Prohibitin promotes apoptosis of promyelocytic leukemia induced by arsenic sulfide

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Received August 8, 2015; Accepted September 22, 2015

DOI: 10.3892/ijo.2015.3217

Abstract. Arsenic sulfide (AsS4), an oral form of arsenic agent, has been shown to have similar efficacy and safety to intravenous arsenic trioxide in the treatment of acute promyelocytic leukemia (APL). The aim of the present study was to identify proteins modulated by AsS4 and to determine their involvement in the apoptotic pathway. We used comparative proteomic analysis to screen and identify the proteins that were differentially expressed with AsS4 treatment. Prohibitin (PHB) was selected for its diverse role and its increased expression in the cells treated with AsS4. To examine whether PHB play a functional role, two clones of PHB-knockdown and PHB-overexpression were generated by transfection of NB4-R1 with vectors containing PHB gene sequences. In comparison with parental NB4-R1 cells, PHB overexpression showed an increase in baseline apoptosis and an enhanced response in AsS4-induced apoptosis. PML-RARα fusion protein was found to be reduced with PHB-overexpression, and following AsS4 treatment, a greater reduction of promyelocytic leukemia-retinoic acid receptor-α (PML-RARα) fusion protein was seen in PHB-overexpression than that in parental cells. Consistently, PHB knockdown presented with a significant reduction in AsS4-induced apoptosis and a lesser degree of PML-RARα degradation. The results indicate the antitumor activity of PHB in promoting apoptosis of APL cells.

Introduction

Acute promyelocytic leukemia (APL) is characterized by specific chromosomal translocations, typically t(15;17), which result in the formation of the promyelocytic leukemia-retinoic acid receptor-α (PML-RARα) fusion gene (1,2). PML-RARα fusion protein forms homo/heterodimers that sequestre RXR and/or PML proteins in a large protein complex and disrupt the retinoic acid (RA) signal pathway. This specific oncogenic lesion determines characteristic cell morphology and clinical presentations, and it also determines the unique response to the treatment with all-trans retinoic acid (ATRA) or arsenic agents (3,4). Both drugs have been demonstrated to target the PML/RARα oncoprotein for proteasome-mediated degradation. Clinically, ATRA induces complete remissions in ~90% of newly diagnosed APL, but many patients eventually experience a relapse and develop ATRA-resistance (5,6). Arsenic trioxide is also shown to be effective in the treatment of APL, especially in relapsed APL with ATRA-resistance (7,8).

Arsenic trioxide has dual effects of inducing differentiation and apoptosis of APL cells. However, there are issues of availability and cost of arsenic trioxide that limit its general applications. The development of oral form of arsenic drug may promote its applications in APL. Arsenic sulfide (AsS4), also known as realgar, is an oral arsenic formulation. This oral arsenic drug has been shown to have similar effect and safety to intravenous arsenic trioxide in the treatment of newly diagnosed and relapsed/refractory APL or ATRA-resistance (9). The therapeutic action of AsS4 is closely associated with its function of inducing apoptosis. Although it is known that AsS4 induces cell apoptosis through degrading PML-RARα fusion protein (10), the definitive molecular mechanisms of action of AsS4 remain unclear and require further investigations.

In the present study, we used a comparative proteomic approach to screen and identify proteins that are differentially expressed in APL cells induced by AsS4. By using two-dimensional gel electrophoresis (2-DE) followed by a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis, we identified prohibitin (PHB)

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Abbreviations: AsS4, arsenic sulfide; APL, acute promyelocytic leukemia; PHB, prohibitin; ATRA, all-trans retinoic acid; PML-RARα, promyelocytic leukemia-retinoic acid receptor-α

Key words: prohibitin, apoptosis, acute promyelocytic leukemia, arsenic sulfide, all-trans retinoic acid
among the differentially expressed proteins, PHB was significantly upregulated in ATRA-resistance APL cells (NB4-R1) by As4S4 treatment. Further studies of PHB-knockdown and PHB-overexpression indicate a functional role of PHB in As4S4-induced apoptosis of NB4-R1 cells.

