BMI-1 is important in bufalin-induced apoptosis of K562 cells

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Received May 21, 2013; Accepted January 23, 2014

DOI: 10.3892/mmr.2014.1980

Abstract. The purpose of this study was to analyze the effects of bufalin on the gene expression of K562 cells and on the expression of BMI-1 pathway constituents in K562 cell apoptosis. K562 cells were treated with bufalin, and the inhibition rate and apoptosis were detected by an MTT assay, flow cytometry and a microarray assay. BMI-1, p16INK4a and p14ARF were examined by quantitative polymerase chain reaction (qPCR). Bufalin induced significant changes in the gene expression of the K562 cells; 4296 genes were differentially expressed, 2185 were upregulated and 2111 were downregulated. The most upregulated genes were associated with transcription regulation, while the most downregulated genes were associated with the non-coding RNA metabolic processes and DNA repair. qPCR analysis demonstrated that BMI-1 was overexpressed in the K562 cells. Bufalin is able to downregulate BMI-1 expression levels in K562 cells prematurely and cause an increase in the expression levels of p16INK4a and p14ARF. Moreover, bufalin downregulated BCR/ABL expression levels in a time-dependent manner, and the expression of BCR/ABL was not associated with the upregulation or downregulation of BMI-1 expression. Bufalin may induce K562 cell apoptosis by downregulating BMI-1 expression levels and accordingly upregulating the expression levels of p16INK4a and p14ARF. Bufalin may also induce K562 cell apoptosis via downregulating BCR/ABL expression levels, and this pathway may be independent of the BMI-1 pathway.

Introduction

Bufalin is a traditional Chinese medicine and it is the major digoxin-like immunoactive component of Chan Su, which is obtained from the skin and parotid venom glands of toads (1). It is a cardioactive C-24 steroid, with the molecular formula C24H34O4 and a relative molecular weight of 386.5. Bufalin exhibits a variety of biological activities, such as cardio-dynamic, anesthetic, blood pressure stimulatory, respiratory and antineoplastic effects (2). In terms of its antitumor activity, bufalin has been demonstrated to inhibit the growth of gastric, colon, breast, prostate, gynecological, hepatocellular and bladder tumors (3). It is also able to induce strong differentiation and apoptosis of myeloid leukemia cells, such as HL60, U937 and K562 cells. The mechanisms by which bufalin induces leukemia cell apoptosis are complex and involve a diverse range of cell signals (4-7). It is capable of inhibiting sodium-potassium-ATPase, and then activating apoptotic pathways (8,9). It is also able to activate the mitogen-activated protein kinase (MAPK) signaling pathway (9); downregulate the expression of the proto-oncogene c-myc and the anti-apoptotic protein Bcl-2 (10); and reduce the quantity, activity and mRNA levels of topoisomerase II (11). However, the precise pathways by which bufalin induces apoptosis remain unclear, and an improved understanding of such pathways is an essential goal of further study. To determine the global molecular mechanisms underlying bufalin-induced leukemia cell apoptosis, the present study analyzed the effects of bufalin on the gene expression of K562 cells using a Microarray assay. Based on this experiment, the expression of the BMI-1 pathway components in K562 cell apoptosis was also analyzed.

BMI-1 is a member of the polycomb-group gene family, that is known to be essential in supporting the self-renewal of stem cells through their epigenetic transcriptional regulation (12-15). BMI-1 is located on chromosome 10p13, a region involved in chromosomal translocations in infant leukemia (16). BMI-1 is a transcriptional repressor, the most widely known of the INK4a/ARF locus, that encodes the cell cycle regulators and tumor suppressors p16INK4a and p14ARF (17,18). Downregulation of p16INK4a and p14ARF inhibits cell apoptosis that is induced by p53 and retinoblastoma protein (pRB). It has become clear that BMI-1 also functions in protecting against oxidative stress. In the absence of BMI-1, reactive oxygen species (ROS) accumulate, which are associated with the activation of DNA damage response pathways and increased apoptosis (19). Therefore, overexpression of BMI-1 results in cell immortalization and tumorigenesis, whereas downregulation in tumor (stem) cells results in impaired self-renewal and proliferation (20,21). It has been demonstrated that BMI-1 is often overexpressed in various types of leukemia and its depletion in those leukemia
Total RNA was amplified from K562 cells using a TRIzol reagent (cat. no. 15596-018; Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Cy3-labeled cRNA was prepared from the labeled RNA following the manufacturer's instructions. Samples were analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) following staining.

