

Arsenic Trioxide Sensitizes Human Glioma Cells, but not Normal Astrocytes, to TRAIL-Induced Apoptosis via CCAAT/Enhancer-Binding Protein Homologous Protein–Dependent DR5 Up-regulation

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Abstract

The current study shows that treatment of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant glioma cells with a combination of TRAIL and subtoxic doses of arsenic trioxide (As₂O₃) induces rapid apoptosis. Whereas TRAIL-mediated proteolytic processing of procaspase-3 was partially blocked in glioma cells, treatment with As₂O₃ efficiently recovered TRAIL-induced activation of caspases. We also found that As₂O₃ treatment of glioma cells significantly up-regulated DR5, a death receptor of TRAIL. Furthermore, suppression of DR5 expression by small interfering RNA (siRNA) inhibited As₂O₃/TRAIL-induced apoptosis of U87MG glioma cells, suggesting that DR5 up-regulation is critical for As₂O₃-induced sensitization of glioma cells to TRAIL-mediated apoptosis. Our results also indicate that an increase in CCAAT/enhancer binding protein homologous protein (CHOP) protein levels precedes As₂O₃-induced DR5 up-regulation. The involvement of CHOP in this process was confirmed by siRNA-mediated CHOP suppression, which not only attenuated As₂O₃-induced DR5 up-regulation but also inhibited the As₂O₃-stimulated TRAIL-induced apoptosis. These results therefore suggest that the CHOP-mediated DR5 up-regulation, brought about by As₂O₃, stimulates the TRAIL-mediated signaling pathway. This in turn leads to complete proteolytic processing of caspase-3, which is partially primed by TRAIL in glioma cells. In contrast to human glioma cells, astrocytes were very resistant to the combined administration of As₂O₃ and TRAIL, demonstrating the safety of this treatment. In addition, As₂O₃-mediated up-regulation of CHOP and DR5, as well as partial proteolytic processing of procaspase-3 by TRAIL, was not induced in astrocytes. Taken together, the present results suggest that the combined treatment of glioma cells with As₂O₃ plus TRAIL may provide an effective and selective therapeutic strategy. [Cancer Res 2008;68(1):266–75]

Introduction

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is considered to be a

promising anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types while having negligible effects on normal cells (1, 2). TRAIL induces apoptosis in tumor cells via the death receptor pathway, using a mechanism similar to that of TNF (3). TRAIL has also been shown to cross-link with the death receptors DR4 or DR5, leading to the aggregation of these receptors, recruitment of the Fas-associated death domain adaptor molecule, and activation of the initiator caspase-8 (4). The activated caspase-8 is then released into the cytoplasm and initiates a protease cascade that activates downstream “effector” caspases, such as caspase-3 (5).

Malignant gliomas are the most common primary brain tumors and are known to invade the surrounding normal brain tissue. This often results in incomplete surgical resection, local recurrence, and a poor response to multimodal therapeutic interventions, such as radiotherapy and chemotherapy (6). Many malignant glioma cells are resistant to TRAIL despite their expression of TRAIL receptors (7, 8). Therefore, the development of a new therapeutic strategy, which restores the TRAIL-induced apoptotic potency in glioma cells, is of utmost importance. One agent that may be of use in sensitizing glioma cells to TRAIL-induced apoptosis is arsenic trioxide (As₂O₃), which has a long history of use as a pharmaceutical agent (9). Recently, As₂O₃ was shown to induce tumor cell apoptosis or differentiation and thus has considerable efficacy in the treatment of patients with acute promyelocytic leukemia (9, 10). Furthermore, As₂O₃ shows anticancer effects not only in hematologic cancers other than acute promyelocytic leukemia (11–13) but also in solid tumors derived from several tissues (14–17). Despite these observations, the mechanisms underlying the antitumor effects of As₂O₃ are not fully understood.

In this study, we show that the combined treatment of TRAIL-resistant glioma cells with subtoxic doses of As₂O₃ and TRAIL dramatically induces glioma cell apoptosis, but is not cytotoxic to normal human astrocytes. This suggests that the combined As₂O₃ and TRAIL treatment may offer an attractive and safe strategy for the treatment of malignant gliomas. The current study also presents the underlying mechanisms by which this combined treatment achieves selective killing of glioma cells. We show that As₂O₃ induces CHOP-dependent DR5 up-regulation and that TRAIL stimulates partial priming of the proteolytic processing of caspase-3. Therefore, the combined treatment with As₂O₃ and TRAIL may synergistically stimulate and accelerate the death receptor-mediated apoptotic signaling pathway. These effects are only induced in glioma cells and not in astrocytes, suggesting that they may be responsible for the effective induction of cancer cell apoptosis.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Chemicals and antibodies. Recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114-281) was from KOMA Biotech, Inc. Calcein acetoxymethyl ester (calcein-AM), ethidium homodimer (EthD-1), 6-carboxy-2',7'-dichlorofluorescein diacetate (H₂DCF-DA), and dihydroethidium were from Molecular Probe. As₂O₃, *N*-acetylcysteine, butyl-hydroxyanisole, Cu(II)-(diisopropylsalicylate)₂, Mn(III) *meso*-tetakis(4-benzoic acid) porphyrin, and 3,4-dihydro-5-[1-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) were from Sigma. The following antibodies were used: anti-caspase-8, caspase-3, survivin, and XIAP from Stressgen; anticaspase-9, caspase-2, FAK, Cdk2, c-IAP1, c-IAP2, DR4, CHOP/growth arrest and DNA damage-inducible gene 153 (GADD153), Bcl-2, and Bcl-xL from Santa Cruz Biotechnologies; anti-PAR and BNIP3 from BD Pharmingen; anti-Bid, phosphorylated Akt, and total Akt from Cell Signaling; anti-c-FLIP (NF6) from Alexis; anti-Flag M2 and FITC-conjugated anti-goat IgG from Sigma; anti-DR5 for Western blotting from KOMA Biotech; anti-DR5 and anti-DR4 antibody for flow cytometry from R&D Systems; and anti-rabbit IgG horseradish peroxidase, mouse IgG, and goat IgG from Zymed Laboratories, Inc.

