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

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Abstract

Arsenic trioxide (As_2O_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 As_2O_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 As_2O_3 in leukemias, including APL; As_2O_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_2O_3 through modulation of the generation of reactive oxygen species, whereas the attenuation of CBR1 was sufficient to sensitize cells to As_2O_3. A combination treatment with the specific CBR1 inhibitor hydroxy-PP-Me remarkably increased As_2O_3-induced apoptotic cell death compared with As_2O_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 As_2O_3 and, therefore, is a rational target for the development of combination chemotherapy with As_2O_3 in diverse leukemias including APL. *Cancer Res; 72(16); 4214–24. ©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 able 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.

As_2O_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 As_2O_3 induces apoptosis and partial differentiation in leukemic promyelocytes (13, 14). The susceptibility of cells to undergo apoptosis in the presence of As_2O_3 appears to be dependent on the intracellular redox homeostasis. In particular, the effectiveness of As_2O_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 As_2O_3 is not restricted

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to APL cells. The ability of As_2O_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_2O_3 is limited in other types of leukemia because of their low susceptibility to it (20, 21). Moreover, As_2O_3 can have resultant side effects or later repercussions in patients with APL (22). Therefore, a strategy to enhance the efficacy of As_2O_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_2O_3 renders leukemia cells resistant to apoptosis. Further, we show that the combination of a CBR1 inhibitor with As_2O_3 substantially increases apoptotic cell death and inhibits tumor growth relative to As_2O_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 (AmpFISTR 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 $2^{-\Delta\Delta C_T}$ method (23), calculations were done using the following equation: R (ratio) $= 2^{-[\Delta C_{T\text{sample}} - \Delta C_{T\text{control}}]}$. 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′-AACAAGTTTGTGGAAGGATACAAAGAAGGGA-3′; CBR1 reverse, 5′-TGTTCAACTCCTTCTCTGAAACAAAT-TGTC-3′; GAPDH forward, 5′-TGACCACAGTCCATGCCAT-3′; GAPDH reverse, 5′-TTCTAGACGGCAGGTCAGGT-3′.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was conducted as described previously (24) using the following oligonucleotides: CBR1/WT, 5′-GATGCCTGTTGACCCACTCCTCTTC-3′; CBR1/Mutant, 5′-GATGCCTGTTGGTCCACTCCTCTTC-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′-TGGAAAATCAGACACCAGACC-CCTCAC-3′; reverse, 5′-GCAGGGAAGAAATGTAACTGCATG-TGG-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-(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 $(L \times l^2)/2$, 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). 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

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effect of hydroxy-PP-Me with As_2O_3 was calculated by using CalcuSyn software (Biosoft).

Results

CBR1 is upregulated at the transcriptional level by As_2O_3

To investigate whether CBR1 is induced by As_2O_3, we first conducted Western blot analysis on total cell lysates from the leukemia cell lines following treatment with As_2O_3. When cells were treated with either 2 μmol/L (U937, K562, and HL-60) or 0.5 μmol/L (NB4) As_2O_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_2O_3-treated cells (Fig. 1B). A similar increase in the CBR1 mRNA levels was observed by qRT-PCR (Fig. 1C).

For determining whether As_2O_3 increased CBR1 mRNA levels through mRNA stabilization or transcriptional activation, U937 cells were grown under As_2O_3 treatment for 48 hours and then incubated in the presence of actinomycin D (5 μg/mL) with or without As_2O_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

