All experiments were conducted at 48 hr time. Data are expressed relatively to mRNA levels in control group (DMSO), arbitrarily set at the value of 1. Data represent the mean±SE from three independent experiments. Significant differences are evaluated using independent samples t-test. *P*-values lower than 0.05 are considered statistically significant. Ns: not statistically significant, * *P*<0.05, ** *P*<0.01, *** *P*<0.001, ****P*<0.0001

BBR downregulates Cdk2 and upregulates p21, p27 and p53 in THP-1 cells

BBR-treated THP-1 cells were selected after 48 hr to evaluate the expression of genes involved in cell cycle progression, as BBR was more effective in suppressing cell growth at this time without exerting cytotoxic effects on normal monocytes. According to the role of CDKs and CDK inhibitors in the regulation of cell cycle progression, the effect of BBR was examined on the mRNA expression of CDK2 (cyclin-dependent kinase 2)(33), p21 (cyclin-dependent kinase inhibitor 1)(34) and p27 (cyclin-dependent kinase inhibitor 1B)(34) genes. The effects of BBR treatment was also evaluated on the expression of p53 as a well-known tumor suppressor (35). Analyzing the expression of p21 and p27 by real-time RT-PCR showed that BBR treatment of THP-1 leukemic cells for 48 hr resulted in a dose-dependent overexpression of these genes (Figure 4A and B). It was also shown that BBR-mediated activation of AhR was involved in the overexpression of p53 in a dose-dependent manner (Figure 4C). Moreover, the effect of BBR was assessed on the expression of CDK2. As compared with the control group (DMSO treated cells alone), CDK2 was markedly downregulated upon BBR treatments (Figure 4D).

Secretion level of IL-10 and IL-12 upon BBR treatment

To evaluate the anti-inflammatory effect of BBR, the secretion level of IL-10 was evaluated in the cell culture

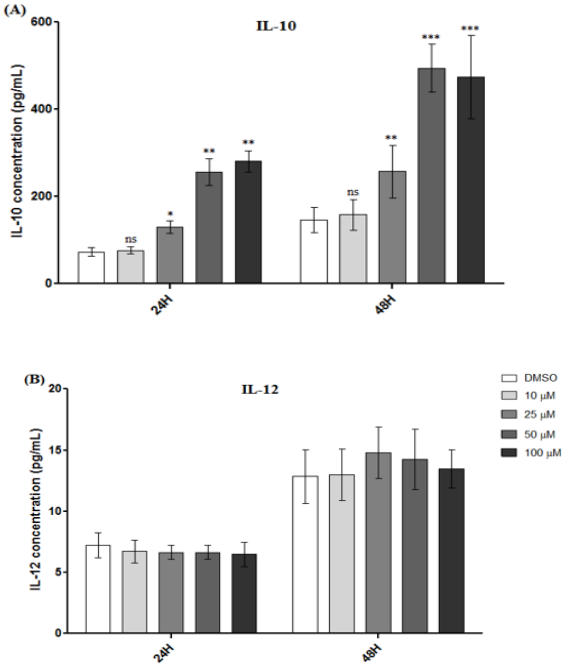

Figure 5. ELISA cytokine assay. The expression of IL-10 is elevated in response to BBR in a dose- and time-dependent manner (A). IL-12 expression levels are not altered in response to BBR significantly (B). Data of each bar demonstrates means±SE from three independent experiments. *P*-values lower than 0.05 were considered as statistically significant. Ns: not significant *P<0.05, **P<0.01, ***P<0.001

supernatant of treated and non-treated cells at different time intervals. It was shown that the secretion of IL-10 is significantly enhanced upon BBR treatment in a dose- and time-dependent manner (Figure 5A). The level of IL-12 was also examined to address the inflammatory effect of BBR. Although, there was a decline in the level of IL-12 followed by BBR treatments, there were no significant differences in all combinations (Figure 5B).

Discussion

Therapy-associated morbidity and mortality still remains the major burden of AML current treatment strategies (1). Investigation of novel therapeutic compounds with minimal side-effects should be seriously considered to enhance the survival of AML patients. Today, using natural and biological components proposed as new and safe strategies for controlling different range of disease from infectious disease to cancer (36, 37)

BBR is a well-known isoquinoline alkaloid isolated from *B. vulgaris*, which is commonly used as a herbal medication in persian traditional medicine and could be introduced as an alternative treatment possibility (4). BBR has been recently studied for its anti-inflammatory (5) and anti-cancer effects (7). Despite proposing different molecular mechanisms, the underlying factors have not been well elucidated.