Cell culture. Human glioma cell lines U87MG, U251MG, T98G, U343, U373MG, and U251N were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies-Bethesda Research Laboratories). The primary cultures of normal human astrocytes were prepared from 14-week gestation of fetal cerebrum tissues as described previously (18). Human astrocyte cultures were grown in DMEM with high glucose supplemented with 10% FBS and 20 µg/mL gentamicin, subcultured every 2 weeks, and cell culture passage number of <5 were used in the present study. Immunofluorescence study indicated that better than 99% of cells expressed glial fibrillary acidic protein, a cell type specific marker for astrocytes. Permission to use human brain tissues for research was granted by the clinical screening committee involving human subjects of the university.

Measurement of cellular viability. Cell viability was assessed by double labeling of cells with 2 µmol/L calcein-AM and 4 µmol/L EthD-1. The calcein-positive cells and EthD-1-positive dead cells were visualized using fluorescence microscope (Axiovert 200M, Zeiss).

Reverse transcription-PCR. Total RNA was extracted from U87MG cells using the TRIzol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) was done, following the manufacturer's protocol (TaKaRa Shuzo Co.). Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for 5 µmol/L As₂O₃. Human DR5 mRNA was amplified using the sense primer 5'-GTCTGCTCT-GATCACCCAAC-3' and the antisense primer 5'-CTGCAAACT-GTGACTCTATG-3' (corresponding to a 424-bp region of DR5). For CHOP, the sense primer 5'-CAACTGCAGAGATGGCAGCTGA-3' and the antisense primer CTGATGCTCCCAATTGTTTCAT-3' (corresponding to a 536-bp region of CHOP) were used. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5'-CGGCCATCACGCCACAGTTT-3' and the antisense primer 5'-CGGCCATCACGCCACAGTTT-3' were used (corresponding to a 310-bp region of GAPDH). The PCR cycling conditions (30 cycles) chosen were as follows: (a) 30 s at 94°C for GAPDH and DR5 and 45 s at 94°C for CHOP; (b) 30 s at 68°C for DR5, 30 s at 60°C for GAPDH, and 45 s at 52.5°C for CHOP; and (c) 1 min 30 s at 72°C for DR5 and GAPDH and 45 s at 72°C for CHOP, with a subsequent 10-min extension at 72°C. Reaction products were analyzed on 2% agarose gels. The bands were visualized by ethidium bromide.

Flow cytometry of death receptors. Cells were analyzed for the surface expression of DR4 and DR5 by indirect staining with primary goat anti-human DR4 and DR5 (R&D Systems), followed by FITC-conjugated rabbit anti-goat IgG (Sigma). Briefly, cells (1×10^6) were stained with 200 µL PBS containing saturating amounts of anti-DR4 or anti-DR5 antibody on ice for 30 min. After incubation, cells were washed twice and reacted with FITC-conjugated rabbit anti-goat IgG on ice for 30 min. After washing with PBS, the expressions of these death receptors were analyzed by a fluorescence-activated cell sorter (FACS; Becton Dickinson and Co.).

Measurement of reactive oxygen species. Cells were plated at a density of 5×10^4 in 24-well plates, allowed to attach overnight, and exposed to 5 µmol/L As₂O₃ for 4 h. The cells were stained with 10 µmol/L H₂DCF-DA for 10 min or 10 µmol/L dihydroethidium for 20 min at 37°C and then reactive oxygen species (ROS) levels were analyzed using a FACS sorter.

Establishment of the stable cell lines overexpressing catalase and CrmA. The cDNAs encoding human catalase were PCR amplified from plasmids containing these sequences (kindly provided by Dr. Akashi, National Institute of Radiological Sciences), with the catalase-specific primers designed to incorporate a 5'-hemagglutinin epitope. The respective PCR products were subcloned into the pcDNA3.1(+) expression vector (Invitrogen), and the resulting constructs were confirmed by nucleotide sequencing. U87MG cells were separately transfected with this generated expression vector encoding catalase or an expression vector containing CrmA cDNA (kindly provided by Prof. V.M. Dixit, University of Michigan Medical School). Stable cell lines overexpressing catalase or CrmA were selected with fresh media containing 500 µg/mL G418 (Calbiochem). Overexpression of catalase was analyzed by Western blotting using anti-5'-hemagglutinin antibody (Covance Research Products). Overexpression of CrmA was analyzed by Western blotting using anti-Crma (BD Pharmingen).