Materials and methods

Materials. Bufalin (10.0 mg/vial) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in anhydrous alcohol to make 0.01 mol/l stock solution. The solution was stored at -20°C and diluted in RPMI-1640 when used. K562 cells were obtained from the central laboratory of The First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). Bone marrow mononuclear cells were acquired from two female healthy iron-deficiency anemia volunteers (ages, 38 and 29 years) as normal controls. The study was approved by the Ethics Committee of Shaanxi Provincial People's Hospital and written informed consent was obtained from the volunteers.

Cell culture. The K562 human erythocyte leukemia cell line was grown in suspension culture in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA), at 37°C in a humidified atmosphere with 5% CO₂. K562 cells at an exponential growth stage were employed in all of the experiments.

MTT assay. Cells were seeded in 96-well plates at a density of 10⁵ cells/ml in 100 µl of medium and cultured overnight. Each experiment was performed in triplicate. Subsequently, the cells in the wells were treated with fresh medium containing different concentrations of bufalin diluted from the stock solution. The resulting concentrations of bufalin were 0.025, 0.05, 0.1, 0.5, 1.0 and 2.0 µmol/l. The culture solution was applied to the wells as a blank control. The cells that were not treated with bufalin served as negative controls. Cells were cultured for 48 or 72 h. At the indicated times, MTT solution [5 mg/ml in 20 µl phosphate-buffered saline (PBS)] was added to each well and incubated for 4 h. Following removal of the medium, 200 µl dimethylsulfoxide was added to each well to dissolve the formazan crystals. The absorbance at 490 nm was determined using a microplate reader (Beijing Pulongxin Technology Co., Ltd., Beijing, China). The inhibition rate of cell proliferation was calculated as follows: Inhibition rate (%) = A490 (control) - A490 (test) / A490 (control) - A490 (blank) x 100.

Flow cytometric analysis. To evaluate the effect of bufalin on K562 cell early apoptosis and to determine a suitable concentration of bufalin for microarray experiments, an Annexin V-Fluorescin isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Immunocytometry Systems, Franklin Lakes, NJ, USA) for flow cytometry was used to examine the apoptosis. K562 cells were treated with 0.025, 0.05, 0.1, 0.5 or 1.0 µmol/l bufalin for 48 h. The cells were washed twice with cold PBS and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. Samples were analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) following staining.

RNA isolation and microarray analysis

RNA extraction and purification. Total RNA was extracted using TRIzol reagent (cat. no. 15596-018; Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions, and checked for an RNA integrity number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Qualified total RNA was further purified using an RNeasy mini kit (cat. no. 74106; Qiagen GmBH, Hilden, Germany) and RNase-Free DNase set (cat. no. 79254; Qiagen GmBH).

RNA amplification and labeling. Total RNA was amplified and labeled using a Low Input Quick Amp Labeling kit, one-color (cat. no. 5190-2305; Agilent Technologies), according to the manufacturer's instructions. Labeled cRNA were purified using the RNeasy mini kit (cat. no. 74106; Qiagen GmBH).

Hybridization. Each slide was hybridized with 1.65 µg Cy3-labeled cRNA using a Gene Expression Hybridization kit (cat. no. 5188-5242; Agilent Technologies) in a hybridization oven (cat. no. G2545A; Agilent Technologies), according to the manufacturer's instructions. Following 17 h of hybridization, slides were washed in staining dishes (cat. no. 121; Thermo Shandon, Waltham, MA, USA) with the Gene Expression Wash Buffer kit (cat. no. 5188-5327; Agilent Technologies), following the manufacturer's instructions.