AhR is a ligand-activated cytoplasmic receptor and transcription factor (13, 14), which is responsible for the regulation of cellular responses (15) by exerting anti-cancer and anti-inflammatory effects (16). AhR as a potential drug target has been recently studied in carcinogenesis, while several investigations revealed that AhR activation is associated with tumor-suppressive properties (17, 19). BBR is an exogenous dietary AhR agonist, which may activate AhR in a ligand-dependent manner (11). In our present investigation, we hypothesized that BBR might exert its anti-cancer and anti-inflammatory activities through AhR activation. Therefore, the aim of this study was to characterize the anti-proliferative effects of BBR on THP-1 leukemic cells and normal monocytes and to address the gene expression alterations involved in AhR activation and cell cycle regulation. The expression of cytokines was also assessed.

Here, we showed that BBR significantly inhibited the viability of AML THP-1 cells without incurring cytotoxicity to normal monocytes under the present experimental conditions, which suggests that BBR could be an effective chemotherapeutic agent against AML leukemic cancer cells. The concentrations of BBR and incubation times used here were in accordance with those reported by others demonstrating significant growth inhibition in different cell lines (4, 9, 34).

It has been suggested that the expression of AhR is low among THP-1 cells (5). However, we realized that PMA-induction as a tumor promoting compound, which is widely used to shape macrophage-like cells in THP-1 cell line could enhance the expression of AhR. In order to evaluate the activation of AhR in response to BBR, the mRNA expression of two major AhR target genes including CYP1A1 and IL1β was quantified. The alterations in the expression of AhR target genes approved AhR activation. CYP1A1 was overexpressed upon BBR treatments, which indicates the effective activation of AHR in a dose- and time-dependent manner. Although, the level of IL1β was increased in response to BBR, it was downregulated gradually. Regarding the anti-inflammatory effects of BBR and inflammatory nature of IL1β, we comprehend that the continuous exposure to BBR has dampened the inflammatory immune response, which is in favor of previous study (38).

Cell cycle regulators are frequently mutated in most common cancers including hematologic malignancies (1). Therefore, one of the potentially effective strategies for the control of cancer cells could be through cell cycle regulation. The role of AhR as a cell cycle mediator is associated with multiple aspects in cell proliferation and differentiation, gene regulation and tumor development (15). Induction of G1-phase cell cycle arrest has been shown as one of the mechanisms by which BBR may act to inhibit the proliferation of cancer cells (33). Therefore, we investigated the expression of genes involved in G1-S cell cycle progression. The CDK inhibitors p21 and p27 regulate the progression of cell cycle in the G0-G1 phase, and overexpression of these factors causes a blockade of the G1 to S transition (28). Our data indicated that treatment of THP-1 cells with BBR resulted in significant overexpression of p21 and p27 genes in a dose-dependent manner. We also investigated the expression of p53 as a tumor suppressor gene, which is also known as a G1-S regulation point upon DNA damage (35). We observed a BBR-mediated overexpression of p53, which was dose-dependent. The CDK inhibitory factors suppress cell cycle progression by binding to CDK-cyclin complex and inhibiting its kinase activity (6, 7, 35). Thus, we addressed the expressional changes of CDK2 as a major cyclin-dependent kinase upon BBR treatment. We found a significant decrease in the expression of CDK2, which may indicate a perturbation of the uncontrolled cell cycle progression. Based on our data, it seems that overexpression of p21 and p27 and downregulation of CDK2 may be responsible for most of the anti-proliferative effects observed in response to BBR because these regulators are markedly regulated even by lowest dose of BBR. Identification of agents that provide an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway is of high priority. G1-phase cell cycle arrest provides such an opportunity for cancer cells, especially in chemo-resistant malig-

nancies. Therefore, BBR could be introduced as a cell cycle regulator by giving rise to the expression of p21, p27 and p53 to exert its anti-proliferative effects by inducing G1-phase cell cycle arrest.

In order to address the anti-inflammatory effects of BBR on THP-1 cells, we evaluated the cytokine expression of IL-10 and IL-12. IL-10 could down-regulate co-stimulatory molecules on macrophages and monocytes and also suppresses the expression of several pro-inflammatory cytokines such as IL-12 (27). Although the expression level of IL-12 as an inflammatory cytokine was not significantly changed, but the level of anti-inflammatory IL-10 was markedly increased. Overexpression of IL-10 represents the possible anti-inflammatory role of BBR (36) on THP-1 cell lines, which could be mediated by the activation of AhR and accompanied by the induction G1-S cell cycle arrest.

Conclusion

On the basis of our findings, we claim that the anti-inflammatory and anti-proliferative effects of BBR are probably mediated by the activation of AhR among THP-1 cells, which results in the overexpression of p21, p27 and p53 and downregulation of CDK2 and probably induction of G1-S cell cycle arrest in a dose-dependent manner. Accordingly, AhR could be targeted in leukemic malignancies and could be introduced as a possible anti-cancer agent, especially when activated by specific ligands such as BBR. Altogether, BBR should be considered in the future assessment of its translational value in AML therapy. However, further *in vivo* and clinical investigations are required.

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