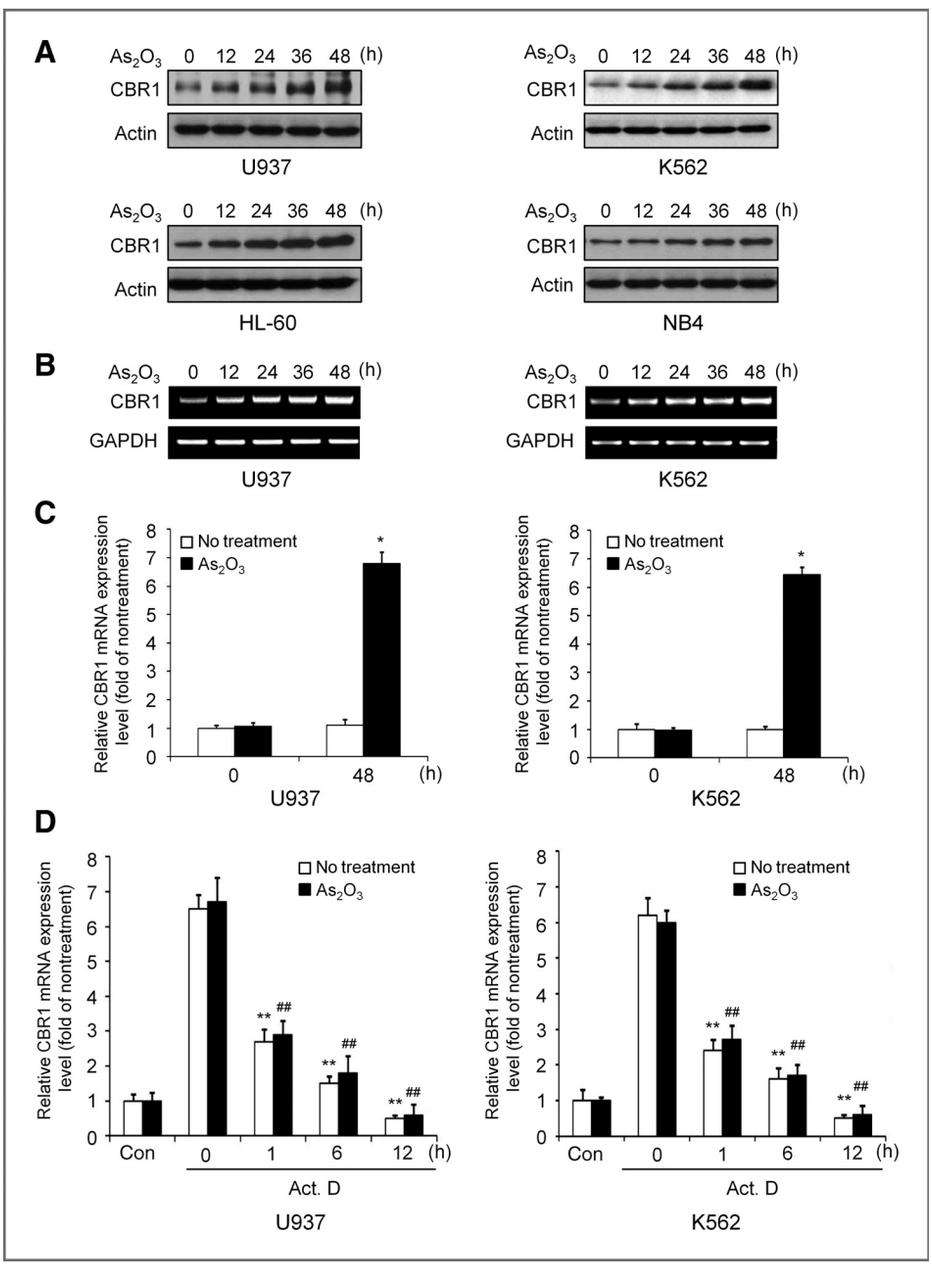

Figure 1. CBR1 is upregulated at the transcriptional level by As_2O_3. A, U937, K562, and HL-60 were treated with 2 μmol/L As_2O_3; NB4 cells were likewise treated with 0.5 μmol/L As_2O_3. Total cell lysates were analyzed using Western blot analysis. B, total RNA was extracted from cells treated with 2 μmol/L As_2O_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, $n = 3$. *, $P < 0.01$ versus untreated cells. D, decay rate of CBR1 mRNA. Cells were treated with 2 μmol/L As_2O_3 for 48 hours and then incubated in the presence of 5 μg/mL actinomycin D (Act. D) with or without As_2O_3. The expression levels of CBR1 mRNA were analyzed by qRT-PCR analysis. Data represent mean ± SE, $n = 3$. **, $P < 0.01$ versus 0 hours in untreated cells; ##, $P < 0.01$ versus 0 hours in As_2O_3-treated cells.