Materials and methods

Cell culture. The ATRA-resistance human APL cell line (NB4-R1), received from Shanghai Institute of Hematology, (Shanghai, China) was maintained in cultures with RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heated-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO2.

Cell viability assay. Cytotoxicity of As4S4 (Xi'an Traditional Chinese Drug Company, Xi'an, China) was assessed by using MTT assay (Sigma, St. Louis, MO, USA) (11). The absorbance was measured at 570 nm using a universal microplate reader (Model ELX800; BioTek Instruments, Inc., Winooski, VT, USA). Experiments were performed in triplicate.

Apoptosis evaluation. Transmission electron microscopy (TEM) and flow cytometric analysis (FCM) were performed to evaluate cell apoptosis. After the various treatments, the cell samples were examined under a JEM-100SX electron microscope (JEOL, Ltd., Tokyo, Japan) and were analyzed in a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) and CellQuest software, respectively. All experiments were performed in triplicate.

2-DE and image analysis. Total cellular proteins were prepared from NB4-R1 cells before and after As4S4 treatment. Protein extraction was performed by sonication in a sample buffer (SB) containing 40 mM Tris base, 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), 1 mM EDTA and protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). For nuclei enrichment cells were dissolved in 200 µl of lysis buffer [10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, in the presence of protease inhibitor cocktail (Sigma), 20 µg/µl DNase and 20 µg/µl RNase] and incubated on ice for 30 min. After incubation, NP-40 (Roche) was added to a final concentration of 0.5% (v/v). After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was used for analysis with the protein concentration determined by the Bradford method. The protein concentration was performed on 13% SDS-polyacrylamide gradient gels. Gel pieces were destained and digestion. In-gel digestion was done according to the protocol of Granvogl et al (16).

MALDI-TOF MS and MALDI-TOF MS/MS analysis. Differentially expressed spots were manually excised from 2-DE gels. Gel pieces were destained and digestion. In-gel digestion was done according to the protocol of Granvogl et al (16).

The protein spots which were not identified by MALDI-TOF-MS were analyzed by MALDI-TOF MS/MS. MALDI-TOF MS/MS analysis was performed in LIFT mode. Precursor ions were selected manually. MS/MS spectra were acquired with a minimum of 4000 and a maximum of 8000 laser shots using the instrument calibration file. The precursor mass window was set automatically after the precursor ion selection. Spectra baseline subtraction, smoothing (Savitsky-Golay) and centroiding was performed by FlexAnalysis software (version 3.0; Bruker Daltonik GmbH, Bremen, Germany).

Western blot analysis. Cell protein extracts were prepared following standard procedures. The protein samples (~20 µg) were separated by SDS-PAGE. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). The filters were washed, blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (25 mM Tris, pH 7.4, 136 mM NaCl, 2.6 mM KCl and 0.5% Tween-20) for 1 h, and incubated overnight with mouse anti-PHB antibody diluted to 1:700 (Abcam, Cambridge, MA, USA) at room temperature. After washing three times with TBST buffer, the membranes were incubated with the secondary HRP-conjugated goat anti-mouse IgG Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:10,000 dilution. Mouse anti-GAPDH antibody (Santa Cruz Biotechnology) was used to ensure equal loading of samples.
Quantitative real-time PCR (qRT-PCR). The total RNA from cells was isolated with TRizol (Life Technologies, Rockville, MD, USA) and reverse-transcribed to cDNA by using the PrimeScript™ RT reagent kit (Takara Bio, Dalian, China). The cDNA was studied using a CFX96 real-time PCR system (Bio-Rad Laboratories) with SYBR-Green PCR Master Mix (Takara) to determine the transcriptional expression of PHB gene. PCR products were electrophoresed on 1.5% agarose gels. The GAPDH was used for normalization, relative gene expression was calculated by the 2^-∆∆Ct method.

