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

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Abstract

Arsenic trioxide (As$_2$O$_3$) is currently used as an effective chemotherapeutic agent for acute promyelocytic leukemia (APL). However, the side effects and relatively low efficacy of As$_2$O$_3$ against other leukemias have limited its wider use in therapeutic applications. In this study, we found that the expression of carbonyl reductase 1 (CBR1) affects the resistance to As$_2$O$_3$ in leukemias, including APL. As$_2$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 As$_2$O$_3$ through modulating the generation of reactive oxygen species, while the attenuation of CBR1 was sufficient to sensitize cells to As$_2$O$_3$. A combination treatment with the specific CBR1 inhibitor hydroxy-PP-Me remarkably increased As$_2$O$_3$-induced apoptotic cell death compared with As$_2$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 suggest that CBR1 contributes to the low efficacy of As$_2$O$_3$ and therefore CBR1 is a rational target for the development of combination chemotherapy with As$_2$O$_3$ in diverse leukemias including APL.
Introduction

Carbonyl reductase 1 (CBR1) is a ubiquitous NADPH-dependent enzyme belonging to the short chain dehydrogenase/reductase (SDR) 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 able to modify proteins and to produce DNA damage within cells (4). Furthermore, 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 ATF (activating transcription factor) families (7). AP-1 regulates gene expression by binding to the DNA sequence at specific AP-1 binding sites. Furthermore, 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 (JNKs)/stress-activated protein kinases (SAPKs), 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.

As₂O₃ 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 As₂O₃ induces apoptosis and partial differentiation in leukemic promyelocytes (13, 14). The susceptibility of cells to undergo apoptosis in the presence of As₂O₃ appears to be dependent on the intracellular redox homeostasis. In particular, the effectiveness of As₂O₃ 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 As₂O₃ is not restricted to APL cells. The ability of As₂O₃ 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 \textit{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 result in 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. Here, we report that the upregulation of CBR1 by As$_2$O$_3$ renders leukemia cells resistant to apoptosis. Furthermore, we demonstrate 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 \textit{in vitro} and \textit{in vivo}. We also show a synergistic cytotoxic effect in a combination treatment in 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 (AmpFISTR Identifiler Kit; Applied Biosystems, Carlsbad, CA, USA) 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 better malignant cells. The isolation of CD34-positive cells from normal bone marrow was conducted using the immunomagnetic microbead method.

Real-time quantitative RT-PCR (qRT-PCR) analysis

The qRT-PCR was performed 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 $2^{-\Delta\Delta CT}$ method (23), calculations were performed using the following equation: $R$ (ratio) = $2^{-(\Delta CT)_{\text{sample}}}$.
ΔCt\text{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′-AACAAGTTTGTGGAAGGATACAAAGGGA-3′; CBR1 reverse, 5′-TGTTCAACTCTCTCTGAAACAAATTGTC-3′; GAPDH forward, 5′-TGACCACAGTCCATGCCAT-3′; GAPDH reverse, 5′-TTCTAGACGGCAGGTAGGTC-3′.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed as described previously (24) using the following oligonucleotides: CBR1/WT, 5′-GATGCGTTGACTACAGCTTCCTT-3′; CBR1/Mutant, 5′-GATGCGTTGACTACAGCTTCCTT-3′.

**Chromatin immunoprecipitation (ChIP)**

Conventional ChIP 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′-TGGAAAATCAGACACCAGACCCCTCAC-3′; reverse, 5′-GCAGGGAAGAAATGTAACTGCATGTGG-3′.

**Lipid peroxidation assay**

Lipid peroxidation was evaluated by measuring MDA levels using the thiobarbituric acid (TBA)-reactive substances assay kit (Enzo Life Sciences, Farmingdale, NY, USA). 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-(7-isopropyl-4-(methylamino)-7H-pyrrolo[2,3-d]pyrimidin-5yl)phenol (hydroxy-PP-Me; 30 mg/kg), As_2O_3 (5 mg/kg), or a combination of both
compounds once every 3 days. Tumor weights were calculated with the formula of \( \frac{L \times l^2}{2} \), where \( L \) is the tumor length and \( l \) 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, San Diego, CA). The immunostained specimens were visualized using the Dako EnVision Detection Kit (Dako, Denmark).

