Carbonyl Reductase 1 Offers a Novel Therapeutic Target to Enhance Leukemia Treatment by Arsenic Trioxide

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Abstract

Arsenic trioxide ($\text{As}_2\text{O}_3$) is used, in current practice, as an effective chemotherapeutic agent for acute promyelocytic leukemia (APL). However, the side effects and relatively low efficacy of $\text{As}_2\text{O}_3$ in treating other leukemias have limited its wider use in therapeutic applications. In the present study, we found that the expression of carbonyl reductase 1 (CBR1) affects the resistance to $\text{As}_2\text{O}_3$ in leukemias, including APL; $\text{As}_2\text{O}_3$ upregulated CBR1 expression at the transcriptional level by stimulating the activity of the transcription factor activator protein-1. Moreover, CBR1 overexpression was sufficient to protect cells against $\text{As}_2\text{O}_3$ through modulation of the generation of reactive oxygen species, whereas the attenuation of CBR1 was sufficient to sensitize cells to $\text{As}_2\text{O}_3$. A combination treatment with the specific CBR1 inhibitor hydroxy-PP-Me remarkably increased $\text{As}_2\text{O}_3$-induced apoptotic cell death compared with $\text{As}_2\text{O}_3$ alone, both in vitro and in vivo. These results were confirmed in primary cultured human acute and chronic myeloid leukemia cells, with no significant cell death observed in normal leukocytes. Taken together, our findings indicate that CBR1 contributes to the low efficacy of $\text{As}_2\text{O}_3$ and, therefore, is a rational target for the development of combination chemotherapy with $\text{As}_2\text{O}_3$ in diverse leukemias including APL. Cancer Res; 72(16); 1–11. ©2012 AACR.

Introduction

Carbonyl reductase 1 (CBR1) is a ubiquitous NADPH-dependent enzyme belonging to the short-chain dehydrogenase/reductase family (1). This enzyme catalyzes a significant number of biologically and pharmacologically active substrates, including a variety of endogenous and xenobiotic carbonyl compounds (2). The best substrates of CBR1 are quinones, including ubiquinone-1 and tocopherolquinone (vitamin E). Ubiquinone (coenzyme Q) is a constitutive factor in the respiratory chain, and tocopherolquinone protects the lipids of biological membranes against lipid peroxidation, indicating that CBR1 plays an important role as an oxidation–reduction catalyst in cellular processes (3). Moreover, CBR1 inactivates highly reactive lipid aldehydes, such as 4-oxonon-2-enal (ONE), 4-hydroxynon-2-enal (HNE), and acrolein, which are allowed to modify proteins and capable of producing DNA damage within cells (4). Further, overexpression of human CBR1 in NIH3T3 cells provides protection from reactive oxygen species (ROS)-induced cellular damage (5), which supports CBR1 as a major contributor to the control of oxidative stress.

Oxidative stress can regulate gene expression by activating several transcription factors (6), including the redox-sensitive activator protein-1 (AP-1). As a heterodimeric protein, AP-1 is composed of subunits belonging to the Fos (c-Fos, FosB, Fra1, and Fra2), Jun (c-Jun, JunB, and JunD), and activating transcription factor families (7). AP-1 regulates gene expression by binding to the DNA sequence at specific AP-1 binding sites. Further, AP-1 activity is partially regulated through the phosphorylation of its various subunits. Importantly, the transcriptional activity of c-Jun is increased through phosphorylation by the Jun NH2-terminal kinases (JNK)/stress-activated protein kinases, which are strongly stimulated by oxidative stress (8). Therefore, AP-1 is known to be important in the regulation of gene expression by oxidative stress.

$\text{As}_2\text{O}_3$ has been reported to be an effective therapeutic agent in both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL) (9–12). This success has prompted an interest in the molecular mechanisms of action underlying the clinical effectiveness of this cytotoxic agent. Previous studies have shown that $\text{As}_2\text{O}_3$ induces apoptosis and partial differentiation in leukemic promyelocytes (13, 14). The susceptibility of cells to undergo apoptosis in the presence of $\text{As}_2\text{O}_3$ appears to be dependent on the intracellular redox homeostasis. In particular, the effectiveness of $\text{As}_2\text{O}_3$ in leading to apoptosis is associated with an increased generation of intracellular ROS in the presence of this cytotoxic agent (15, 16). The therapeutic potential of $\text{As}_2\text{O}_3$ is not restricted...
to APL cells. The ability of As$_2$O$_3$ to induce cell death has been observed in other malignant cells, including non-APL acute myeloid leukemia cells, myeloma cells, and chronic myeloid leukemia cells, as well as various solid tumor cells in vitro (17–19).