Small interfering RNAs. The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and has the following sequences: DR5, UACAAUCACCGACCUUGACCAUCCC; CHOP, UUCACCAU UCGGUCAUCAGAGCUC. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Plasmids, transfection, and luciferase assay. The DR5/*Sac*I plasmid [containing DR5 promoter sequence (-2,500/+3)] and pDR5/-605 [containing DR5 promoter sequence (-605/+3)] were gifts from Dr. T. Sakai (Kyoto Prefectural University of Medicine). Point mutation of the CHOP binding sites to the DR5/-605 promoter was generated by a two-step PCR method using the following primers: mCHOP (5'-CTTGGGAGGAGG-TAGTTGACGA and 5'-TCGTCAACTACCTCCTCCGCAAG). Clone representing this point mutation was sequenced to ensure the accuracy of the PCR amplification procedure. The CHOP promoter-luciferase construct (19) was generously provided by Dr. P. Fournoux (U.R. 238 de Nutrition Cellulaire et Moléculaire, France). For transfection, in brief, cells were plated onto 60-mm dishes at a density of 5×10^5 cells and grown overnight. Cells were cotransfected with 2 µg of various plasmid constructs and 1 µg of the pCMV-β-galactosidase plasmid for 5 h using Lipofectamine plus reagent (Invitrogen) following the manufacturer's protocol. After incubation for 24 h, transfected cells were further treated with or without As₂O₃. Luciferase and β-galactosidase activities were assayed following the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysates and expressed as an average of three independent experiments.

Statistical analysis and determination of synergy. All data are presented as means ± SE of at least three independent experiments. The statistical significance of differences was assessed using ANOVA (GraphPad software; GraphPad), followed by Student-Newman-Keuls multiple comparison tests. $P < 0.05$ was considered significant. Synergy of As₂O₃ and TRAIL was evaluated by the isobologram method (20). The cells were treated with different concentrations of each agent (As₂O₃ or TRAIL) alone or with the two agents in combination for 24 h. The relative survival was assessed, and the EC₅₀ values for each drug given alone or in combination with a fixed concentration of the second agent were established from the concentration-effect curves. The EC₅₀ values of cotreatment were divided by the EC₅₀ value of each drug in the absence of the other drug. In a graphical presentation, the straight line connecting the EC₅₀ values of the two agents when applied alone corresponds to additivity or independent effects of both agents. Values below this line indicate synergy, and values above this line indicate antagonism.

Results

As₂O₃ sensitizes human glioma cells to TRAIL-induced apoptosis. As we have previously reported, many human glioma

cell lines are resistant to TRAIL (7, 8). In this study, we initially investigated whether As₂O₃ could sensitize these glioma cells to TRAIL-induced apoptosis. As such, six different glioma cell lines were treated with As₂O₃ alone, TRAIL alone, or with both As₂O₃ and TRAIL for 24 h. Calcein-AM and EthD-1 assessment showed that cell viability was not significantly decreased by treatment with As₂O₃ alone or by TRAIL alone (Fig. 1A). However, the viability of cells significantly decreased when these factors were used in combination, both when fixing the concentration of TRAIL and varying the concentrations of As₂O₃ and conversely when fixing the concentration of As₂O₃ and varying TRAIL concentrations (Fig. 1A). Furthermore, synergistic effect, as determined by isobologram analysis (20), was observed for As₂O₃ and TRAIL in U87MG cells and U251MG cells (Fig. 1B). These results show that the combined treatment of TRAIL-resistant glioma cells with As₂O₃ and TRAIL very effectively induces cell death.

Cotreatment with As₂O₃ and TRAIL recovers incomplete activation of caspases in TRAIL-resistant glioma cells. Next, we examined whether the sensitizing effect of As₂O₃ on TRAIL-mediated cell death was associated with the activation of caspases. To do this, U251MG or U87MG cells were treated with 5 μmol/L As₂O₃ or 100 ng/mL TRAIL alone or pretreated with As₂O₃ followed by TRAIL exposure for the indicated times. Treatment of cells with 5 μmol/L As₂O₃ alone did not induce any proteolytic processing of caspases in either cell line (Fig. 1C). In contrast, a 8-h exposure of U87MG to TRAIL led to a partial cleavage of the 32-kDa procaspase-3 into a 20-kDa intermediate form (p20), but not to further cleavage of this form into the active p17 subunit. In

addition, processing events of other caspases were not evident in these cell lines. In contrast, treatment of U87MG cells with As₂O₃ plus TRAIL induced the cleavage of caspase-3 into the p20 intermediate form by 8 h and its subsequent cleavage into the active p17 and p12 subunit after 12 h. In addition, precursor forms of caspase-2, caspase-8, and caspase-9 progressively decreased after this combined treatment. We further assessed the cleavage of several key death substrates, such as FAK and Bid, which indicate activation of caspases (21). Our results show that both FAK and Bid were degraded from 12 h after the combined treatment, whereas degradation was not observed after treatment with TRAIL or As₂O₃ alone. In addition, similar but faster activation patterns of caspases were observed in U251MG cells in response to As₂O₃ and TRAIL. These results suggest that, in these glioma cells lines, TRAIL resistance may be associated with a blockade in the proteolytic processing of procaspase-3 and a failure to stimulate the subsequent caspase signaling cascade. Cotreatment of glioma cells with As₂O₃ may contribute to the relief of the proteolytic processing blockade. To investigate the functional role of caspases, we examined the effect of overexpressing the viral caspase-8 inhibitor CrmA (22) on As₂O₃/TRAIL-induced apoptosis. We found that overexpression of CrmA completely inhibited the cell death induced by the combined treatment (Fig. 1D). These results show that caspases play a critical role in the As₂O₃/TRAIL-induced apoptosis.