**In situ apoptosis assay**

Tumor tissue samples from mice subjected to different treatments were sectioned by using a cryostat and mounted on silanecoated slides. The in situ apoptosis assay was performed by using the DeadEnd colorimetric TUNEL system (Promega, Madison WI). The positive apoptotic nuclei were stained dark brown.

**Statistical analysis**

The results were expressed as the mean ± SE of at least three independent experiments.
The difference between two 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, Cambridge, UK).
Results

**CBR1 is up-regulated at the transcriptional level by As$_2$O$_3$**

To investigate whether CBR1 is induced by As$_2$O$_3$, we first performed 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 µM (U937, K562, and HL-60) or 0.5 µM (NB4) As$_2$O$_3$, the CBR1 protein levels were continuously increased 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 4–5-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. As shown by 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 up-regulate 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 phosho-c-Jun levels but also As$_2$O$_3$-induced CBR1 protein levels (Fig. 2B). Knockdown of endogenous c-Jun and c-Fos also 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 no-treatment. In contrast, cells transfected with pGL3-CBR1/1000 showed a ~7-fold increase in luciferase activity under As$_2$O$_3$ treatment, while cells carrying
pGL3-CBR1/1000M showed quite less activity (Fig. 2D). Next, EMSA 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 performed ChIP assays. AP-1 complex (phospho-c-Jun and phospho-c-Fos) directly bound to the CBR1 promoter (Fig. 2F). Collectively, these results indicate 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$_3$**

Since CBR1 was directly induced by As$_2$O$_3$ (Fig. 1), we hypothesized the upregulation of CBR1 is involved in the resistance to As$_2$O$_3$ treatment in leukemia cells. To assess 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 ~2-3 fold in three independent CBR1/WT clones (Fig. 3A). In contrast, CBR1 expression was strongly suppressed by
the CBR1 shRNA in three independent CBR1 shRNA clones (Fig. 3A). To observe the
effect of CBR1 on cell survival against As$_2$O$_3$ treatment, we performed a cell viability
assay. After 4 µM As$_2$O$_3$ treatment, the CBR1/WT transfected cell lines demonstrated
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). Furthermore, 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 As$_2$O$_3$ alone treatment or 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
A combined treatment with hydroxy-PP-Me and As$_2$O$_3$ enhances cell death in leukemia cells

Recently, hydroxy-PP-Me was 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 µM (U937, K562, and HL-60) or 1 µM (NB4) As$_2$O$_3$ alone or in combination with 20 µM hydroxy-PP-Me for 48 hours. Co-treatment with hydroxy-PP-Me and As$_2$O$_3$ significantly enhanced cell death relative to As$_2$O$_3$ alone (Fig. 4A). Also, the combined treatment also 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. 4C (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 suggest 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.

The inhibition of CBR1 amplifies As$_2$O$_3$-induced ROS generation through NADPH oxidase (NOX) activation

As$_2$O$_3$ induces ROS generation through increasing expression levels of 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 up to 36 hours and, relative to As$_2$O$_3$ treatment alone, caused a 2-fold enhanced increase in ROS generation. To test whether the enhanced ROS production in the combined treatment was also mediated by the NOX pathway, cells were pretreated with 200 µM 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$_3$. Increased expression was also 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 >4-fold compared with that of the non-treated cells but significantly decreased in the apocynin-treated cells (Fig. 5C), providing additional support for 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 with As$_2$O$_3$ alone or in combination with hydroxy-PP-Me. Both antioxidants protected cells from As$_2$O$_3$ alone or in the combined treatment (Fig. 5E). Finally, to examine the direct involvement of NOX in the production of ROS in treatments with As$_2$O$_3$ alone or in combination 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 As$_2$O$_3$ alone and combination. Furthermore, transfection with either p47phox or p67phox siRNA resulted in a substantial protection against As$_2$O$_3$ alone or combination with hydroxy-PP-Me (Fig. 5F). Collectively, these observations suggest the enhanced ROS generation by the CBR1 inhibitor in combination with As$_2$O$_3$ is caused via NOX activation.