Knockdown and overexpression of PHB. Lentiviral vector-mediated shRNA targeting human PHB mRNA (named pGCSIL-GFP-PHB) was previously described (17). The target sequences on the human PHB gene (GeneBank accession number NM_002634) for RNAi were designed using an internet application system as follows: 5'-GAGTTCACAGACCCGCTGTT-3'. A shRNA which had no significant homology to any known human gene (5'-TTCTCCGAACTGTCACTG-3') was used as a negative control. Oligonucleotides were ligated into the AgeI and EcoRI sites of pGCSIL-GFP vector (BD Biosciences, San Jose, CA, USA) to generate a pGCSIL-GFP-PHB, which was then transformed into E. coli. Positive recombinant clones were selected by PCR (upstream primer: 5'-CTATTCTCCCATGGATTCTTTCA-3'; downstream primer: 5'-GTAATACGCTATTTGACAAGC-3') and DNA sequencing. The recombinant lentivirus vector was produced by co-transfecting 293T cells with the lentivirus expression plasmid and packaging plasmids (pHelper 1.0 and pHelper 2.0) with Lipofectamine 2000 (Invitrogen). Infectious lentivirus vector was harvested at 48 h post-transfection and then concentrated. The infectious titer was determined by the GFP-tagged positive rate in 293T cells. NB4-R1 cells were cultured at a density of 6x10^5/well in 6-well plates and infected with lentivirus in RPMI-1640 media containing 10% FBS and 8 µg/ml of polybrene (Sigma), at the multiplicity of infection (MOI) 20, according to the pre-experimental results. After 48 h of culture, the transduction efficiency was ascertained on the basis of GFP expression under a fluorescence microscope. The knockdown efficiency of PHB was analyzed by real-time quantitative PCR and western blot analysis. NB4-R1 cells transfected with vector containing pGCSIL-GFP-PHB were designated as PHB-knockdown (KD).

The PHB gene overexpression vector (named pEGFP-N1-3FLAG-PHB) was also established. Briefly, the cDNA fragment of PHB was amplified using a PCR-based approach (upstream primer: 5'-CGGCTCGAGATGGCTCGCAAAATGTGTTTG; downstream primer: 5'-GGGGTACCGTCTTCTGGGCACAGTGGAGGAG) from a cDNA library. The PCR fragment of confirmed sequences was ligated into the XhoI and KpnI sites of overexpression vector pEGFP-N1-3FLAG (BD Biosciences). The resultant construct, pEGFP-N1-3FLAG-PHB, was transformed into E. coli. Positive recombinant clones were selected by PCR and DNA sequencing (upstream primer: 5'-CGGCTCGAGATGGCTCGCAAAATGTGTTTG; downstream primer: 5'-CGGCTCGAGATGGCTCGCAAAATGTGTTTG). The expression of PHB was analyzed by real-time quantitative PCR and western blot analysis. The NB4-R1 cell clone transfected with the vector containing pEGFP-N1-3FLAG-PHB were designated as PHB-overexpression (OE).

Results

AsS4 inhibits the growth of ATRA-resistant NB4-R1 cells. We started with MTT assay to evaluate the cytotoxicity of AsS4 on ATRA-resistant NB4-R1 cells. The results demonstrated that AsS4 inhibited the growth of NB4-R1 cells in a dose- and time-dependent manner (Fig. 1). The IC50 values of AsS4 were determined at 43.04±0.11 µM for 12 h, 25.07±0.27 µM for 24 h, 9.70±0.13 µM for 48 h and 6.38±0.09 µM for 72 h in culture. The concentration of 25 µM, the IC50 of AsS4 at 24 h, was chosen for subsequent experiments.

AsS4 induces apoptosis of NB4-R1 cells. AsS4-induced apoptosis was assessed by using TEM and FCM analysis. The NB4-R1 cells treated with AsS4 showed morphological features of cytoplasmic vacuolization, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (Fig. 2A). The apoptotic cells were quantified by FCM assay for Annexin V+ cells. The percentage of apoptotic cells was significantly increased with AsS4 treatment for 24 and 48 h (Fig. 2B).