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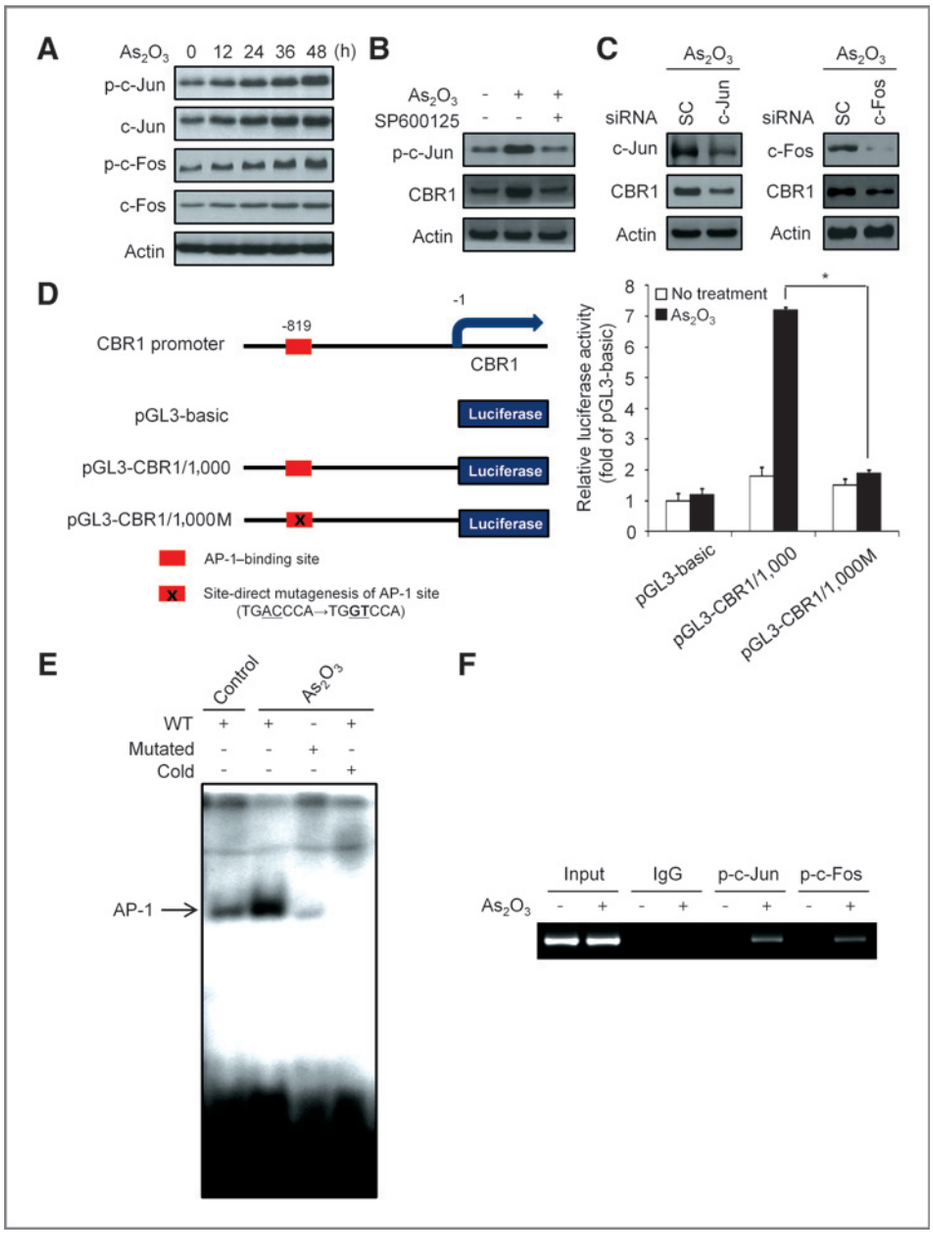

Figure 2. CBR1 upregulation during exposure to As_2O_3 is mediated by AP-1 in leukemia cells. A, total cell lysates were extracted from cells treated with 2 μmol/L As_2O_3 and were subjected to Western blot analysis. B, cells were pretreated with the JNK inhibitor SP600125 at 20 μmol/L, and further incubated with As_2O_3 for 48 hours. C, cells were transfected with c-Jun and c-Fos siRNAs and followed by treatment with 2 μmol/L As_2O_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 μmol/L As_2O_3. The relative luciferase activities are expressed in comparison with the activity of the pGL3-Basic construct. Data are the mean ± SEM from 6 independent experiments. *, $P < 0.05$ versus pGL3-CBR1/1000 in As_2O_3-treated cells. E, nuclear extracts were obtained from U937 cells treated with As_2O_3, and then incubated with ^{32}P-labeled wild-type (WT) and mutated oligonucleotide probes. A 100-fold molar excess of unlabeled WT probe (cold) was used for the competitive study. F, a ChIP assay was analyzed in U937 cells treated with As_2O_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.

that As_2O_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_2O_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_2O_3. The phosphorylation and total protein levels of c-Fos and c-Jun were increased by As_2O_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_2O_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_2O_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_2O_3 treatment or in the control. In contrast, cells transfected with pGL3-CBR1/1000 showed an ∼7-fold increase in luciferase activity under As_2O_3 treatment,

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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_2O_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_2O_3.

Overexpression of CBR1 enhances cell survival in the presence of As_2O_3 but knockdown of CBR1 sensitizes cells to As_2O_3

As CBR1 was directly induced by As_2O_3 (Fig. 1), we hypothesized that the upregulation of CBR1 could be involved in the resistance to As_2O_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_2O_3 treatment, we conducted a cell viability assay. Following treatment with 4 μmol/L As_2O_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_2O_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_2O_3, we examined cell survival rates in c-Jun and c-Fos knockdown cells after treatment with As_2O_3 alone or with combined treatment. The c-Jun and c-Fos knockdown cells were more sensitive to As_2O_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_2O_3 alone were similar to those treated with a combination of As_2O_3 and the CBR1 inhibitor (Fig. 3E). Collectively, these findings indicate that CBR1 protects cells against the apoptosis induced by As_2O_3 and provides resistance to As_2O_3.

A combined treatment with hydroxy-PP-Me and As_2O_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_2O_3-induced cell death, we treated cells with As_2O_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_2O_3 alone or in combination with 20 μmol/L hydroxy-PP-Me for 48 hours. Co-treatment with hydroxy-PP-Me and As_2O_3 significantly enhanced cell death relative to treatment with As_2O_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_2O_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_2O_3. Overall, these data indicate that the inhibition of CBR1 in combination with As_2O_3 is a new means to promote the efficacy of As_2O_3-based regimens.