Data acquisition. Slides were scanned with an Agilent Microarray Scanner (cat. no. G2565CA; Agilent Technologies) with the following default settings: Dye channel, green; scan resolution, 5 µm; PMT, 100%, 10%. Additionally, 16-bit Feature Extraction software 10.7 (Agilent Technologies) was used, and raw data were normalized by a quantile algorithm (GeneSpring software 11.0; Agilent Technologies).

qPCR. Total RNA was isolated and purified as previously described. Reverse transcription of the total RNA was conducted using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA). qPCR was performed using SYBR-Green chemistry in an ABI StepOnePlus system (Applied Biosystems, New York, NY, USA). GAPDH served as a housekeeping gene. PCR primer sequences were as follows: Forward: 5’-TGGGTTGTGAACCATGAGAAGT-3’ and reverse:
5'-TGAGTCTTCCAGATACCAA-3' for human GAPDH; forward: 5'-CTTGCGCTGGATTCTCTTTTCT-3' and reverse: 5'-CTCTGATGCTTGTGAGCTTTTG-3' for BMI-1; forward: 5'-GACAAGAAAAACGCAACAAATC-3' and reverse: 5'-CTGAAACTTGTACCTGACCCG-3' for MDM2; forward: 5'-GTCAGGACCTTCGAGCATTG-3' and reverse: 5'-CTCAGGACAGCACAAGAAAATC-3' for E2F; forward: 5'-GAGGGCTCCCTCTGGACCCG-3' and reverse: 5'-TCTTTTCAATCGGGAGATTCTT-3' for p16<sup>INK4a</sup>; forward: 5'-TGTCGAGTGGCTGACTGAATGCT-3' and reverse: 5'-TGACAAGGTCTTCTTCTCAATTGTT-3' for p14<sup>ARF</sup>; and forward: 5'-GGAGGAAATACAGGCCCCACGG-3' and reverse: 5'-AGGAGGTCCCCGAGGTG-3' for BCR/ABL.

Triplicates were made for each sample. For each sample, an amplification plot and corresponding dissociation curves were examined. Relative quantification analysis was performed using the comparative CT method (2<sup>ΔΔCT</sup>). The formula was as follows: 2<sup>ΔΔCT</sup> = 2[CT gene of interest - CT internal control) sample A - (CT gene of interest - CT internal control) sample B].

Statistical Analysis. The data are expressed as the mean ± standard deviation from experiments performed in triplicate. Student's t-test was used to identify statistically significant differences between the experimental and control groups. The statistical analyses were performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of bufalin on the growth of K562 cells.

To investigate the effects of bufalin on K562 cells and to determine a suitable concentration for microarray experiments, the effect of various doses of bufalin on the viability of K562 cells was tested using an MTT assay and flow cytometry.

MTT results. The cells were treated for 48 and 72 h as shown in Fig. 1A, and cell growth was inhibited by bufalin in a dose- and time-dependent manner (Fig. 1A-C). The calculated IC<sub>50</sub> values (50% inhibitory concentration) were 0.153 and 0.028 μmol/l for cells treated for 48 and 72 h, respectively. The difference in the apoptosis rate between bufalin treatment at a concentration of 0.025 and 0.05 μmol/l at 48 h was significant (P<0.05).

Flow cytometric analysis. K562 cells were treated with 0.025, 0.05, 0.5 or 1.0 μmol/l bufalin for 48 h. Flow cytometric analysis showed that bufalin induced the apoptosis of cells in a dose-dependent manner (Fig. 2A-E).