Neither BNIP3 induction nor poly(ADP-ribose) polymerase activation is important for As₂O₃/TRAIL-mediated apoptosis. Next, we investigated the underlying mechanisms by which As₂O₃

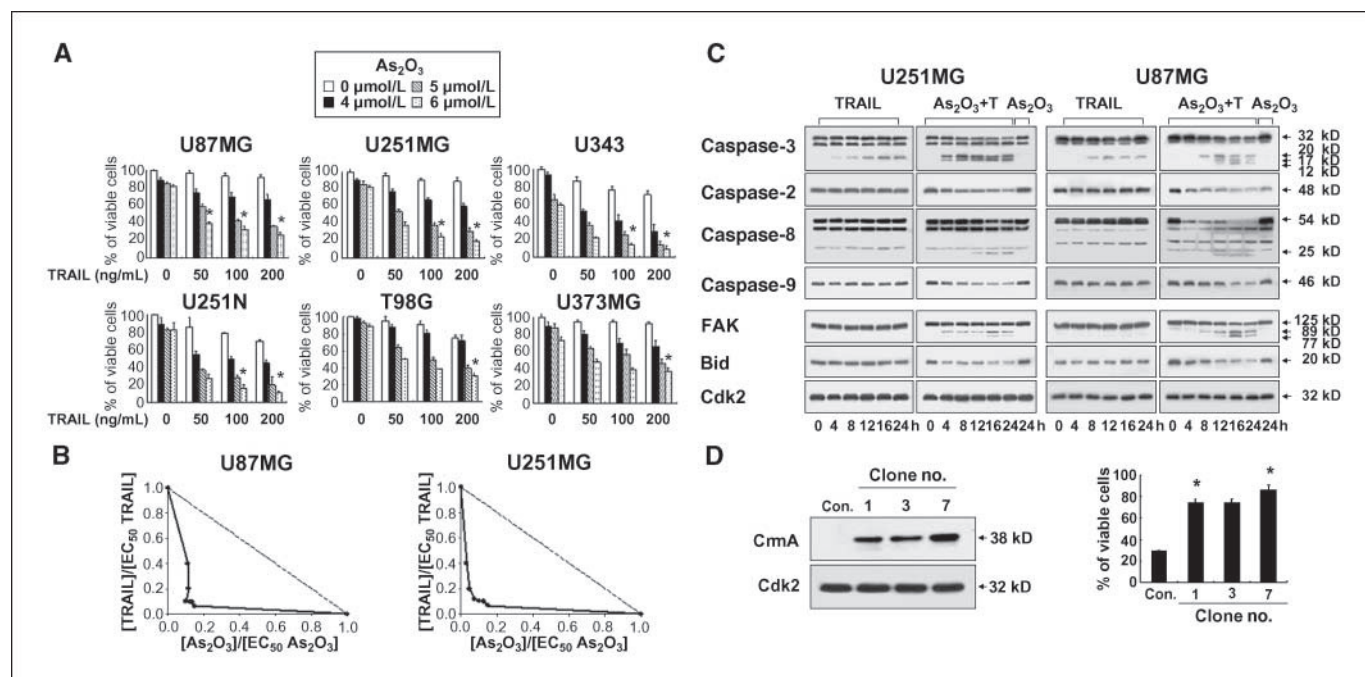


Figure 1. As₂O₃ sensitizes human glioma cells to TRAIL-induced apoptosis. **A**, effect of As₂O₃ and/or TRAIL on the viability of human glioma cell lines and astrocytes. Six different human glioma cell lines and astrocytes were treated with As₂O₃ for 30 min and then further treated with TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1. Columns, average of three independent experiments; bars, SE. *, *P* < 0.05 compared with untreated cells. **B**, synergistic induction of cell death by As₂O₃ and TRAIL. U87MG or U251MG cells were treated for 24 h with increasing concentrations of As₂O₃ and TRAIL. Isobologram analysis was performed as described in Materials and Methods. **C**, cotreated As₂O₃ effectively restores the proteolytic processing of caspase-3 incompletely by TRAIL alone. U251MG or U87MG cells were treated with 5 μmol/L As₂O₃ alone, 100 ng/mL TRAIL alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting to detect the changes in the expression of caspases. To confirm the activation of caspases, Western blotting of FAK and Bid, the substrate proteins of caspases, was performed. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. **D**, overexpression of CrmA blocks As₂O₃-sensitized TRAIL-induced apoptosis. Protein levels of CrmA in the respectively selected stable cell lines (left). The sublines overexpressing CrmA were treated with 5 μmol/L As₂O₃ plus 100 ng/mL TRAIL for 24 h, and the cellular viability was measured using calcein-AM and EthD-1 (right). Columns, average of three independent experiments; bars, SE. *, *P* < 0.05 compared with cells treated with As₂O₃ plus TRAIL.

enhances TRAIL-induced apoptosis in TRAIL-resistant glioma cells. Recent reports have shown that several intracellular proteins, including survivin, XIAP, Bcl-2, and Bcl-xL, are capable of inhibiting TRAIL-mediated cancer cell apoptosis when present at sufficient levels (23, 24). We first examined the possibility that As₂O₃ might down-regulate the expression levels of these antiapoptotic proteins. However, significant differences in the levels of the tested inhibitor of apoptosis proteins (survivin, XIAP, c-IAP1, and c-IAP2) or antiapoptotic Bcl-2 family proteins (Bcl-2 and Bcl-xL) were not observed after treatment with 5 μmol/L As₂O₃ (Fig. 2A). These results suggest that the sensitizing effect of As₂O₃ on TRAIL-mediated apoptosis is independent of these antiapoptotic proteins.