PHB is an upregulated protein induced by AsS4. We next used proteomic approaches to screen and identify proteins that were differentially expressed following AsS4 treatment. The comparison of 2-DE protein profiles of NB4-R1 cells at 0 h with that at 24 and 48 h AsS4 treatment were performed, and 22 protein spots with at least a 2-fold increase or decrease in density were selected for further analysis (Fig. 3A and B). These spots were cut out, followed by in-gel trypsin digestion and MALDI-TOF MS analysis. The protein spots which were not identified by MALDI-TOF-MS were further
Table I. Identification of differentially expressed protein spots by MALDI-TOF-MS and MALDI-TOF-MS/MS.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein name</th>
<th>NCBInr ID no.</th>
<th>Function classification</th>
<th>Mr (Da)</th>
<th>pI</th>
<th>Peptides (MALDI/MS)</th>
<th>Sequence coverage (%)</th>
<th>Protein expression&lt;sup&gt;b&lt;/sup&gt; R24/R48</th>
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<tr>
<td>D1</td>
<td>Poly C binding protein 1 (PCBP1)</td>
<td>gi6754994</td>
<td>Regulates gene expression</td>
<td>37474</td>
<td>6.66</td>
<td>17</td>
<td>28</td>
<td>52</td>
</tr>
<tr>
<td>D2</td>
<td>Acidic leucine-rich nuclear phosphoprotein 32 family member A (ANP32A)</td>
<td>gi5453880</td>
<td>Cell proliferation, differentiation, apoptosis</td>
<td>28568</td>
<td>3.99</td>
<td>8</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>D3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SET/protein phosphatase 2A inhibitor (SET/I2PP2A)</td>
<td>gi70763500</td>
<td>Multitasking protein</td>
<td>33469</td>
<td>4.23</td>
<td>7</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>D4</td>
<td>Eukaryotic translation initiation factor 4H isofrom 1 (eIF4H-I)</td>
<td>gi1155923</td>
<td>Protein synthesis</td>
<td>27368</td>
<td>6.67</td>
<td>14</td>
<td>29</td>
<td>48</td>
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<tr>
<td>D5</td>
<td>60S acidic ribosomal protein P2 (RPP2)</td>
<td>gi4506671</td>
<td>Protein synthesis</td>
<td>11658</td>
<td>4.42</td>
<td>7</td>
<td>20</td>
<td>37</td>
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<tr>
<td>U1</td>
<td>High mobility group protein B1 (HMGB1)</td>
<td>gi4504425</td>
<td>Signal transduction</td>
<td>24878</td>
<td>5.62</td>
<td>11</td>
<td>20</td>
<td>48</td>
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<tr>
<td>U2</td>
<td>Transgelin-2 (TAGLN2)</td>
<td>gi4507357</td>
<td>Not be determined</td>
<td>22377</td>
<td>8.41</td>
<td>15</td>
<td>19</td>
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<tr>
<td>U3</td>
<td>Eukaryotic translation initiation factor5A) (eIF5A-1)</td>
<td>gi83448388</td>
<td>Protein synthesis, cellular growth, differentiation and proliferation</td>
<td>16821</td>
<td>5.08</td>
<td>9</td>
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<td>U4</td>
<td>Transcription factor(TF)</td>
<td>gi388307</td>
<td>Transcription</td>
<td>20700</td>
<td>6.28</td>
<td>4</td>
<td>33</td>
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<tr>
<td>U5</td>
<td>α-tubulin</td>
<td>gi37492</td>
<td>Cellular motility and transportation</td>
<td>50126</td>
<td>5.02</td>
<td>26</td>
<td>9</td>
<td>6.18/19.98</td>
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<tr>
<td>U6</td>
<td>Histone H2B type 1-M (H2B1M)</td>
<td>gi4504263</td>
<td>Transcription, DNA repair</td>
<td>13981</td>
<td>10.31</td>
<td>12</td>
<td>31</td>
<td>67</td>
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<tr>
<td>U7</td>
<td>Rho GDP dissociation inhibitor β 2 (RhoGD12)</td>
<td>gi56676393</td>
<td>Signal transduction and regulates Rho GTPases</td>
<td>22974</td>
<td>5.10</td>
<td>8</td>
<td>33</td>
<td>54</td>
</tr>
<tr>
<td>U8</td>
<td>Prohibitin (PHB)</td>
<td>gi4505773</td>
<td>Cell proliferation, tumor suppressor</td>
<td>29786</td>
<td>5.57</td>
<td>13</td>
<td>14</td>
<td>61</td>
</tr>
<tr>
<td>U9</td>
<td>Ribosomal phosphoprotein P0 (RPP0)</td>
<td>gi4506667</td>
<td>Protein synthesis and apoptosis</td>
<td>34252</td>
<td>5.71</td>
<td>14</td>
<td>19</td>
<td>46</td>
</tr>
<tr>
<td>U10</td>
<td>Heat shock 27 kDa protein (HSP27)</td>
<td>gi4504517</td>
<td>Stress resistance</td>
<td>22768</td>
<td>5.98</td>
<td>14</td>
<td>18</td>
<td>46</td>
</tr>
<tr>
<td>U11</td>
<td>Elongation factor 1-β (EF-1-β)</td>
<td>gi18203449</td>
<td>Protein synthesis</td>
<td>24748</td>
<td>4.50</td>
<td>6</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>U12</td>
<td>Keratin-2</td>
<td>gi47132620</td>
<td>Proliferation and keratinization</td>
<td>65393</td>
<td>8.07</td>
<td>11</td>
<td>38</td>
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<tr>
<td>U13</td>
<td>ERP29</td>
<td>gi5803013</td>
<td>Protein processing</td>
<td>28975</td>
<td>6.77</td>
<td>12</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td>U14</td>
<td>β-actin (ACTB)</td>
<td>gi4501885</td>
<td>Cellular motility</td>
<td>41710</td>
<td>5.29</td>
<td>10</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>U15</td>
<td>GTPase-activating protein</td>
<td>gi62911375</td>
<td>Increase GTP hydrolysis</td>
<td>23439</td>
<td>5.21</td>
<td>6</td>
<td>17</td>
<td>30</td>
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<tr>
<td>U16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Neuropolyptide h3</td>
<td>gi913185</td>
<td>Serine protease inhibitor</td>
<td>20913</td>
<td>7.42</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>U17</td>
<td>Proteasome β 4 subunit (PSMB4)</td>
<td>gi22558467</td>
<td>Proteolysis</td>
<td>29185</td>
<td>5.72</td>
<td>13</td>
<td>25</td>
<td>35</td>
</tr>
</tbody>
</table>