Microarray analysis of bufalin-induced K562 cell apoptosis. As there is no standard concentration for the use of bufalin in cell culture for microarray experiments, several different concentrations were used to determine the effect of bufalin on the growth of K562 cells at various time points. Chen et al (5) used IC<sub>50</sub> values as their standard concentration for microarray analysis of bufalin-induced HL-60 apoptosis. In the present study, based on the results of the MTT and flow cytometry assays, a concentration of 0.5 μmol/l for 48 h was selected for the microarray experiments. Cells that were not treated with bufalin served as the control. Triplicates were made for the samples treated with bufalin. Gene functional annotation analysis and gene network analysis were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/). All data underwent log transformation. A ratio of ≥1 was regarded as upregulated and a ratio of ≤1 was considered as downregulated. The results showed that bufalin induced significant changes in the gene expression of K562 cells; 4296 genes were differentially expressed, 2185 were upregulated and 2111 were downregulated. As the focus of the study was on enriched functional categories rather than on individual genes, DAVID Annotation Cluster was employed to analyze the molecular mechanisms of bufalin-induced apoptosis. Among the genes with high fold-change values, the most upregulated genes were associated with transcription regulation (Table I), while the most downregulated genes were associated with the non-coding RNA (ncRNA) metabolic process and DNA repair (Table II). Moreover, a significant signaling pathway, the MAPK pathway, was identified, which had been verified as a pathway in bufalin-induced apoptosis (9). Of the genes overexpressed by bufalin in K562 cells, BMI-1, E2F and MDM2 were selected for qPCR analysis and the results were in agreement with the microarray data (Table III).

Effect of bufalin on the BMI-1, p16<sup>INK4a</sup>, E2F, p14<sup>ARF</sup>, MDM2 and Brc/Abi expression. K562 cells were treated with 0.5 or 0.05 μmol/l bufalin for 3, 6, 18, 24 or 48 h, respectively, and then qPCR was performed. The results indicated that BMI-1, E2F and MDM2 expression levels were upregulated in the K562 cells treated with 0.5 μmol/l bufalin for 48 h.
The expression of BMI-1 was decreased relative to the baseline level at 18 and 24 h following treatment with a concentration of 0.5 µmol/l bufalin, and then increased 1.63-fold 48 h after treatment. The minimum expression of the gene was at 24 h after bufalin treatment. In addition, the expression of BMI-1 target genes p16INK4a and p14ARF, and the effect of bufalin on BCR/ABL expression was investigated.

The results indicated that the expression of p16INK4a began to increase 18 h following bufalin treatment, and increased 107.60-fold 48 h after bufalin treatment. The rate of increase of p14ARF expression was slower than that of p16INK4a, and p14ARF expression initially decreased and returned to the original level at 48 h after treatment (Fig. 3A). The expression of BCR/ABL decreased gradually in a time-dependent manner, and had decreased 6.73-fold 48 h after bufalin treatment (Fig. 3B).

Table I. Most upregulated functional annotation clusters of bufalin-induced apoptosis of K562 cells.

<table>
<thead>
<tr>
<th>Category</th>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>GOTERM_BP_FAT</td>
<td>GO:0006350; transcription</td>
<td>258</td>
<td>19.06874</td>
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<tr>
<td>GOTERM_BP_FAT</td>
<td>GO:0045449; regulation of transcription</td>
<td>297</td>
<td>21.95122</td>
<td>3.82 E-20</td>
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<td>GOTERM_BP_FAT</td>
<td>GO:0006355; regulation of transcription, DNA-dependent</td>
<td>204</td>
<td>15.07761</td>
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<tr>
<td>GOTERM_BP_FAT</td>
<td>GO:0051252; regulation of RNA metabolic process</td>
<td>206</td>
<td>15.22542</td>
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Annotation Cluster 1. Enrichment Score, 16.253795458938388.

Table II. Most downregulated functional annotation clusters of bufalin-induced K562 cell apoptosis.