Recently, BNIP3, a proapoptotic member of the Bcl-2 family (25), was proposed to be involved in As₂O₃-induced autophagic cell death in glioma cells (26). We found that the protein level of BNIP3, in particular the monomer form of BNIP3, was rapidly and significantly increased by treatment with As₂O₃. We therefore tested whether BNIP3 is also required for As₂O₃-stimulated TRAIL-induced apoptosis. However, siRNA-mediated BNIP3 suppression did not affect the As₂O₃/TRAIL-induced apoptosis (Fig. 2B), suggesting that the As₂O₃-induced BNIP3 up-regulation is not associated with the sensitizing effect of As₂O₃ on TRAIL-induced glioma cell apoptosis.

Kang et al. recently reported that As₂O₃ induces cell death in human cervical cancer cells through poly(ADP-ribose) polymerase-1 (PARP-1) activation (27). We therefore examined whether PARP-1 is involved in As₂O₃-facilitated TRAIL-induced apoptosis and found that As₂O₃ induced significant activation of PARP-1 in U87MG cells (Fig. 2C). However, pretreatment of U87MG cells with the PARP-1-specific inhibitor DPQ did not attenuate As₂O₃-sensitized TRAIL-mediated apoptosis (Fig. 2C). This shows that PARP activation is not important for As₂O₃-facilitated TRAIL-induced apoptosis. Taken together, these results suggest that neither BNIP3 up-regulation nor PARP-1 activation is responsible for the recovery of As₂O₃-mediated TRAIL-sensitivity in glioma cells.

DR5 up-regulation plays a critical role in As₂O₃-induced sensitization of various glioma cells to TRAIL-induced apoptosis. Very recently, Szegezdi et al. showed that As₂O₃ specifically enhances TRAIL-induced apoptosis in leukemic cell lines, including ML-1, K562, and Jurkat, but not in other tumor cell lines (28). These researchers showed that, in this process, As₂O₃ induced an inhibition of Akt, a down-regulation of the short isoform of c-FLIP (c-FLIP_s) and an increase in the cell surface expression of DR5 (28). However, the functional roles of these factors in As₂O₃-stimulated TRAIL-mediated apoptosis were not clarified. We therefore examined whether these signaling molecules are similarly regulated by As₂O₃ in glioma cells. We show that Akt activity is down-regulated in U87MG, but not in U251MG cells (Fig. 3A). Furthermore, the protein levels of the long isoform of c-FLIP (c-FLIP_l) were down-regulated in As₂O₃-treated U87MG cells, and the protein levels of c-FLIP_s were reduced in U251MG cells. As such, neither Akt inhibition nor down-regulation of a certain isoform of c-FLIP is a common response of glioma cells to As₂O₃. In contrast, As₂O₃ treatment produced a progressive and significant increase in the DR5 protein level, both in U251MG and in U87MG glioma cells. However, this treatment did not alter the protein levels of TRAIL or the levels of the DR4 TRAIL death receptor (Fig. 3A). We further examined whether DR5 is also up-regulated by As₂O₃ in other glioma cells and found that, indeed, the protein levels of DR5 were commonly and dose-dependently up-regulated by As₂O₃ in U343, U251N, and T98G cells (Fig. 3B).

FACS analysis also showed that the cell surface expression of DR5, but not DR4, was significantly increased in U87MG cells treated with As₂O₃ (Fig. 3C). To clarify whether DR5 up-regulation is critical for As₂O₃-stimulated TRAIL-induced apoptosis, we examined the effect of siRNA-mediated knockdown of DR5 expression on this process. We found that suppression of DR5 expression by transfection with three kinds of siRNAs effectively inhibited As₂O₃-stimulated TRAIL-induced cell death in U87MG cells (Fig. 3D). These results support the idea that As₂O₃-induced up-regulation of DR5 is critical for establishing of TRAIL sensitivity in these glioma cells.

ROS generation is not involved in the As₂O₃/TRAIL-mediated apoptosis. Recently, we reported that sulforaphane sensitizes TRAIL-induced apoptosis via ROS-mediated DR5 up-regulation (29). Hence, we examined whether As₂O₃-induced DR5 up-regulation is also dependent on ROS in this system. We performed flow cytometric analysis to quantitatively detect ROS in As₂O₃-treated cells using H₂DCF-DA, the dye which fluoresces on oxidation by ROS. Our results revealed that the fluorescence of DCF significantly increased in U87MG cells treated with 5 μmol/L As₂O₃ for 4 h. This increase was similar to that seen in U87MG cells treated with 250 μmol/L H₂O₂ (Fig. 4A). Flow cytometric analysis using the fluorescent probe dihydroethidium, which detects intracellular O₂⁻ production, also showed the significant increase in O₂⁻ levels after As₂O₃ treatment. Interestingly, however, pretreatment with the antioxidant *N*-acetylcysteine failed to inhibit As₂O₃-induced DR5 up-regulation (Fig. 4B). Furthermore, pretreatment with a variety of antioxidants [*N*-acetylcysteine, vitamin C, catalase, reduced glutathione, butyl-hydroxyanisole, Cu(II)-(diisopropylsalicylate)₂, and Mn(III) *meso*-tetrakis(4-benzoic acid) porphyrin] had no effect on the cell death induced by combined treatment with As₂O₃ and TRAIL (Fig. 4C). Overexpression of catalase also did not block the cell death induced by this combined treatment (Supplementary Fig. S1). Taken together, these results show that As₂O₃-generated ROS is not required for As₂O₃/TRAIL-induced cell death.