Inhibition of CBR1 amplifies As_2O_3-induced ROS generation through NADPH oxidase activation

As_2O_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_2O_3-mediated cell death, we measured ROS levels in a time-dependent manner in U937 cells treated with As_2O_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_2O_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_2O_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_2O_3 more strongly increased the expression and the translocations of the NOX subunits p47phox and p67phox from the cytosol to the membrane than As_2O_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_2O_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_2O_3 alone or in combination with hydroxy-PP-Me. Both

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antioxidants protected cells from As_2O_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_2O_3 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 generaton of ROS compared with both the non-transfected and scrambled siRNA-transfected cells after treatment with As_2O_3 alone and combination therapy. Further, transfection with either p47phox or p67phox siRNA resulted in a substantial protection against As_2O_3 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 As_2O_3 is caused via NOX activation.

Inhibition of CBR1 increases the antitumor activity of As_2O_3 in a U937 xenograft model

Further, to test whether CBR1 expression is increased in response to As_2O_3 *in vivo* and whether As_2O_3-induced cytotoxicity is enhanced by CBR1 inhibition, we injected 2×10^7

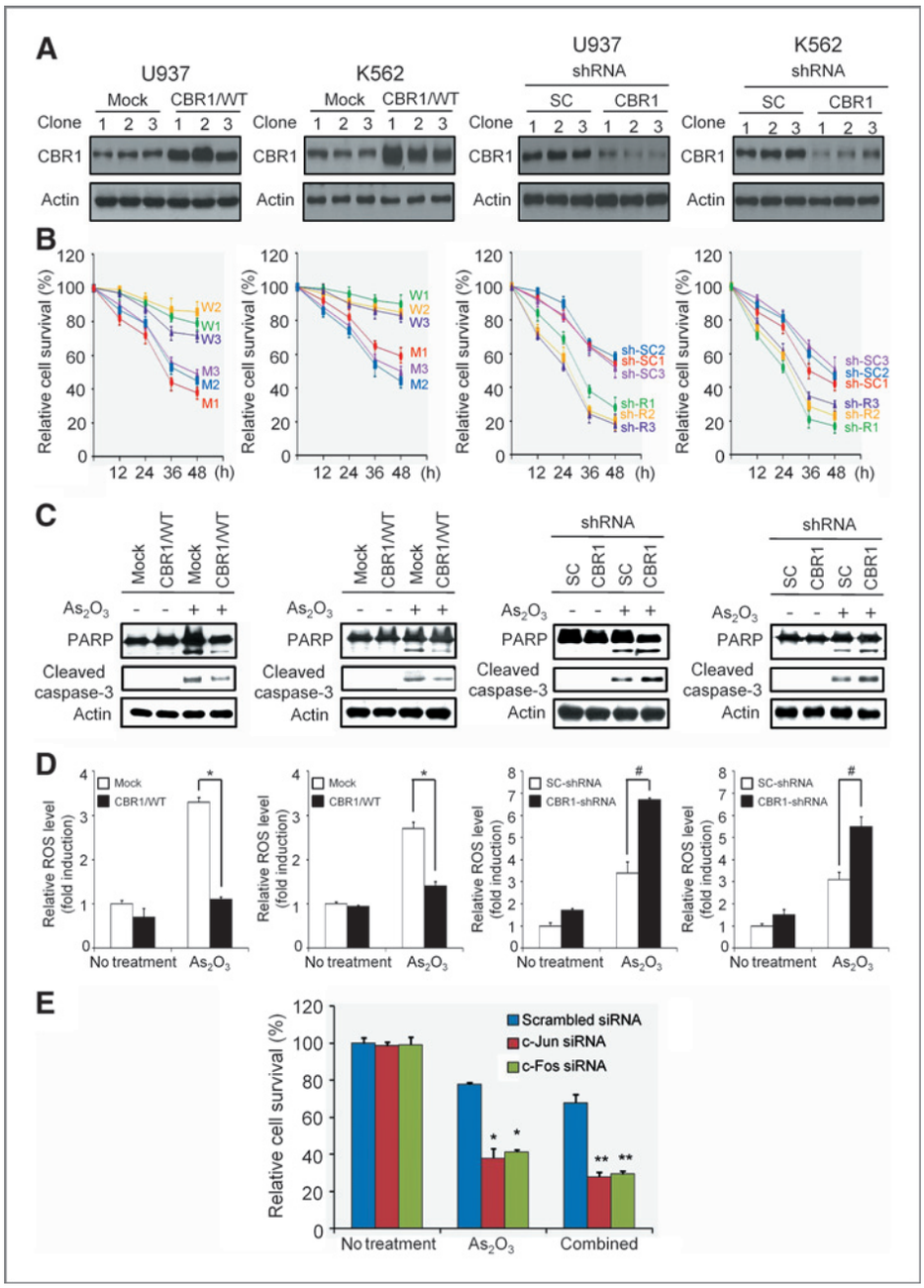

Figure 3. Effects of CBR1 overexpression and knockdown on As_2O_3-induced cell death. A–D, left two, U937 and K562 cells transfected with Mock and CBR1/WT vectors; right two, U937 and K562 cells carrying the SC-shRNA and CBR1-shRNA vectors. A, clones of stably transfected cells carrying Mock, CBR1/WT, 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 μmol/L As_2O_3 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 carried out for processed PARP and caspase-3 in transfectants treated with 4 μmol/L As_2O_3 for 48 hours. The typical data are shown for all clones. D, each transfectant was treated with 4 μmol/L As_2O_3 for 24 hours. The ROS level was measured by flow cytometry. The typical data are shown for all clones. Data represent mean ± SE, $n = 3$. *, $P < 0.01$ versus mock treated with As_2O_3. #, $P < 0.01$ versus SC-shRNA treated with As_2O_3. E, cells were transfected with c-Jun, c-Fos, and scrambled siRNA, and then treated with As_2O_3 alone or in combination with the CBR1 inhibitor for 48 hours. The cell viability was measured. Data represent the mean ± SE, $n = 3$. *, $P < 0.01$ versus scrambled siRNA-transfected cells treated with As_2O_3 alone; **, $P < 0.01$ versus scrambled siRNA-transfected cells treated with both As_2O_3 and the CBR1 inhibitor.

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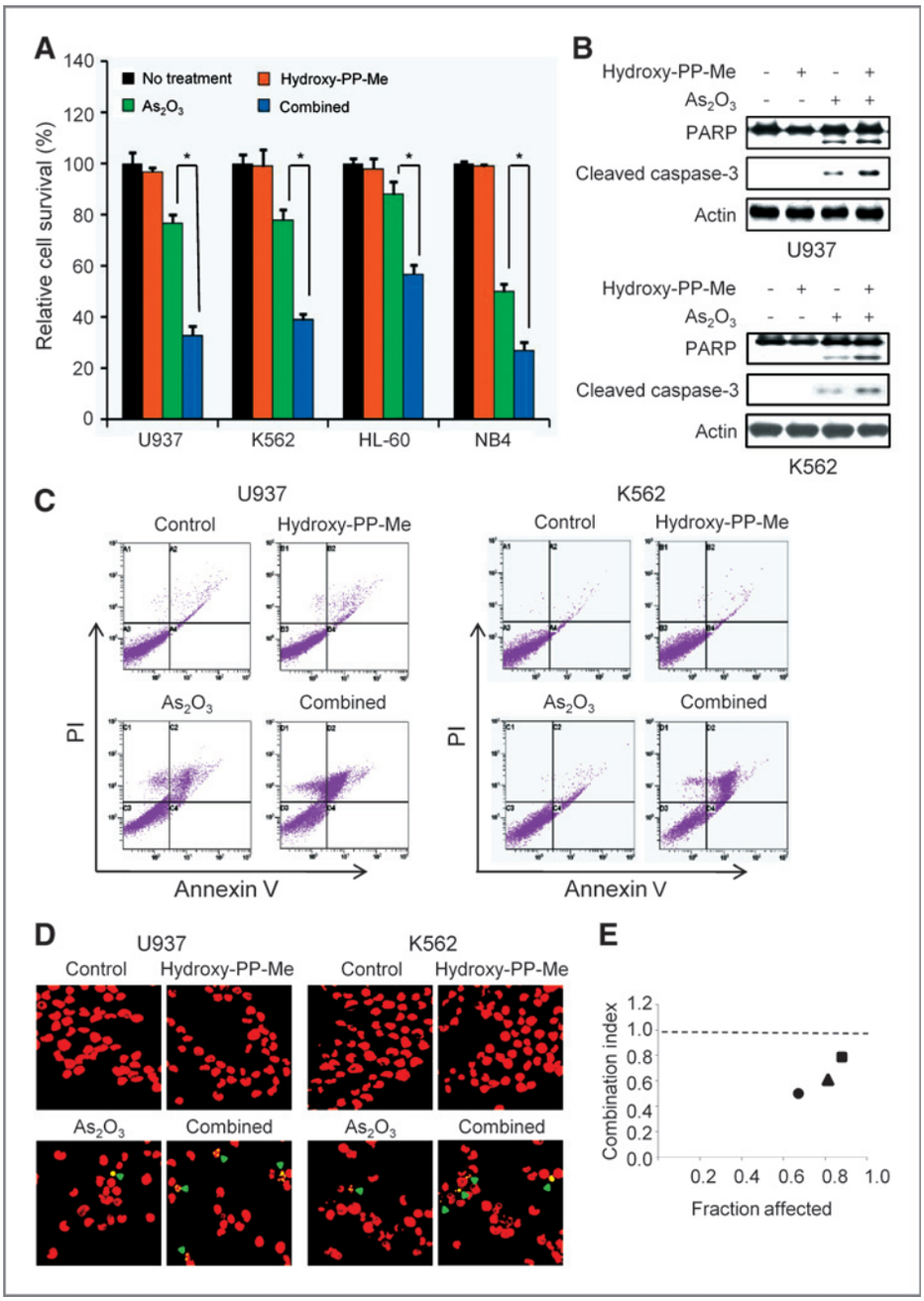