A, Annotation cluster 1a

<table>
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<tr>
<td>GOTERM_BP_FAT</td>
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<td>GOTERM_BP_FAT</td>
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<td>GOTERM_BP_FAT</td>
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<td>GOTERM_BP_FAT</td>
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<td>GOTERM_BP_FAT</td>
<td>GO:0042254; ribosome biogenesis</td>
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<td>1.509872</td>
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<td>GOTERM_BP_FAT</td>
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<td>1.916376</td>
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<td>1.219512</td>
<td>2.12 E-05</td>
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<td>GOTERM_BP_FAT</td>
<td>GO:0016072; rRNA metabolic process</td>
<td>21</td>
<td>1.219512</td>
<td>4.07 E-05</td>
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B, Annotation cluster 2b

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<td>GOTERM_BP_FAT</td>
<td>GO:0006281; DNA repair</td>
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<td>2.206736</td>
<td>0.001353</td>
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<td>GOTERM_BP_FAT</td>
<td>GO:0006974; response to DNA damage stimulus</td>
<td>45</td>
<td>2.61324</td>
<td>0.003657</td>
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<td>GOTERM_BP_FAT</td>
<td>GO:0033554; cellular response to stress</td>
<td>53</td>
<td>3.077816</td>
<td>0.121174</td>
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</table>

*Enrichment Score, 6.320442125275494; †Enrichment Score: 2.29263824337844.

which was in agreement with the results of the microarray experiment (Table III).
The effect of bufalin treatment at a concentration of 0.05 µmol/l on K562 cells was the same as that of 0.5 µmol/l, except that the minimum expression of BMI-1 was at 18 h following treatment. p16\(^{INK4a}\) expression levels began to increase 6 h following bufalin treatment and increased 30.18-fold 48 h after treatment. p14\(^{ARF}\) expression began to increase 24 h following bufalin treatment, and increased 1.26-fold 48 h after the treatment.

In conclusion, p16\(^{INK4a}\) expression began to increase as BMI-1 expression decreased, and the increase in p14\(^{ARF}\) expression lagged behind that of p16\(^{INK4a}\). The expression of BCR/ABL does not appear to be correlated with the upregulation or downregulation of BMI-1. Bufalin-induced apoptosis was 39%.

Table III. Results of qPCR confirmation of differentially-expressed genes compared with microarray data.

<table>
<thead>
<tr>
<th>Gene</th>
<th>qPCR</th>
<th>Microarray</th>
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<tr>
<td>BMI-1</td>
<td>1.63</td>
<td>1.11</td>
</tr>
<tr>
<td>E2F-1</td>
<td>4.89</td>
<td>1.45</td>
</tr>
<tr>
<td>MDM2</td>
<td>1.04</td>
<td>1.00</td>
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qPCR, quantitative polymerase chain reaction.
may be mediated by downregulating the expression of BMI-1, and accordingly upregulating the expression of p14ARF and p16INK4a, and downregulating the expression of BCR/ABL in K562 cells.

In addition, BMI-1 expression was observed in K562 cells treated with bufalin for 24 h. Bone marrow mononuclear cells from healthy iron-deficiency anemia volunteers served as the normal control. BMI-1 expression levels in the untreated control cells were higher than those in the normal control cells, and they were downregulated in the drug-treated cells (Fig. 4).

Discussion

The potential antitumor activity of natural products used in traditional Chinese medicines has been noted (24). Cinobufacini (Huachansu), a Chinese medicine prepared from dried toad skin, has been widely used for the treatment of various types of cancer in China (25). Bufalin, which is the main active component of cinobufacini, has attracted much attention from researchers since the 1990's (2). Experiments have demonstrated that it is capable of inducing apoptosis in a variety of types of tumor cell, including leukemia cells such as U937, HL-60, ML1 and THP1 (5,6,9-11,26). The mechanisms by which bufalin induces apoptosis are complex and involve a diverse range of cell signals (2,3). Data suggest that activation of the MAPK signaling pathway is one of the most important signal transduction pathways for the induction of apoptosis by bufalin (2). Bufalin is also able to induce apoptosis of prostate cancer cells via p53- and Fox-mediated apoptotic pathways (27). In addition, bufalin induces lung cancer A549 cell apoptosis via inhibition of the PI3K/Akt signaling pathway (28). Recently, it has been indicated that bufalin-induced apoptosis is associated with the disruption of the mitochondrial membrane potential, cytochrome c release, ROS generation and caspase activation (29).