The As₂O₃-induced DR5 up-regulation is CHOP-dependent in glioma cells. To further investigate the underlying mechanisms by which As₂O₃ induces DR5 up-regulation, the CHOP protein was examined. CHOP has recently been reported to be involved in the DR5 up-regulation mediated by several TRAIL sensitizers, such as proteasome inhibitor and tunicamycin (30, 31). Our results indicate that CHOP protein levels are significantly increased from 4 h, both in U87MG and U251MG cells treated with As₂O₃, and that this increase precedes As₂O₃-mediated DR5 induction (Fig. 5A). Furthermore, As₂O₃ induced CHOP up-regulation in a dose-dependent manner in the U343, U251N, and T98G cell lines, suggesting that DR5 up-regulation is also a common response of glioma cells to As₂O₃. To clarify the functional role of CHOP in As₂O₃-induced DR5 up-regulation, CHOP siRNA was used. Whereas DR5 was up-regulated by As₂O₃ in U87MG cells transfected with scrambled negative control RNA, transfection with CHOP siRNA significantly abrogated the up-regulation of DR5 (Fig. 5B). Moreover, As₂O₃/TRAIL-mediated cell death was significantly reduced by transfection with CHOP siRNA, but not by scrambled negative control RNA (Fig. 5B). These results therefore show that CHOP-dependent DR5 up-regulation contributes to the sensitizing effect of As₂O₃ on TRAIL-induced apoptosis.

We next investigated whether the As₂O₃-induced CHOP up-regulation is controlled at the transcriptional level. Luciferase