Figure 4. The combination of hydroxy-PP-Me and As_2O_3 shows synergistic antitumor effects in leukemia cells. A, cells were treated with 20 μmol/L hydroxy-PP-Me alone, As_2O_3 alone (4 μmol/L in U937, K562, and HL-60 cells; 1 μmol/L in NB4 cells), or in combination for 48 hours, and then the relative cell survival was analyzed. Data represent mean ± SE, $n = 3$. *, $P < 0.01$ versus cells treated with As_2O_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_2O_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_2O_3 was analyzed by the median dose–effect method. CI values were obtained from 3 different combinations. ●, 6 μmol/L As_2O_3 + 20 μmol/L hydroxy-PP-Me; ▲, 4 μmol/L As_2O_3 + 20 μmol/L hydroxy-PP-Me; ■, 2 μmol/L As_2O_3 + 20 μmol/L hydroxy-PP-Me. The drug effect on cell viability was determined by the MTT assay. CI < 1, synergisitic effect; CI = 1, additive effect; and CI > 1, antagonist effect.

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_2O_3 (5 mg/kg) alone, or with combination therapy that was repeated once every 3 days. On Western blot analysis, it was observed that the CBR1 protein levels in grafted tumor tissues were upregulated ~5-fold after a single intraperitoneal injection of As_2O_3 (Fig. 6A). This strong induction was further corroborated by immunohistochemistry results (Fig. 6B). To determine the combined effects of hydroxy-PP-Me and As_2O_3 on tumor growth *in vivo*, tumor growth was measured for up to 18 days after commencement of the treatments. The results showed that the combination therapy with hydroxy-PP-Me plus As_2O_3 markedly suppressed tumor growth compared with treatment using As_2O_3 alone (Fig. 6C). In addition, these results were confirmed by measuring 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_2O_3 alone (Fig. 6E and 6F). Collectively, these data indicate that As_2O_3 effectively suppresses tumor growth when combined with the CBR1 inhibitor.

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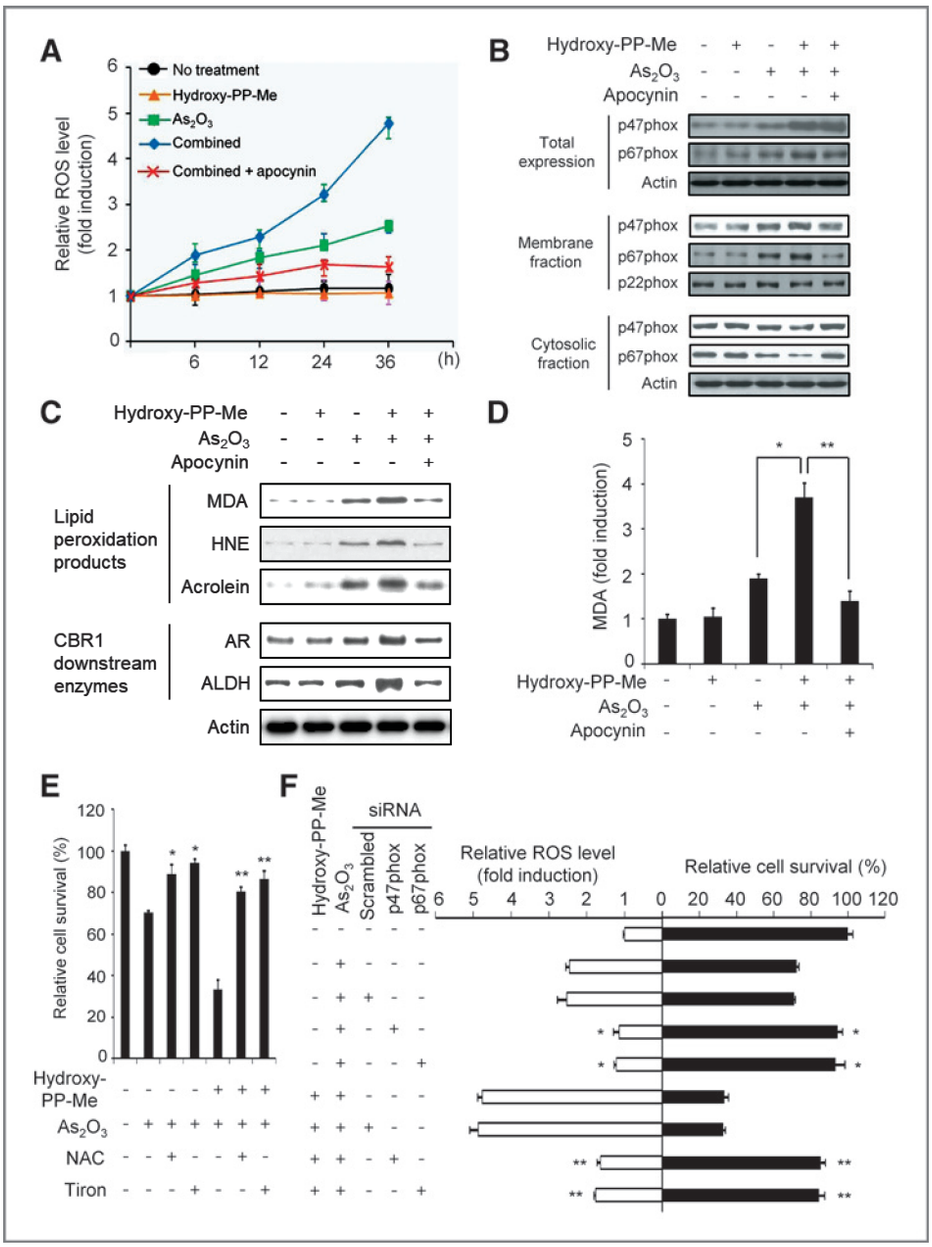