In the present study, it was demonstrated that bufalin is able to induce K562 cell apoptosis in a time- and dose-dependent manner, and the microarray analysis results suggest that bufalin is able to induce significant changes in the gene expression of K562 cells. The most upregulated functional annotation cluster of bufalin-induced apoptosis of K562 cells was associated with transcription regulation. For example, BMI-1, AFF4, DDIT3, ING3, E2F1, E2F8, DENND4A, ATF, CCNL1, CCNL2, CCT1, CCT2, CDK9, JUN, CDK11B and certain zinc finger proteins were upregulated in K562 cells treated with bufalin. DDIT3, also known as CCAAT enhancer binding protein ζ, has been demonstrated to be involved in the regulation of cellular growth and differentiation (30). The levels of DDIT3 transcript have been demonstrated to be downregulated in myeloid malignancies (31), and overexpression of DDIT3 transcripts has been shown to induce increased apoptosis of myeloid cells and block cell progression from the G1 to S phase (32,33). ING3, a member of the ING family, has been reported to be involved in p53-mediated transcription modulation, cell cycle control and the induction of apoptosis. It was demonstrated that the expression levels of ING3 are decreased in melanoma and head and neck squamous cell carcinoma, and predict a poor prognosis (34). DENND4A, also known as C-myc promoter-binding protein MBP-1, acts as a general transcriptional repressor (35). It binds to c-myc promoter sequences and transcriptionally represses the c-myc gene (36-38). It has been demonstrated that overexpression of the MBP-1 gene induces apoptosis in several types of cancer cell line (39). The family of zinc finger proteins is composed of regulatory proteins that participate in a number of molecular and cellular pathways, and is considered to be one of the most profuse regulatory protein families in eukaryotic cells. Their functions are extremely diverse and include DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (40-41).

The present study demonstrated that bufalin downregulated certain genes participating in ncRNA metabolic processes, such as DGCR8, FTSJ3, MINA, NSUN2, U2AF1, CPSF3, GEMIN4, LMT2 and PRMT5. DGCR8 is an RNA-binding protein that assists the RNase III enzyme Drosha in the processing of microRNAs (miRNAs); it is required for the processing of pri-miRNAs to pre-miRNAs. Using RNA from wild-type embryonic stem cells as a reference, Wang et al (42) observed a global loss of miRNAs in Dgcr8 knockout cells but identified normal expression in heterozygous Dgcr8 cells, by using miRNA microarray analysis. As a result, bufalin is able to interfere with the processing of miRNAs via downregulating certain genes, such as DGCR8.

FTSJ3 is a human NIP7-interacting protein. NIP7 is one of the numerous trans-acting factors required for eukaryotic ribosome biogenesis, which interacts with nascent pre-ribosomal particles and dissociates as they complete maturation and are exported to the cytoplasm. Conditional knockdown revealed that depletion of FTSJ3 affects cell proliferation and causes pre-rRNA processing defects (43). Mina53 is involved in cell division (44) and is directly involved in ribosome biogenesis, most likely during the assembly process of pre-ribosomal particles (45). It may be important in cell growth and survival. The inhibition of Mina53 expression results in the suppression of cell proliferation in certain cell lines (46). PRMT5 is a type II PRMT that catalyzes the symmetrical dimethylation of arginine residues within target proteins (47). PRMT5 is highly conserved among yeast, animals and higher plants, and has been implicated in diverse cellular and biological processes including transcriptional regulation, RNA metabolism, ribosome biogenesis, Golgi apparatus structural maintenance and cell cycle progression (48). Overexpression of PRMT5 promotes tumor cell growth and is associated with poor disease prognosis (49).