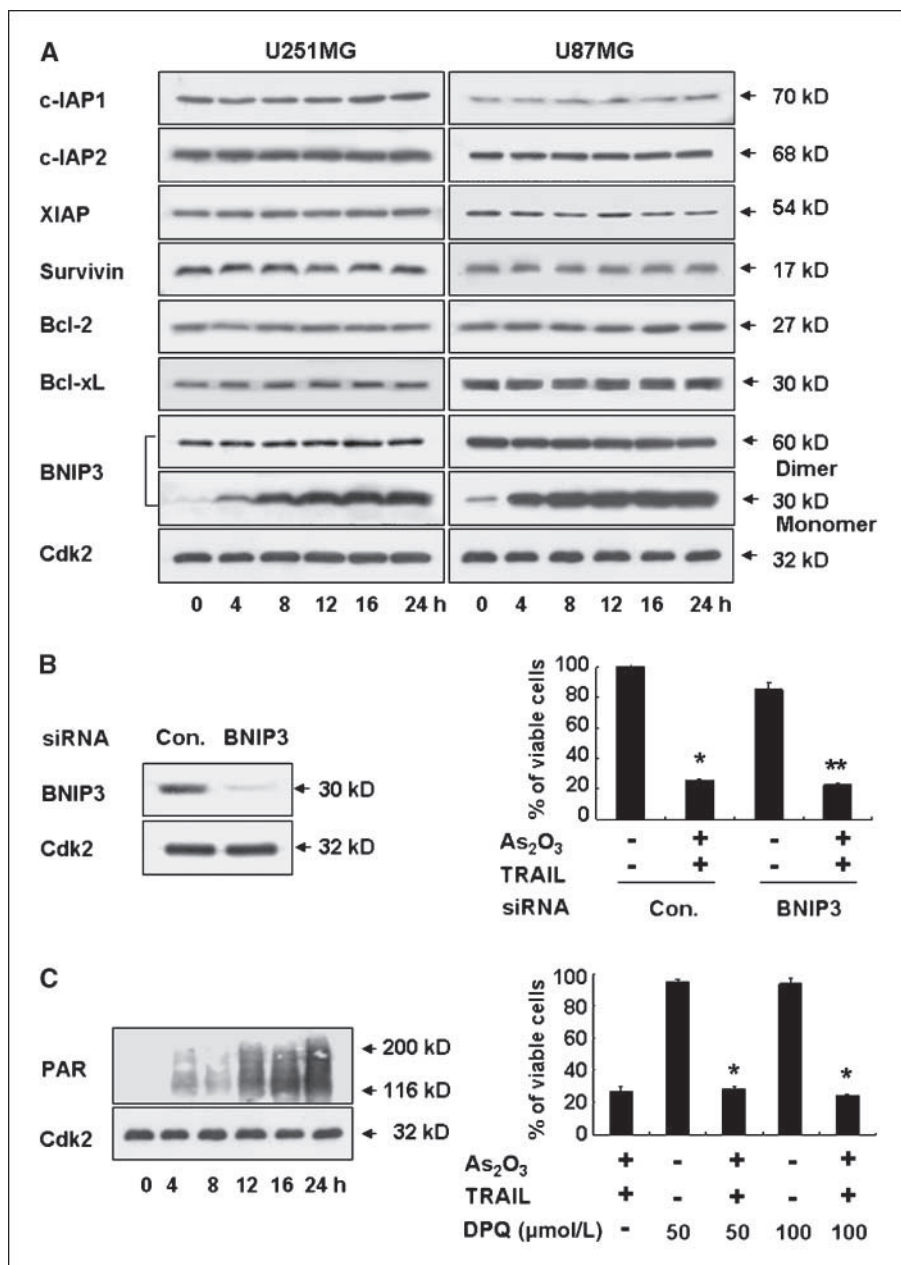


Figure 2. Inhibitor of apoptosis proteins, Bcl-2, Bcl-xL, BNIP3, and PARP may not be involved in the sensitizing effect of As₂O₃ on TRAIL-induced apoptosis. **A**, changes in the expression of various intracellular regulators of cell death. Cell extracts were prepared from U251MG or U87MG cells treated with As₂O₃ for the indicated times, and Western blotting of inhibitors of apoptosis, Bcl-2, Bcl-xL, BNIP3, and Cdk2 was performed. **B**, siRNA-mediated suppression of BNIP3 expression does not affect As₂O₃-stimulated TRAIL-induced apoptosis in U87MG cells. U87MG cells were transfected with scrambled negative control RNA or siRNA duplexes against BNIP3 mRNA. Twenty-four hours after transfection, Western blotting of BNIP3 was done to confirm the down-regulation of BNIP3 by siRNA transfection (*left*). Cdk2 levels were assessed to show equal gel loading. To examine the effect of BNIP3 down-regulation on As₂O₃/TRAIL-induced apoptosis, U87MG cells were transfected with siRNAs, incubated for 24 h, and then further treated with or without 5 μmol/L As₂O₃ plus 100 ng/mL TRAIL for 24 h. Cellular viability was determined using calcein-AM and EthD-1 (*right*). *Columns*, average of three independent experiments; *bars*, SE. *, *P* < 0.005 compared with untreated cells; **, *P* < 0.05 compared with cells treated with As₂O₃ plus TRAIL. **C**, PARP activation is not required for the As₂O₃-sensitized TRAIL-induced apoptosis. Cell extracts were prepared from U87MG cells treated with As₂O₃ for the indicated times and Western blotting of PAR was performed (*left*). Cdk2 levels were assessed to show equal gel loading. U87MG cells were pretreated with DPQ at the indicated concentrations for 30 min and further treated with 5 μmol/L As₂O₃ plus 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1 (*right*). *Columns*, average of three independent experiments; *bars*, SE. *, *P* < 0.05 compared with cells treated with As₂O₃ plus TRAIL.

assays, performed after transfection of U87MG cells with the CHOP promoter-luciferase construct, showed that As₂O₃ increased the CHOP promoter activity in a dose-dependent manner (Fig. 5C). Furthermore, RT-PCR analysis showed that the mRNA levels of both CHOP and DR5 were significantly increased after a 4-h As₂O₃ treatment (Fig. 5C). We also examined whether the CHOP transcriptional factor activity is critical for As₂O₃-induced DR5 up-regulation. To address this issue, we compared the activities of three types of DR5 promoters after As₂O₃ treatment. These promoters were the pDR5-SacI plasmid containing 2.5 kb of the upstream sequence of the DR5 promoter, the pDR5-605 plasmid containing 605 bp of the upstream DR5 promoter sequence, and the pDR5-605-mCHOP plasmid containing a mutation at the CHOP binding site of the pDR5-605 plasmid. Whereas the promoter activities of

pDR5-SacI and pDR5-605 increased in a dose-dependent manner with As₂O₃ treatment, the promoter activity of pDR5-605-mCHOP was not enhanced by the addition of As₂O₃ (Fig. 5D). These results show that the As₂O₃-induced DR5 up-regulation is transcriptionally controlled by CHOP and that the expression of CHOP is also induced by As₂O₃.

Human astrocytes are resistant to As₂O₃-induced DR5 up-regulation and to TRAIL-induced partial proteolytic processing of procaspase-3. Next, we investigated whether the combined As₂O₃ and TRAIL treatment of normal astrocytes affected their viability. Our results indicate that these astrocytes are resistant to either TRAIL or As₂O₃ alone (Fig. 6A). Furthermore, the combined treatment of human astrocytes with As₂O₃ and TRAIL did not induce any significant cell death, suggesting that the sensitizing As₂O₃ and TRAIL regimen may be preferentially toxic to glioma