However, the clinical application of As$_2$O$_3$ is limited in other types of leukemia because of their low susceptibility to it (20, 21). Moreover, As$_2$O$_3$ can have resultant side effects or later repercussions in patients with APL (22). Therefore, a strategy to enhance the efficacy of As$_2$O$_3$ is required to enable its use in a wide variety of medical applications and to reduce its side effects. In this article, we report that the upregulation of CBR1 by As$_2$O$_3$ renders leukemia cells resistant to apoptosis. Further, we show that the combination of a CBR1 inhibitor with As$_2$O$_3$ substantially increases apoptotic cell death and inhibits tumor growth relative to As$_2$O$_3$ alone in vitro and in vivo. In addition, we show a synergistic cytotoxic effect in a combination treatment on primary leukemia cells.

Materials and Methods

Cell culture

Human myeloid leukemia cell lines (U937, K562, HL-60, and NB4) were obtained from the Korean Cell Line Bank (Seoul, Korea). All of the cell lines were regularly passaged and routinely mycoplasma-tested and genotyped (AmpliFISTR Identifiler Kit; Applied Biosystems) to verify their identity. Cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Primary leukemia cells were obtained from bone marrow samples of patients with newly diagnosed AML or CML. Mononuclear cells were isolated by Ficoll density-gradient centrifugation and were at least 80% or higher malignant cells. The isolation of CD34-positive cells from normal bone marrow was conducted using the immunomagnetic microbead method.

Real-time quantitative reverse transcriptase PCR analysis

The real-time quantitative reverse transcriptase PCR (qRT-PCR) was carried out by using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI PRISM 7300 real-time PCR system (Applied Biosystems), according to the manufacturer’s instructions. Based on the $2^{-ΔΔCt}$ method (23), calculations were done using the following equation: $R$ (ratio) = $2^{-[ΔCt_{sample} - ΔCt_{control}]}$. The integrity of the amplified DNA was confirmed by determining the melting temperature. The data were expressed as the fold changes in the treatment groups in relation to the control groups and were normalized to GAPDH levels. The primer sequences were designed by Primer 3 and UCSC In-Silico PCR and were as follows: CBR1 forward, 5'-AACAAAGTTTGTGAAAGATACAAAGGGA-3'; CBR1 reverse, 5'-TGTTCAACTCTCTCCTGGAACAAAAT-TGTGTC-3'; GAPDH forward, 5'-TGACCCACAGCTGCCATC-3'; GAPDH reverse, 5'-TTCTAGACGGGAGGTCCAGGT-3'.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was conducted as described previously (24) using the following oligonucleotides:

- CBR1/WT, 5'-GATGCTGTTCGCCACACTGTCCTTC-3'; CBR1/Mutant, 5'-GATGCTGTTCGCCACACTTTCCTTC-3'.

Chromatin immunoprecipitation

Conventional chromatin immunoprecipitation (ChIP) assay was conducted as described previously (25). Cross-linked U937 chromatin was subjected to immunoprecipitation with antibodies against p-c-Jun and p-c-Fos. The primers used in the PCR to detect AP-1 protein binding to the CBR1 promoter were as follows: forward, 5'-TTGAAAATTCACAGACCAGACC-CTTCAC-3'; reverse, 5'-CGAGGAAAGATACTGCATGTG-3'.

Lipid peroxidation assay

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels using the Thiobarbituric Acid Reactive Substances Assay Kit (Enzo Life Sciences). The final MDA levels were expressed as fold changes in the treatment groups relative to the control group.