D, downregulation; U, upregulation; pI, isoelectric point; Mr, molecular weight. *The spot is identified by MALDI-TOF-MS and MALDI-TOF-MS/MS; <sup>a</sup>As4S4-treated (R24 and R48) divided by untreated (R0); all values are statistically significant, P<0.05.
analyzed by MALDI-TOF MS/MS. PMF and peptide amino acid sequence were analyzed for protein identification using the Mascot search program. Fig. 3C showed the PMF of spot U8 analyzed by MALDI-TOF-MS, spot U8 was identified as prohibitin (PHB) and the corresponding protein sequence is shown in Fig. 3D. The annotation of the 22 identified proteins is shown in Table I.

PHB was identified from the spot U8, which was upregulated induced by As\_4S\_4. The increase in PHB protein was confirmed by western blot analysis. As shown in Fig. 3E, there was a 2.0- and 3.9-fold increase in PHB protein with As\_4S\_4 for 24 and 48 h, respectively. At mRNA level, PHB expression was increased by 1.8- and 3.2-fold with As\_4S\_4 for 24 and 48 h, respectively (Fig. 3F). The results indicate an upregulation of PHB gene expression at both mRNA and protein levels.

**Generation of PHB-overexpression and PHB-knockdown NB4-R1 cells.** To investigate whether PHB plays a functional role in NB4-R1 cell apoptosis, we used the PHB gene overexpressing vector (pEGFP-N1-3FLAG-PHB) to generate PHB-overexpression NB4-R1 cells (OE group). The PHB-overexpression efficiency was then validated by qRT-PCR and western blot analysis, respectively. Our results showed that PHB expression in OE group was increased by 3.8-fold in comparison with the parental NB4-R1 cells (26.73±6.53 vs. 7.11±1.02%, P<0.01) (Fig. 5A), and the PML-RAR\_α fusion protein was reduced by 1.5-fold in comparison with the control (34.21±3.81 vs. 51.31±8.55%, P<0.01) (Fig. 5B).