**Inhibition of CBR1 increases the antitumor activity of As$_2$O$_3$ in a U937 xenograft model**

To test whether CBR1 expression is also increased in response to As$_2$O$_3$ *in vivo* and whether As$_2$O$_3$-induced cytotoxicity is enhanced by CBR1 inhibition, we injected $2 \times 10^7$ 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 (30 mg/kg) alone, As$_2$O$_3$ (5 mg/kg) alone, or in combination once every 3 days. As shown by Western blot analysis, the CBR1 protein levels in grafted tumor tissues were up-regulated ~5-fold after a single intraperitoneal injection of As$_2$O$_3$ (Fig. 6A). This strong induction was further corroborated by immunohistochemistry (Fig. 6B). To determine the combined effects of hydroxy-PP-Me and As$_2$O$_3$ on tumor growth *in vivo*, tumor growth was
measured up to 18 days after starting of the treatments. The results showed that the combination therapy with hydroxy-PP-Me plus As$_2$O$_3$ markedly suppressed tumor growth compared with As$_2$O$_3$ alone (Fig. 6C). The results were also confirmed by measuring the tumor weight (Fig. 6D). Finally, we observed enhanced expression of apoptotic markers and apoptotic nuclei in the tumor specimens from mice injected with both compounds compared with As$_2$O$_3$ alone (Fig. 6E and 6F). Collectively, these data indicate that As$_2$O$_3$ effectively suppresses tumor growth when combined with the CBR1 inhibitor.

The 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 µM hydroxy-PP-Me alone, 4 µM As$_2$O$_3$ alone, or in combination for 48 hours. The hydroxy-PP-Me alone resulted in no cell death in all of the samples. Meanwhile, As$_2$O$_3$ resulted in a cell death rate of ~20% in the AML and CML samples. However, the co-treatment with both compounds
significantly enhanced cell death in all of the primary cultured cells. In contrast, even the combined treatment resulted in a low rate of cell death in the normal CD34-positive bone marrow cells (Fig. 7).
Discussion

As$_2$O$_3$ is a widely used drug 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/day, side effects such as leukocytosis, headache, fatigue, fever, cough, dyspnea, and gastrointestinal disorders are commonly observed (22). Furthermore, 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, though 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 suggested to induce CBR1 (39). We previously demonstrated that
CBR1 is transcriptionally induced in response to hypoxia (40). Based on our results, we here argue 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. As well, the As$_2$O$_3$-induced CBR1 activation was blocked by SP600125 and 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 As$_2$O$_3$-induced AP-1 activation.

There is a report that As$_2$O$_3$ produces ROS via increasing expression of NOX subunits (30). In this study, we showed that the combined treatment of As$_2$O$_3$ with hydroxy-PP-Me further enhanced ROS production and p47phox and p67phox expression compared with As$_2$O$_3$ alone. We also showed that NOX inhibitor apocynin and knockdown of NOX subunits diminished ROS production almost to the basal level in not only As$_2$O$_3$ alone treated cells but also combined treated cells with CBR1 inhibitor, indicating that NOX is a main enzyme that produces ROS under both conditions. Since 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 highly possible that the combined treatment enhances ROS production by As$_2$O$_3$ via
augmenting expression of NOX subunits in the cells. However, the complete molecular mechanism that coordinates all these events must be clarified.

Although As$_2$O$_3$ increases apoptotic cell death in various tumors, the antitumor effects of As$_2$O$_3$ in other leukemia cells and in solid tumor cells were lower than those in APL (20, 21). To improve the therapeutic efficacy of As$_2$O$_3$ in As$_2$O$_3$-resistant tumors, focus of interest has been to combine As$_2$O$_3$ 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 As$_2$O$_3$ and that suppression of either its expression or enzymatic activity increases sensitivity to As$_2$O$_3$ in vitro and in vivo. Moreover, we showed that the combined treatment of hydroxy-PP-Me with As$_2$O$_3$ induced synergistic apoptosis in primary leukemia cells. Therefore, we suggest that CBR1 will be an important potential target for novel therapeutic drug development against diverse leukemias as well as APL.
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Authorship

Contribution: K.Shokat. provided hydroxy-PP-Me, CBR1 inhibitor. Y.H.M. provided primary leukemia cells. M.J. and S.S.K. designed the experiments, analyzed the data, and wrote the manuscript. M.J., Y.K., H.W., S.L., J.K.R, and A.D. performed all the experiments. J.H. analyzed the data.