Animals and xenograft model

Female athymic BALB/c nude mice (5–6 weeks old) were purchased from Orient Bio, Inc. (Sungnam, Korea). The animals were placed in a pathogen-free environment and allowed to acclimate for a week before being used in the study. The experimental protocol [KHUASP(SE)-10-018] was approved by the Institutional Animal Care and Use Committee of Kyung Hee University. U937 cells (2 × 10$^7$) were injected subcutaneously into the mice (n = 5 mice/group). The mice were then injected intraperitoneally with 3-(3-isopropyl-4-(methylamo)-7H-pyrrolo[2,3-d]pyrimidin-5yl)phenol (hydroxy-PPMe; 30 mg/kg), As$_2$O$_3$ (5 mg/kg), or a combination of both compounds once every 3 days. Tumor weights were calculated with the formula of ($L 	imes W^2$)/2, where L is the tumor length and W is the tumor width, both of which were measured with a set of calipers.

Immunohistochemistry

Small pieces of tumor tissues were fixed in 4% paraformaldehyde overnight and sectioned at a thickness of 6 μm. The sections were incubated overnight at 4°C with a monoclonal antibody against CBR1 (Imgenex). The immunostained specimens were visualized using the Dako EnVision Detection Kit (Dako).

In situ apoptosis assay

Tumor tissue samples from mice subjected to different treatments were sectioned by using a cryostat and mounted on silane-coated slides. The in situ apoptosis assay was conducted by using the DeadEnd Colorimetric TUNEL System (Promega). The positive apoptotic nuclei were stained dark brown.

Statistical analysis

The results were expressed as the mean ± SE of at least 3 independent experiments. The difference between 2 means was analyzed with the Student’s t test and considered statistically significant when $P < 0.05$. The cytotoxic combination...
effect of hydroxy-PP-Me with As$_2$O$_3$ was calculated by using CalcuSyn software (Biosoft).

Results

**CBR1 is upregulated at the transcriptional level by As$_2$O$_3$**

To investigate whether CBR1 is induced by As$_2$O$_3$, we first conducted Western blot analysis on total cell lysates from the leukemia cell lines following treatment with As$_2$O$_3$. When cells were treated with either 2 μmol/L (U937, K562, and HL-60) or 0.5 μmol/L (NB4) As$_2$O$_3$, the CBR1 protein levels were continuously increased for up to 48 hours in a time-dependent manner (2.8-fold for U937, 2.6-fold for K562, 3.0-fold for HL-60, and 2.1-fold for NB4 cells; Fig. 1A). To confirm this result, we conducted RT-PCR. The CBR1 mRNA levels showed a substantial 4- to 5-fold increase in the As$_2$O$_3$-treated cells (Fig. 1B). A similar increase in the CBR1 mRNA levels was observed by qRT-PCR (Fig. 1C).

For determining whether As$_2$O$_3$ increased CBR1 mRNA levels through mRNA stabilization or transcriptional activation, U937 cells were grown under As$_2$O$_3$ treatment for 48 hours and then incubated in the presence of actinomycin D (5 μg/mL) with or without As$_2$O$_3$ for an additional 12 hours. On the real-time qRT-PCR results, similar decay rates were observed for the CBR1 mRNA levels under both conditions (Fig. 1D), indicating...
that As$_2$O$_3$ induced an increase in CBR1 mRNA levels through transcriptional activation rather than mRNA stabilization.

The CBR1 promoter is activated by AP-1

AP-1 is stimulated by diverse stimuli including oxidative stress (8, 26). Moreover, As$_2$O$_3$ has been shown to induce apoptosis through ROS production in a number of different cancer cell lines, ranging from diverse leukemia cell lines to solid tumor cell lines (19, 27, 28). Therefore, we explored whether AP-1 would upregulate CBR1 expression in the presence of As$_2$O$_3$. The phosphorylation and total protein levels of c-Fos and c-Jun were increased by As$_2$O$_3$ in U937 cells (Fig. 2A). Moreover, SP600125, an inhibitor of c-Jun N-terminal kinase, abrogated not only phospho-c-Jun levels but also As$_2$O$_3$-induced CBR1 protein levels (Fig. 2B). In addition, knockdown of endogenous c-Jun and c-Fos showed the same results as the inhibitor (Fig. 2C). Bioinformatic analysis revealed the CBR1 promoter contained an AP-1 binding site located at -819 bp upstream of the transcriptional initiation site. To determine whether the AP-1 site was involved in the response to As$_2$O$_3$ treatment, we designed several luciferase reporter constructs and conducted luciferase assays. No changes in luciferase activity were observed in cells transfected with the empty pGL3-basic vector under the As$_2$O$_3$ treatment or in the control. In contrast, cells transfected with pGL3-CBR1/1000 showed an ~7-fold increase in luciferase activity under As$_2$O$_3$ treatment.
while cells carrying pGL3-CBR1/1000M showed significantly less activity (Fig. 2D). Next, an electrophoretic mobility shift assay was performed to assess the binding affinity of AP-1 to its binding site in the CBR1 promoter. The wild-type oligonucleotide incubated with nuclear extracts from U937 cells treated with As$_2$O$_3$ exhibited strong mobility-shifted bands, whereas the mutated oligonucleotide and a 100-fold excess of cold oligonucleotide showed either weak or no mobility-shifted bands (Fig. 2E). To verify these results, we conducted ChIP assays. AP-1 complex (phospho-c-Jun and phospho-c-Fos) directly bound to the CBR1 promoter (Fig. 2F). Collectively, these results indicate that CBR1 expression can be induced at the transcriptional level by AP-1 in response to As$_2$O$_3$.