The response of the OE cells to As\_4S\_4 was evaluated in comparison with parental NB4-R1 cells. OE cells showed an increase in As\_4S\_4-induced apoptosis. With As\_4S\_4 at the concentration of 25 \(\mu\)M for 48 h, the apoptotic cells in NB4-R1 and OE cells were 48.33±9.84 and 58.71±11.74%, respectively (Fig. 5A). PML-RAR\_α fusion protein was assessed by western blot analysis, and the results showed that As\_4S\_4 treatment led to greater reduction of PML-RAR\_α protein in OE cells than that in NB4-R1 cells. In comparison with untreated NB4-R1 cells, As\_4S\_4 treatment reduced PML-RAR\_α protein by 51.0 and 76.9% in NB4-R1 and OE cells, respectively (the grayscale ratios of PML-RAR\_α/GAPDH: 25.14±2.87 and 11.86±2.99%, P<0.05) (Fig. 5B).

**PHB-knockdown reduces As\_4S\_4-induced apoptosis and degradation of PML-RAR\_α protein.** PHB-knockdown NB4-R1 cells (KD) was evaluated in comparison with parental NB4-R1 cells. With no As\_4S\_4 treatment, there was no significant difference in the baseline apoptotic cells between KD and NB4-R1 cells. Similarly, no significant difference was seen between KD and the NB4-R1 in the expression of PML-RAR\_α fusion proteins, as determined by PML-RAR\_α/GAPDH (53.16±7.83 vs. 49.78±1.89%) (Fig. 6A and B).

The KD cells were then used to examine its response to As\_4S\_4 treatment. As\_4S\_4-induced apoptosis was evaluated with As\_4S\_4 at the concentration of 25 \(\mu\)M for 48 h. In comparison with parental NB4-R1 cells, the KD showed a lesser degree of cellular apoptosis. The percentages of apoptotic cells in NB4-R1 and KD were determined to be 45.17±5.43 and

**Figure 2. Evaluation of apoptosis of NB4-R1 cells induced by As\_4S\_4.** (A) Ultrastructural changes. Cells treated with As\_4S\_4 for 24 h showed cytoplasmic vacuolization (Vac) and chromatin condensation, and cells treated with As\_4S\_4 for 48 h showed nuclear fragmentation and formation of apoptotic bodies (Apo). Original magnification, x5000. (B) Flow cytometric analysis of NB4-R1 cells apoptosis induced by As\_4S\_4. The percentage of apoptotic cells (Annexin V\(^+\)) was 2.7, 33.4 and 44.5% in control, As\_4S\_4 24 and 48 h, respectively.
22.16±3.92%, respectively (Fig. 6A). Thus, there was a 2.0-fold less As$_4$S$_4$-induced apoptosis in KD than that in parental NB4-R1.

**Discussion**

Arsenic agents have been proved highly effective in the treatment of APL. It is particularly useful for relapsed/refractory APL with ATRA-resistance (18). As$_4$S$_4$, is a new and prom-
ising oral arsenic formulation. A multicenter study in China has shown that a complete remission (CR) rate of 99.1% and a disease-free survival (DFS) rate of 98.1% at 2 years were achieved in 108 APL cases treated with an oral As$_4$S$_4$ combined with ATRA (19, 20). In the present study, we demonstrated that As$_4$S$_4$ inhibited the growth and induced apoptosis of ATRA-resistant NB4-R1 cells. The result is consistent with previous findings (21, 22). By using comparative proteomic approach, we identified PHB was significantly upregulated during As$_4$S$_4$-induced NB4-R1 apoptosis. As PHB is of particular interest, further experiments were performed to modulate the gene expression, either PHB overexpression or PHB knockdown. The results with modulation of PHB expression implicate its activity in promoting As$_4$S$_4$-induced apoptosis.