Bufalin also downregulated certain genes associated with DNA repair, such as RUVBL1, Cdc7, ERCC5, Gtx2, MSH6, MUTYH, NEIL1, NTHL1, PRMT6, tyrosyl-DNA phosphodiesterase I (Tdp1) and MYC. RUVBL1 is a member of the AAA+ (ATPase associated with diverse cellular activities) family of proteins that is associated with several chromatin-remodeling complexes and is important in transcriptional regulation, the DNA damage response, telomerase activity, snoRNP assembly, cellular transformation and cancer metastasis (50-52). RUVBL1 is overexpressed in a number of different types of cancer and interacts with major oncogenic factors, such as by regulating the function of β-catenin and c-Myc (50-51). RUVBL1 blocks p53-mediated apoptosis by repressing the expression of p53 and its target genes, and siRUVBL1 is also able to induce the apoptotic death of HCT116 (p53-/-) cells, suggesting that RUVBL1 is
able to suppress apoptosis that is mediated by p53 and other molecules (53). Cdc7 is a conserved serine-threonine kinase, as well as an essential replication regulator (54). Knockdown of Cdc7 has been demonstrated to cause the death of cancer cells, but not normal cells, via p53-dependent pathways which are able to arrest the cell cycle, presumably in the G1 phase (55,56). It has also been reported that Cdc7 knockdown induced p38-dependent cell death in HeLa cells (57). Cdc7 depletion is able to induce DNA damage in cancer cells (58), and activated Chk2 is capable of stabilizing the FoxM1 transcription factor, which induces the expression of cyclin B1 (59). Thus, anticancer drugs, such as bufalin, that facilitate Cdc7 depletion are able to induce cancer cell death via numerous pathways.

Grx2 belongs to the oxidoreductase family and it has been reported that Grx2 protects cells against a variety of oxidative insults (60-62). Conversely, knockdown of Grx2 sensitizes HeLa cells to anticancer drugs (63). A study indicated that Grx2 also has an anti-apoptotic function. Grx2 was demonstrated to protect cells against H2O2-induced apoptosis via its peroxidase and dethiolase activities; in particular, Grx2 prevents complex I inactivation and preserves mitochondrial function (64).

The DNA mismatch repair (MMR) system is one of a number of DNA repair mechanisms. MSH6 is an MMR-related protein that belongs to the MutS family. MSH proteins recognize errors in the genome sequence during replication, and prevent the duplication of the damaged strand and repair single strand breaks (65). Bufalin also downregulates certain genes involved in the base excision repair and nucleotide excision repair pathways of oxidization-induced DNA damage, such as MUTYH, NEIL1 and NTHL1. Bufalin is capable of inducing apoptosis via a ROS-dependent mitochondrial death pathway (29) and as a result, bufalin induces apoptosis by inducing ROS production and inhibiting repair of DNA damage induced by oxidation.

Tdp1 is a cellular enzyme that repairs the irreversible topoisomerase I (Top1)-DNA complexes and confers chemotherapeutic resistance to Top1 inhibitors. Inhibiting Tdp1 provides an attractive approach to potentiating clinically used Top1 inhibitors (66,67). Bufalin is able to reduce the quantity, activity and mRNA levels of topoisomerase II (11). Thus, bufalin is a Top II inhibitor and a sensitizer that increases quantity, activity and mRNA levels of topoisomerase II (11).

The qPCR analysis of the present study demonstrated that bufalin is able to downregulate BMI-1 expression levels in K562 cells after 3 h. The expression levels in the K562 cells were higher than in the normal controls and decreased following treatment with bufalin. BMI-1 is required for maintenance and self-renewal of normal and leukemia stem and progenitor cells. Leukemia stem and progenitor cells lacking BMI-1 eventually undergo proliferation arrest and exhibit signs of differentiation and apoptosis, leading to transplant failure of the leukemia (20). Rizo et al (68) suggested that repression of BMI-1 in cord blood CD34+ cells impaired their long-term expansion and progenitor-forming capacity, in cytokine-driven liquid cultures and in bone marrow stromal co-cultures. In addition, the long-term culture-initiating cell frequencies were markedly decreased upon knockdown of BMI-1, indicating an impaired maintenance of stem and progenitor cells. The reduced progenitor and stem cell frequencies were associated with increased expression levels of p14ARF and p16INK4a and enhanced apoptosis (68). It has been verified that BMI-1 is overexpressed in hematological malignancies and is correlated with a poor prognosis (22,69). The findings of the present study also demonstrated that BMI-1 is overexpressed in K562 cells, and that the increase in p16INK4a and p14ARF expression levels is associated with the downregulation of BMI-1 expression levels. We propose that bufalin may induce K562 cell apoptosis via downregulating BMI-1 expression and accordingly upregulating the expression of p16INK4a and p14ARF.