**Overexpression of CBR1 enhances cell survival in the presence of As$_2$O$_3$ but knockdown of CBR1 sensitizes cells to As$_2$O$_3$**

As CBR1 was directly induced by As$_2$O$_3$ (Fig. 1), we hypothesized that the upregulation of CBR1 could be involved in the resistance to As$_2$O$_3$ treatment in leukemia cells. To verify this hypothesis, we generated stably transfected cell lines by transfecting U937 and K562 cells with Mock, CBR1/WT, SC-shRNA, or CBR1 shRNA vectors. Compared with the Mock clones, CBR1 was overexpressed (~2- to 3-fold in 3 independent CBR1/WT clones (Fig. 3A). In contrast, CBR1 expression was strongly suppressed by the CBR1 shRNA in 3 independent CBR1 shRNA clones (Fig. 3A). To observe the effect of CBR1 on cell survival against As$_2$O$_3$ treatment, we conducted a cell viability assay. Following treatment with 4 μmol/L As$_2$O$_3$, the CBR1/WT transfected cell lines showed better survival rates than the Mock-transfected cell lines in an expression level–dependent manner in both cell lines (Fig. 3B). However, the cell lines transfected with CBR1 shRNA exhibited a marked reduction in cell survival in a suppression-dependent manner, compared with the cell lines transfected with scrambled shRNA (Fig. 3B). Similar results were observed for the levels of the apoptotic markers poly (ADP-ribose) polymerase (PARP), and caspase-3 (Fig. 3C). Further, we assessed the antioxidant activity of CBR1 in both transfected cell lines. As$_2$O$_3$ resulted in the enhanced generation of ROS (Fig. 3D). Importantly, CBR1 overexpression significantly suppressed enhanced ROS generation (Fig. 3D), while CBR1 knockdown aggravated ROS generation (Fig. 3D). Finally, to confirm the impact of CBR1 expression levels on sensitivity to As$_2$O$_3$, we examined cell survival rates in c-Jun and c-Fos knockdown cells after treatment with As$_2$O$_3$ alone or with combined treatment. The c-Jun and c-Fos knockdown cells were more sensitive to As$_2$O$_3$ alone or combination with the CBR1 inhibitor than the scrambled siRNA-transfected cells. However, the trends in the survival rates of cells treated with As$_2$O$_3$ alone were similar to those treated with a combination of As$_2$O$_3$ and the CBR1 inhibitor (Fig. 3E). Collectively, these findings indicate that CBR1 protects cells against the apoptosis induced by As$_2$O$_3$ and provides resistance to As$_2$O$_3$.