PHB was selected in this study for its diverse roles in the regulation of proliferation, apoptosis and gene transcription (23-27). PHB proteins have been found to localize in the mitochondria, nucleus and plasma membrane of mammalian cells. PHB is implicated in diverse cellular processes, including mitochondrial biogenesis, cell death and replicative senescence. A functional role for PHB as a regulator of transcription has been shown for its interactions with p53, E2F and Rb (28-30). PHB has been associated with various types of cancer. The role of PHB in cancer cell proliferation or tumor suppression is considered controversial. PHB was shown to be necessary for the activation of C-Raf by the oncogene Ras in HeLa cells (31). However, many reports have shown evidence that PHB has antitumorigenic activity in prostate, gastric and ovarian cancer (32-35). PHB overexpression was shown to result in the inhibition of prostate cancer cell growth and the knockdown of PHB by siRNA accelerates tumor growth (33).

In the present study, stable clones of KD (PHB-knockdown NB4-R1 cells) and OE (PHB-overexpression NB4-R1 cells) were established and used to determine the cellular response to As$_4$S$_4$. The results showed that PHB overexpression enhanced apoptosis of NB4-R1 cells, and reduction of PML-RARα fusion protein. Although PHB knockdown had no significant effect on baseline apoptosis and PML-RARα fusion protein, a downregulation of PHB was associated with an attenuated apoptosis and lesser reduction of PML-RARα protein in the cells treated with As$_4$S$_4$. These results strongly support that PHB has antitumorigenic activity.

Figure 4. RT-PCR and western blot analysis of PHB overexpression and knockdown in NB4-R1 cells. (A) RT-PCR analysis of PHB overexpression. (B) Western blot analysis of PHB overexpression. (C) RT-PCR analysis of PHB knockdown. (D) Western blot analysis of PHB knockdown. *P<0.01, CON, control (NB4-R1 cells); NC, negative control (NB4-R1 cells transfected with empty vector); KD, PHB-knockdown NB4-R1 cells; OE, PHB-overexpression NB4-R1 cells.
The effects of PHB on cellular processes may be due to its subcellular localization in different type cells. The subcellular localization of PHB has been shown to affect cell fate, specifically apoptosis (36). PHB has been shown to increase on the cell membrane that facilitates tumorigenesis through its interaction with c-Raf induced by the Ras oncoprotein (37,38), whereas increased levels of PHB in the nucleus induce apoptosis by increasing the transcriptional activity of...
p53 and its translocation to the cytoplasm (39). We have found the increased levels of PHB, either modulated by As$_2$S$_3$ or by PHB overexpression vectors, in the nucleus locations of APL cells.

The PML-RAR$\alpha$ fusion protein is the key molecule that drives APL cells. This fusion protein also serves as the therapeutic target of ATRA and arsenic agents (40). While ATRA induces APL to undergo differentiation by targeting the RAR$\alpha$ moiety, arsenic agents induce apoptosis through SUMO-1-mediated degradation of the PML moiety of the fusion protein (41). However, other molecules involved in the process remain to be identified. In this study, we showed a close relationship of upregulation of PHB with reduction of PML:RAR$\alpha$ during As$_2$S$_3$-induced apoptosis. Consistently, PHB knockdown experiments showed a reduced degradation of PML:RAR$\alpha$ protein. These results indicate that PHB is involved in the APL cell apoptosis. However, the biochemical pathway of PHB activity in relation to PML:RAR$\alpha$ remains the subject of investigations.

In conclusion, PHB was identified among the upregulated proteins associated with As$_2$S$_3$-induced apoptosis of NB4-R1 cells. The experiments with modulation of PHB expression indicate that PHB overexpression enhances apoptosis and degradation of PML:RAR$\alpha$ fusion protein, and consistently PHB knockdown attenuated the cellular response to As$_2$S$_3$ treatment.

Acknowledgements

The present study is supported by a research grant from the Natural Science Foundation of China (NSFC, grant no. 30701133), the Shaanxi Province Science and Technology Development Fund (SPSTDF, grant no. 2012KTC03-12). The authors thank Dr Qunling Zhang from Shanghai Institute of Advanced Technology, Chinese Academy of Sciences, for their technological assistance; and Dr Byron Wang from the First Affiliated Hospital, Xi'an Jiaotong University for their technological assistance; and Dr Byron Song from University of Tronto, Ontario, Canada for critically reading the manuscript.

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