To date, it has not been demonstrated that expression of BMI-1 alone is sufficient to induce leukemia. However, various studies suggest that BMI-1 may be an important collaborating factor in the transformation process. For example, BMI-1 is able to cooperate with H-RAS to induce aggressive breast cancer with brain metastases (70,71). Using qPCR, Merkerova et al (72) demonstrated significantly increased levels of BMI-1 transcripts in chronic myelogenous leukemia (CML) cells. Using siRNA silencing, the impact of BMI-1 inhibition on the growth and cell cycle of BCR/ABL-positive CML cell lines was measured, and it was demonstrated that BMI-1 inhibition did not affect the proliferation rate or the cell cycle of these cells. As it is well-known that the growth deregulation in CML cells is caused by the BCR/ABL oncogene, BMI-1 may have a secondary role in the proliferation of these cells (73). Merkerova et al (72) also reduced BCR/ABL expression by siRNA in K562 cells to determine if BMI-1 upregulation was dependent on BCR/ABL expression, and demonstrated that the BMI-1 transcript levels did not change after BCR/ABL inhibition, which suggests that the increased expression levels of BMI-1 are not directly associated with BCR/ABL. In the present study, the expression of BCR/ABL did not appear to be correlated with the upregulation or downregulation of BMI-1 expression levels. Bufalin may induce K562 cell apoptosis via downregulating BCR/ABL expression levels, and this pathway may occur independently of the BMI-1 pathway.

It has become clear that BMI-1 also functions in protecting against oxidative stress. In the absence of BMI-1, ROS accumulate and are associated with the activation of DNA damage response pathways and increased apoptosis. BMI-1-mediated control over ROS levels may occur via impaired mitochondrial functions, independent of the INK4a/ARF pathway (19). BMI-1 may be required to protect hematopoietic stem/progenitor cells from apoptosis induced by oxidative stress conditions (68). As mentioned previously, bufalin is able to induce apoptosis via a ROS-dependent mitochondrial death pathway and inhibit repair of DNA damage induced by oxidation (29). The association between bufalin, BMI-1 and DNA damage repair genes requires further investigation.

The signaling pathways that regulate BMI-1 have not been extensively studied. SALL4, an oncogene that is expressed in AML, is capable of upregulating BMI-1 expression by directly binding to its promoter (74). Furthermore, BMI-1 expression appears to be under the control of miRNAs, including miRNA-128. miRNA-128 causes a marked decrease in the expression levels of BMI-1 by direct regulation of the BMI-1 mRNA 3 untranslated region, through a single miRNA-128 binding site (75,76). miRNA-128a increases intracellular ROS levels by targeting BMI-1 and inhibits medulloblastoma
cancer cell growth by promoting senescence (77). However, BMI-1 is not expressed in a cell cycle-regulated manner in various types of primary and transformed cells and is not affected by overexpression of p16INK4a (19). In the present study, BMI-1 expression levels increased 48 h following bufalin treatment. Although the mechanism requires in-depth research, we propose certain potential possibilities: i) Bufalin may upregulate genes upstream of BMI-1, such as SALL4; ii) bufalin may downregulate mRNAs and eliminate their suppression of BMI-1, for example, bufalin is capable of interfering with the processing of miRNA via downregulating DGCR8; and iii) transcription factor E2F-1 may directly regulate BMI-1, which represses the INK4A gene, thereby promoting phosphorylation of the pRb and activation of E2F, as has been demonstrated previously (78). The present study showed that bufalin is able to upregulate E2F-1 expression and this may be a reason why BMI-1 increased 48 h after drug treatment. The phenomena of BMI-1 expression levels increasing at later time also suggests that BMI-1 alone cannot induce apoptosis of K562 cells.

References