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Figure Legends

**Figure 1.** CBR1 is up-regulated at the transcriptional level by As$_2$O$_3$. A, U937, K562, and HL-60 were treated with 2 µM As$_2$O$_3$; NB4 cells were likewise treated with 0.5 µM As$_2$O$_3$. Total cell lysates were analyzed using Western blot analysis. B, Total RNA was extracted from cells treated with 2 µM As$_2$O$_3$ and subjected to RT-PCR analysis. C, The expression levels of CBR1 mRNA were confirmed by the qRT-PCR analysis. Data represent mean ± SE, n = 3. *P < 0.01 vs. untreated cells. D, Decay rate of CBR1 mRNA. Cells were treated with 2 µM As$_2$O$_3$ for 48 hours and then incubated in the presence of 5 µg/ml actinomycin D (Act. D) with or without As$_2$O$_3$. The expression levels of CBR1 mRNA were analyzed by qRT-PCR analysis. Data represent mean ± SE, n = 3. **P < 0.01 vs. 0 hour in untreated cells, ##P < 0.01 vs. 0 hours in As$_2$O$_3$-treated cells.

**Figure 2.** CBR1 up-regulation during exposure to As$_2$O$_3$ is mediated by AP-1 in leukemia cells. A, Total cell lysates were extracted from cells treated with 2 µM As$_2$O$_3$ and were subjected to Western blot analysis. B, Cells were pretreated with the JNK inhibitor SP600125 at 20 µM, and further incubated with As$_2$O$_3$ for 48 hours. C, The
cells were transfected with c-Jun and c-Fos siRNAs and followed by treatment with 2 µM As$_2$O$_3$ for 48 hours. SC, scrambled siRNA. D, Luciferase reporter assay. The cells were transfected with the luciferase reporter constructs shown on the left and then exposed to 2 µM As$_2$O$_3$. The relative luciferase activities are expressed in comparison with the activity of the pGL3-Basic construct. Data are the mean ± SEM from six independent experiments. *P < 0.05 vs. pGL3-CBR1/1000 in As$_2$O$_3$-treated cells. E, Nuclear extracts were obtained from U937 cells treated with As$_2$O$_3$, and then incubated with $^{32}$P-labeled wild-type (WT) and mutated oligonucleotide probes. A 100-fold molar excess of unlabeled wild type probe (cold) was used for the competition study. F, A ChIP assay was analyzed in U937 cells treated with As$_2$O$_3$. Input, amplified CBR1 from a 1:100 dilution of total input chromatin as a positive control; Immunoglobulin G (IgG), immunoprecipitation with nonspecific IgG as a negative control.

**Figure 3.** Effects of CBR1 overexpression and knockdown on As$_2$O$_3$-induced cell death.

A to D, left two panels, U937 and K562 cells transfected with Mock and CBR1/WT vectors; right two panels, U937 and K562 cells carrying the SC-shRNA and CBR1-shRNA vectors. A, Clones of stably transfected cells carrying Mock, CBR1/WT, scrambled shRNA, or CBR1-shRNA vectors were selected with G418 or puromycin and
obtained by the limiting-dilution technique. The expression level of CBR1 was
monitored by Western blot analysis. B, Each transfectant was treated with 4 μM As₂O₃
for 48 hours, and the relative cell survival was analyzed. M, Mock; W, CBR1/WT; sh-
SC, scrambled shRNA; sh-R, CBR1 shRNA. C, For monitoring apoptotic cell death,
Western blot analysis was performed for processed PARP and caspase-3 in transfectants
treated with 4 μM As₂O₃ for 48 hours. The typical data are shown for all clones. D, Each
transfectant was treated with 4 μM As₂O₃ for 24 hours. The ROS level was measured by
flow cytometry. The typical data are shown for all clones. Data represent mean ± SE, n
= 3. *P < 0.01 vs. Mock treated with As₂O₃. #P < 0.01 vs. SC-shRNA treated with
As₂O₃. E, Cells were transfected with c-Jun, c-Fos, and scrambled siRNA, and then
treated with As₂O₃ alone or in combination with the CBR1 inhibitor for 48 hours. The
cell viability was measured. Data represent the mean ± SE, n = 3. *P < 0.01 vs.
scrambled siRNA-transfected cells treated with As₂O₃ alone, **P < 0.01 vs. scrambled
siRNA-transfected cells treated with both As₂O₃ and the CBR1 inhibitor.