**A combined treatment with hydroxy-PP-Me and As$_2$O$_3$ enhances cell death in leukemia cells**

Recently, hydroxy-PP-Me has been reported to be a selective inhibitor of CBR1 (29). To further investigate the role of CBR1 in the resistance to As$_2$O$_3$-induced cell death, we treated cells with As$_2$O$_3$ alone or in combination with hydroxy-PP-Me. We conducted a cell viability assay after exposure to 4 μmol/L (U937, K562, and HL-60) or 1 μmol/L (NB4) As$_2$O$_3$ alone or in combination with 20 μmol/L hydroxy-PP-Me for 48 hours. Co-treatment with hydroxy-PP-Me and As$_2$O$_3$ significantly enhanced cell death relative to treatment with As$_2$O$_3$ alone (Fig. 4A). Moreover, the combined treatment increased the cleavages of PARP and caspase-3 (Fig. 4B). The combined effect of hydroxy-PP-Me with As$_2$O$_3$ was further confirmed in an Annexin V/PI assay (Fig. 4C). Moreover, a TUNEL assay provided similar results to those shown in Fig. 4D. Finally, we calculated the drug combination index and observed it to be less than 1.0 (Fig. 4E), indicating a synergistic effect between hydroxy-PP-Me and As$_2$O$_3$. Overall, these data indicate that the inhibition of CBR1 in combination with As$_2$O$_3$ is a new means to promote the efficacy of As$_2$O$_3$-based regimens.

**Inhibition of CBR1 amplifies As$_2$O$_3$-induced ROS generation through NADPH oxidase activation**

As$_2$O$_3$ induces ROS generation through increasing expression levels of NADPH oxidase (NOX) subunits (30). To investigate the molecular mechanism by which the inhibition of CBR1 increases As$_2$O$_3$-mediated cell death, we measured ROS levels in a time-dependent manner in U937 cells treated with As$_2$O$_3$ alone or in combination with hydroxy-PP-Me. The combined treatment resulted in a continuous increase in ROS levels for up to 36 hours and, relative to As$_2$O$_3$ treatment alone, caused a 2-fold increase in ROS generation. To test whether the enhanced ROS production in the combined treatment could also be mediated by the NOX pathway, cells were pretreated with 200 μmol/L apocynin, a NOX inhibitor. ROS was significantly reduced in cells treated with As$_2$O$_3$ alone or in combination with hydroxy-PP-Me (Fig. 5A and data not shown). Consistent with these results, the combination of hydroxy-PP-Me and As$_2$O$_3$ more strongly increased the expression and the translocations of the NOX subunits p47phox and p67phox from the cytosol to the membrane than As$_2$O$_3$ alone. Further, these translocations to the membrane were almost completely abrogated by apocynin in both cases (Fig. 5B). Moreover, the expression levels of the lipid peroxidation products HNE, MDA, and acrolein were all increased in the cells co-treated with hydroxy-PP-Me and As$_2$O$_3$. In addition, increased expression was observed for ALDH and AR, which were known to detoxify HNE (31). This increased expression for both the lipid peroxidation products and ALDH and AR was greater than 4-fold when compared with that of the non-treated cells but was significantly decreased in the apocynin-treated cells (Fig. 5C), providing additional evidence supporting NOX involvement. Finally, we tested the formation of MDA, a lipid peroxidation indicator, and observed the same patterns of change (Fig. 5D). To determine whether increased ROS generation induces cell death, we pretreated cells with the ROS scavengers N-acetyl l-cysteine (NAC) or 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron, a superoxide scavenger) for 30 minutes and then measured cell viability after 48 hours on treatment with As$_2$O$_3$ alone or in combination with hydroxy-PP-Me. Both
antioxidants protected cells from As₂O₃ alone or in the combined treatment (Fig. 5E). Finally, to examine the direct involvement of NOX in the production of ROS in treatments with As₂O₃ alone or in combination therapy with hydroxy-PP-Me, we transfected cells with p47phox- or p67phox-specific siRNA or a scrambled siRNA. The cells transfected with p47phox or p67phox siRNA showed a significantly lower generation of ROS compared with both the non-transfected and scrambled siRNA-transfected cells after treatment with As₂O₃ alone and combination therapy. Further, transfection with either p47phox or p67phox siRNA resulted in a substantial protection against As₂O₃ alone or combination with hydroxy-PP-Me (Fig. 5F). Collectively, these observations indicate that the enhanced ROS generation by the CBR1 inhibitor in combination with As₂O₃ is caused via NOX activation.