Figure 5. The inhibition of CBR1 enhances NOX-derived ROS generation by As_2O_3 in U937 cells. A to D, the cells were treated with 20 μmol/L hydroxy-PP-Me alone, 4 μmol/L As_2O_3 alone, or a combination of both. Cells were pretreated with 200 μmol/L apocynin for 2 hours before drug administration. A, 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_2O_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 μmol/L hydroxy-PP-Me alone, 4 μmol/L As_2O_3 alone, or both compounds. Relative cell survival was analyzed. Data represent mean ± SE, $n = 3$. *, $P < 0.01$ versus cells treated with As_2O_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_2O_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_2O_3 alone; **, $P < 0.01$ versus scrambled siRNA-transfected cells treated with both compounds.

Effects of hydroxy-PP-Me and As_2O_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_2O_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 μmol/L hydroxy-PP-Me alone, 4 μmol/L As_2O_3 alone, or with combination therapy for 48 hours. Treatment with hydroxy-PP-Me alone resulted in no cell death in all of the samples. Meanwhile, As_2O_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_2O_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_2O_3 in patients with APL, especially in high-risk patients (32–34). However, two common and potentially serious side effects of As_2O_3 treatment, such as APL differentiation syndrome

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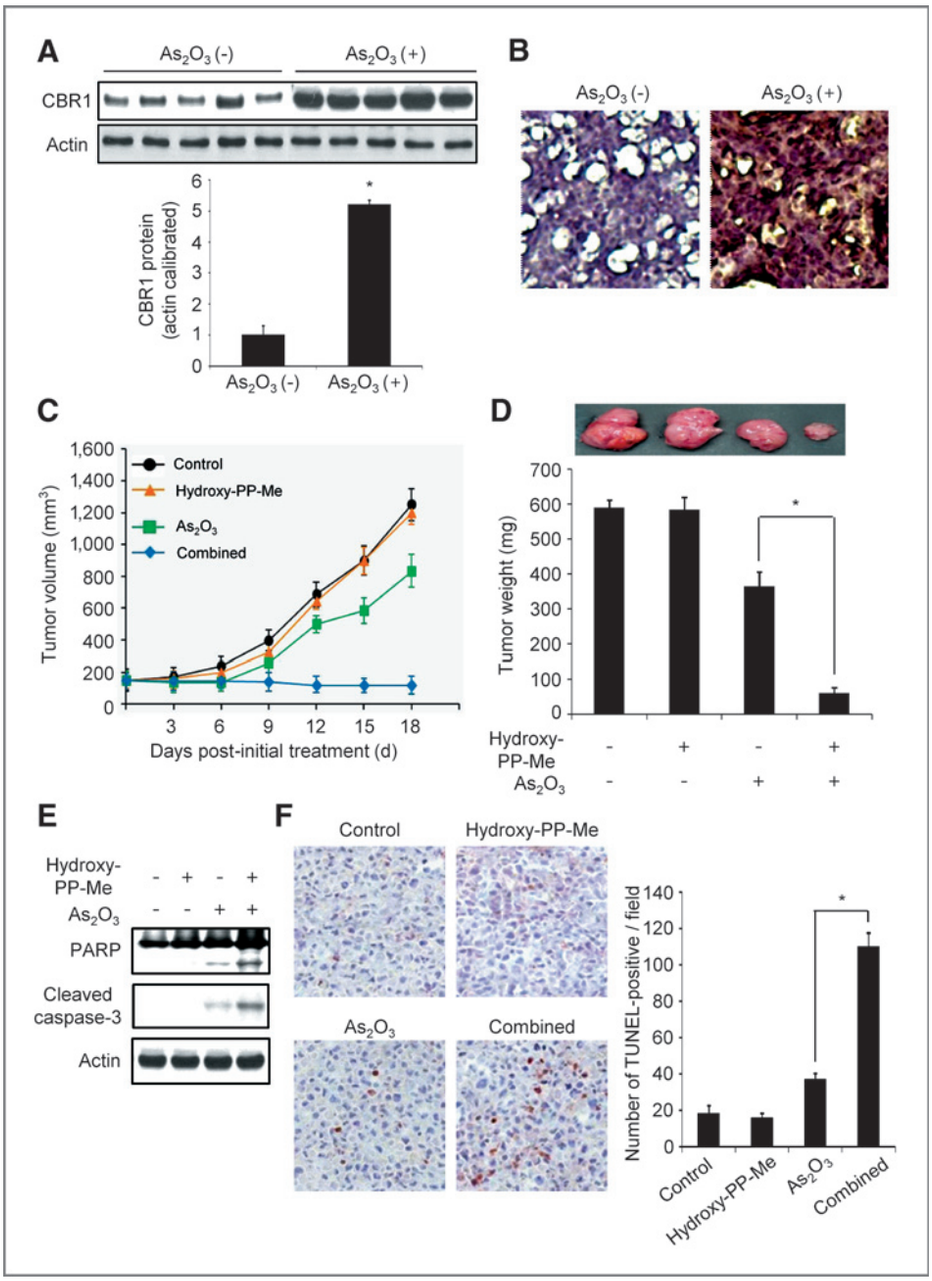