**Figure 4.** The combination of hydroxy-PP-Me and As₂O₃ shows synergistic antitumor
effects in leukemia cells. A, Cells were treated with 20 μM hydroxy-PP-Me alone,
As₂O₃ alone (4 μM in U937, K562 and HL-60 cells; 1 μM 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 vs. cells treated with As₂O₃ 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 µM hydroxy-PP-Me alone, 4 µM As₂O₃ alone, or in combination 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 As₂O₃ was analyzed by the median dose-effect method. CI values were obtained from three different combinations. ●, 6 µM As₂O₃ + 20 µM hydroxy-PP-Me; ▲, 4 µM As₂O₃ + 20 µM hydroxy-PP-Me; ◇, 2 µM As₂O₃ + 20 µM hydroxy-PP-Me.

The drug effect on cell viability was determined by the MTT assay. CI < 1, synergistic effect; CI = 1, additive effect; CI > 1, antagonist effect.

**Figure 5.** The inhibition of CBR1 enhances NOX-derived ROS generation by As₂O₃ in U937 cells. A to D, The cells were treated with 20 µM hydroxy-PP-Me alone, 4 µM As₂O₃ alone, or in combination. Cells were pretreated with 200 µM 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. AR, aldose reductase; ALDH, aldehyde dehydrogenase. D, The lipid peroxidation product MDA was measured by a lipid peroxidation assay. Data represent mean ± SE, n = 3. *P < 0.01 vs. cells treated with As$_2$O$_3$ alone, **P < 0.01 vs. cells treated with both compounds. E, Cells were pretreated with NAC or Tiron for 30 minutes, and then were treated with 20 µM hydroxy-PP-Me alone, 4 µM As$_2$O$_3$ alone, or both compounds. Relative cell survival was analyzed. Data represent mean ± SE, n = 3. *P < 0.01 vs. cells treated with As$_2$O$_3$ alone, **P < 0.01 vs. 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 vs. scrambled siRNA-transfected cells treated with As$_2$O$_3$ alone, **P < 0.01 vs. scrambled siRNA-transfected cells treated with both compounds.

**Figure 6.** The CBR1 inhibitor in combination with As$_2$O$_3$ sensitizes tumor cells to apoptosis in U937 xenograft model. U937 cells (2 × 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 in combination once every 3 days. A, CBR1 overexpression was detected by Western blot analysis. *$P < 0.01$ vs. untreated tissues B, CBR1 overexpression was detected by immunohistochemistry. Original magnification, ×100. C, Tumor volume was calculated with the formula of $(L \times l^2)/2$, where $L$ is the tumor length and $l$ is the tumor width. D, Tumor weight was measured in the indicated groups. *$P < 0.01$ vs. 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 ± SE, n = 3. *$P < 0.05$ vs. tissues treated with As$_2$O$_3$ alone. Scale bar, 100 µm.

Figure 7. Cytotoxicity of hydroxy-PP-Me, As$_2$O$_3$, and their combination in primary human leukemia cells and normal CD34-positive bone marrow cells. Primary leukemia cells and normal CD34-positive bone marrow cells were isolated from 3 APL, 10 AML, 10 CML patients and healthy donors, respectively. These cells were treated with hydroxy-PP-Me alone, As$_2$O$_3$ alone, or in combination for 48 hours. Cell viability was determined by an annexin V/PI assay.
Figure 1.

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Figure 4.
Figure 7.

- **non-APL AML**
  - Relative cell survival (%)
  - Control, Hydroxy-PP-Me, As₂O₃, Combined

- **CML**
  - Relative cell survival (%)
  - Control, Hydroxy-PP-Me, As₂O₃, Combined

- **APL**
  - Relative cell survival (%)
  - Control, Hydroxy-PP-Me, As₂O₃, Combined

- **Normal**
  - Relative cell survival (%)
  - Control, Hydroxy-PP-Me, As₂O₃, Combined
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Miran Jang, Yeonghwan Kim, Hyeran Won, et al.

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