**Inhibition of CBR1 increases the antitumor activity of As₂O₃ in a U937 xenograft model**

Further, to test whether CBR1 expression is increased in response to As₂O₃ in vivo and whether As₂O₃-induced cytotoxicity is enhanced by CBR1 inhibition, we injected $2 	imes 10^7$...
U937 cells into 5 athymic nude mice per group. Two weeks after the inoculation of U937 cells, mice were injected intraperitoneally with hydroxy-PP-Me alone, As$_2$O$_3$ alone (4 µmol/L in U937, K562, and HL-60 cells; 1 µmol/L in NB4 cells), or in combination for 48 hours, and then the relative cell survival was analyzed. Data represent mean ± SE, n = 3. * P < 0.01 versus cells treated with As$_2$O$_3$ alone. B, apoptotic cell death was monitored by Western blot analysis of processed PARP and caspase-3 in each group of cells. C, the induction of apoptosis in cells treated with 20 µmol/L hydroxy-PP-Me alone, 4 µmol/L As$_2$O$_3$ alone, or a combination of both for 48 hours was analyzed by flow cytometry after double staining with Annexin V/PI. D, the arrows indicate TUNEL-positive cells. Original magnification, ×400. E, the combination index (CI) of hydroxy-PP-Me and As$_2$O$_3$ was analyzed by the median dose–effect method. CI values were obtained from 3 different combinations.

CBR1 in As$_2$O$_3$ Treatment

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Effects of hydroxy-PP-Me and As$_2$O$_3$ alone or in combination on cell death in myeloid primary leukemia cells

To explore the clinical applicability of CBR1 inhibition, the effect of co-treatment with As$_2$O$_3$ and hydroxy-PP-Me were tested in primary leukemia cells isolated from 3 APL, 10 AML, and 10 CML patients and in normal CD34-positive bone marrow cells isolated from healthy donors. The cells were treated with 20 $\mu$mol/L hydroxy-PP-Me alone, 4 $\mu$mol/L As$_2$O$_3$ alone, or a combination of both. Cells were pretreated with 200 $\mu$mol/L apocynin for 2 hours before drug administration. A, ROS levels were measured in a time-dependent manner by flow cytometry. B, the total cell lysates and the cellular fractions were subjected to Western blot analysis. C, the lipid peroxidation products and enzymes downstream of CBR1 were analyzed by Western blot analysis. ALDH, aldehyde dehydrogenase; AR, aldose reductase. D, the lipid peroxidation product MDA was measured by a lipid peroxidation assay. Data represent mean ± SE, n = 3. *, P < 0.01 versus cells treated with As$_2$O$_3$ alone; **, P < 0.01 versus cells treated with both compounds. E, cells were pretreated with NAC or Tiron for 30 minutes, and then were treated with 20 $\mu$mol/L hydroxy-PP-Me alone, 4 $\mu$mol/L As$_2$O$_3$ alone, or both compounds. Relative cell survival was analyzed. Data represent mean ± SE, n = 3. *, P < 0.01 versus cells treated with As$_2$O$_3$ alone; **, P < 0.01 versus cells treated with both compounds. F, cells were transfected with p47phox, p67phox, and scrambled siRNAs, and further treated with As$_2$O$_3$ alone or both compounds. The level of intracellular ROS and cell viability were monitored. Data represent mean ± SE, n = 3. *, P < 0.01 versus scrambled siRNA-transfected cells treated with As$_2$O$_3$ alone; **, P < 0.01 versus scrambled siRNA-transfected cells treated with both compounds.

Discussion

As$_2$O$_3$ is a drug that is widely used for the clinical treatment of leukemia. A reduction in the relapse rate and an improvement in survival are achieved by using As$_2$O$_3$ in patients with APL, especially in high-risk patients (32–34). However, two common and potentially serious side effects of As$_2$O$_3$ treatment, such as APL differentiation syndrome
and electrocardiogram abnormalities, have been observed. Moreover, when As$_2$O$_3$ is administered intravenously at a conventional dose of 0.15 mg/kg per day, side effects such as leukocytosis, headache, fatigue, fever, cough, dyspnea, and gastrointestinal disorders are commonly observed (22). Further, although As$_2$O$_3$ had been investigated in the treatment of other types of leukemia, most of these studies showed considerably reduced antitumor effects at clinically achievable concentrations of As$_2$O$_3$. Therefore, the development of more effective treatment modalities for As$_2$O$_3$ is urgent for the treatment of diverse leukemias and solid tumors in addition to APL.