Figure 6. The CBR1 inhibitor in combination with As_2O_3 sensitizes tumor cells to apoptosis in the U937 xenograft model. U937 cells (2×10^7) were injected subcutaneously into mice. The mice were then injected intraperitoneally with hydroxy-PP-Me (30 mg/kg) alone, As_2O_3 (5 mg/kg) alone, or a combination 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, ×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. *, $P < 0.01$ versus tissues treated with As_2O_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, $n = 3$. *, $P < 0.05$ versus tissues treated with As_2O_3 alone. Original magnification, ×100.

and electrocardiogram abnormalities, have been observed. Moreover, when As_2O_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_2O_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_2O_3. Therefore, the development of more effective treatment modalities for As_2O_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_2O_3 exposure. In the presence of As_2O_3, the Fos and Jun subunits were increased. In addition, the As_2O_3-induced CBR1 activation was blocked by SP600125 and

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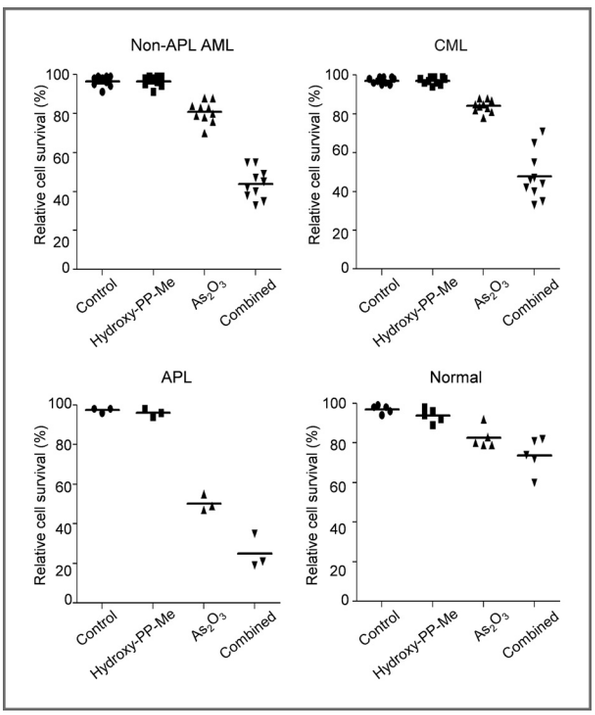

Figure 7. Cytotoxicity of hydroxy-PP-Me, As_2O_3, and their combination in primary human leukemia cells and normal CD34-positive bone marrow cells. Primary leukemia cells and normal CD34-positive bone marrow cells were isolated from 3 APL, 10 AML, 10 CML patients and healthy donors, respectively. These cells were treated with hydroxy-PP-Me alone, As_2O_3 alone, or a combination for 48 hours. Cell viability was determined by an Annexin V/PI assay.

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_2O_3-induced AP-1 activation.

There is a report that As_2O_3 produces ROS via increasing expression of NOX subunits (30). In this study, we showed that the combined treatment of As_2O_3 with hydroxy-PP-Me further enhanced ROS production and p47phox and p67phox expression when compared with As_2O_3 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 As_2O_3 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 As_2O_3 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 As_2O_3 increases apoptotic cell death in various tumors, the antitumor effects of As_2O_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_2O_3 in As_2O_3-resistant tumors, interest has been focused on combination therapy of As_2O_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_2O_3 and the suppression of either its expression or enzymatic activity increases sensitivity to As_2O_3 *in vitro* and *in vivo*. Moreover, we showed that the combined treatment of hydroxy-PP-Me with As_2O_3 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
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Jang, Y. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Jang, Y. Kim, S. Lim, J. K. R, A. Dashdorj, S.S. Kim
Writing, review, and/or revision of the manuscript: M. Jang, H. Won, S.S. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Jang, Y. Kim, Y.H. Min, K.M. Shokat
Study supervision: M. Jang, J. Ha, S.S. Kim

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

In the original version of this article (1), the incorrect actin Western blot image was used in Fig. 5C. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

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1. Jang M, Kim Y, Won H, Lim S, K R J, Dashdorj A, et al. Carbonyl reductase 1 offers a novel therapeutic target to enhance leukemia treatment by arsenic trioxide. Cancer Res 2012;72:4214–24.

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

Miran Jang, Yeonghwan Kim, Hyeran Won, et al.

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