The transcriptional regulatory mechanisms for the induction of CBR1 have rarely been studied, although microarray studies identified CBR1 as one of the responsive proteins to several inducers, including phenethyl isothiocyanate (35), D3T (36), and sulphorophane (37, 38). The transcription factor Nrf2 and the aryl hydrocarbon receptor ligand have been hypothesized to induce CBR1 (39). We previously showed that CBR1 is transcriptionally induced in response to hypoxia (40). Based on our results, we propose that AP-1 is a transcription factor for CBR1 induction under As$_2$O$_3$ exposure. In the presence of As$_2$O$_3$, the Fos and Jun subunits were increased. In addition, the As$_2$O$_3$-induced CBR1 activation was blocked by SP600125 and

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**Figure 6.** The CBR1 inhibitor in combination with As$_2$O$_3$ sensitizes tumor cells to apoptosis in the U937 xenograft model. U937 cells ($2 \times 10^7$) were injected subcutaneously into mice. The mice were then injected intraperitoneally with hydroxy-PP-Me (30 mg/kg) alone, As$_2$O$_3$ (5 mg/kg) alone, or a combination of both once every 3 days. A, CBR1 overexpression was detected by Western blot analysis. *, $P < 0.01$ versus untreated tissues. B, CBR1 overexpression was detected by immunohistochemistry. Original magnification, $\times 100$. C, tumor volume was calculated with the formula of $L \times l^2 / 2$, where $L$ is the tumor length and $l$ the tumor width. D, tumor weight was measured in the indicated groups. *, $P < 0.01$ versus tissues treated with As$_2$O$_3$ alone. E, apoptotic cell death was monitored by Western blot analysis for PARP and caspase-3 cleavage in the tumor specimens. F, TUNEL staining was conducted to detect apoptotic cells in the xenografted tumor derived from U937 cells. TUNEL-positive cells were counted. Data represent mean $\pm$ SE, $n = 3$. *, $P < 0.05$ versus tissues treated with As$_2$O$_3$ alone. Original magnification, $\times 100$. 

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an siRNA that targeted c-Fos and c-Jun. Finally, AP-1 regulated CBR1 expression by binding to the AP-1 binding site within the CBR1 promoter. Collectively, we show for the first time that CBR1 is a direct target of As2O3-induced AP-1 activation.

There is a report that As2O3 produces ROS via increasing expression of NOX subunits (30). In this study, we showed that the combined treatment of As2O3 with hydroxy-PP-Me further enhanced ROS production and p47phox and p67phox expression when compared with As2O3 alone. In addition, we showed that treatment with the NOX inhibitor apocynin and knockdown of NOX subunits diminished ROS production almost to the basal level in cells that were treated with As2O3 alone as well as in cells treated with combined therapy of CBR1 inhibitor, indicating that NOX is the main enzyme that produces ROS under both conditions. Because CBR1 contributes to the detoxification of the reactive carbonyls by reducing the carbonyl group to an alcohol (3, 4) and reactive carbonyls are well known to upregulate the expression of diverse genes at the transcriptional level (41, 42), it is quite possible that the combined treatment enhances ROS production by As2O3 via augmenting expression of NOX subunits in the cells. However, the complete molecular mechanism that coordinates all these events is yet to be determined.

Although As2O3 increases apoptotic cell death in various tumors, the antitumor effects of As2O3 in other leukemia cells and in solid tumor cells were lower than those in APL (20, 21). To improve the therapeutic efficacy of As2O3 in As2O3-resistant tumors, interest has been focused on combination therapy of As2O3 with other compounds, such as cisplatin (43, 44), l-buthionine sulfoximine (45), docosahexaenoic acid (46), sulindac (47), and anthraquinones (48). Our results indicate that CBR1 is a cellular defense protein against oxidative stress induced by As2O3 and the suppression of either its expression or enzymatic activity increases sensitivity to As2O3 in vitro and in vivo. Moreover, we showed that the combined treatment of hydroxy-PP-Me with As2O3 induced synergistic apoptosis in primary leukemia cells. Therefore, we hypothesize that CBR1 is an important potential target for novel therapeutic drug development against diverse leukemias as well as APL.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M. Jang, Y. Kim, H. Won, S.-Y. Kim, J. Ha, S.S. Kim
Development of methodology: M. Jang, Y. Kim
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