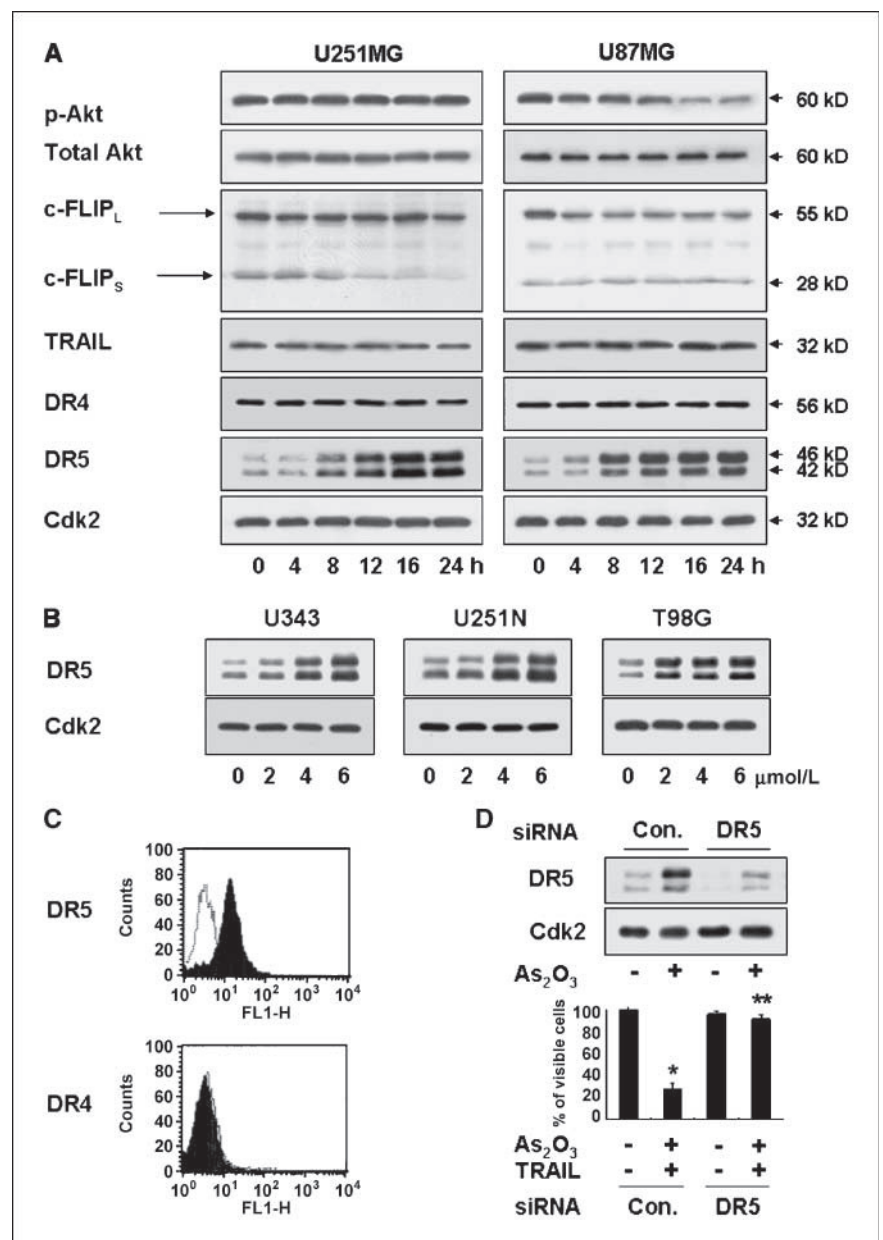
cells and not to normal astrocytes. We then examined whether the resistance of astrocytes to the combined treatment is associated with a differential regulation of CHOP and DR5. Results indicate that in response to As_2O_3 , neither CHOP nor DR5 was up-regulated in human astrocytes, whereas both CHOP and DR5 were dose-dependently up-regulated by As_2O_3 in U87MG and U251MG cells (Fig. 6B). We further investigated whether there is difference in the TRAIL-induced proteolytic processing patterns of procaspase-3 between human glioma cells and astrocytes. We found that, although the proteolytic processing of procaspase-3 was partially primed into the p20 intermediate form in glioma cells, this process was almost completely blocked in human astrocytes (Fig. 6C). Taken together, these results suggest that the human glioma cell-specific effects, which include As_2O_3 -induced DR5 up-regulation and TRAIL-mediated priming of the proteolytic processing of caspase-3, may explain the preferential cytotoxicity of the com-

bined treatment with As_2O_3 and TRAIL in human glioma cells, sparing the astrocytes.

Discussion

Previous studies have shown that As_2O_3 has a good therapeutic effect with only mild side effects in patients with relapse/refractory acute promyelocytic leukemia and multiple myeloma (10, 32, 33). Clinical activity and tolerance studies have indicated that As_2O_3 -based chemotherapy is a promising treatment option for patients who are unable to respond to and/or tolerate other chemotherapy regimens. Recently, As_2O_3 was further shown to have potential as a novel treatment against various malignant solid tumors (14–17). However, higher concentrations of As_2O_3 were required to show the anticancer effect on malignant solid tumors compared with hematopoietic cancers. Because high concentrations of As_2O_3 have been associated with clinical risks (34), the use of lower doses of

Figure 3. As_2O_3 -induced DR5 up-regulation is critical for As_2O_3 -sensitized TRAIL-mediated apoptosis. **A**, neither Akt inhibition nor c-FLIP_S down-regulation is a common response of glioma cells to As_2O_3 . U251MG or U87MG cells were treated with 5 μ mol/L As_2O_3 for the indicated time points and Western blotting of phosphorylated Akt, total Akt, c-FLIP, TRAIL, DR4, and DR5 was performed. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. **B**, As_2O_3 dose-dependently up-regulates DR5 in various glioma cells. U343, U251N, or T98G were treated with As_2O_3 at the indicated concentrations for 12 h and Western blotting of DR5 was performed. **C**, As_2O_3 increases the surface expression levels of DR5 but not DR4. U87MG cells were incubated with or without 5 μ mol/L As_2O_3 for 16 h, and the surface expression of DR5 and DR4 proteins was analyzed by flow cytometry. *X axis*, fluorescence intensity; *Y axis*, relative number of cells. *Black histograms*, treated with As_2O_3 ; *white histograms*, untreated cells. **D**, suppression of DR5 expression by siRNA reduces As_2O_3 -stimulated TRAIL-induced apoptosis in U87MG cells. U87MG cells were transfected with scrambled negative control RNA or siRNA duplexes against DR5 mRNA. Twenty-four hours after transfection, cells were further treated with or without 5 μ mol/L As_2O_3 for 12 h. Western blotting of DR5 was done to confirm the down-regulation of DR5 by siRNA transfection. Cdk2 levels were assessed to show equal gel loading (*top*). To examine the effect of DR5 down-regulation on As_2O_3 /TRAIL-induced apoptosis, U87MG cells were transfected with siRNAs, incubated for 24 h, and further treated with or without 5 μ mol/L As_2O_3 plus 100 ng/mL TRAIL for 24 h. Cellular viability was determined using calcein-AM and EthD-1 (*bottom*). *Columns*, average of three independent experiments; *bars*, SE. *, $P < 0.005$ compared with untreated cells; **, $P < 0.05$ compared with cells treated with As_2O_3 plus TRAIL.



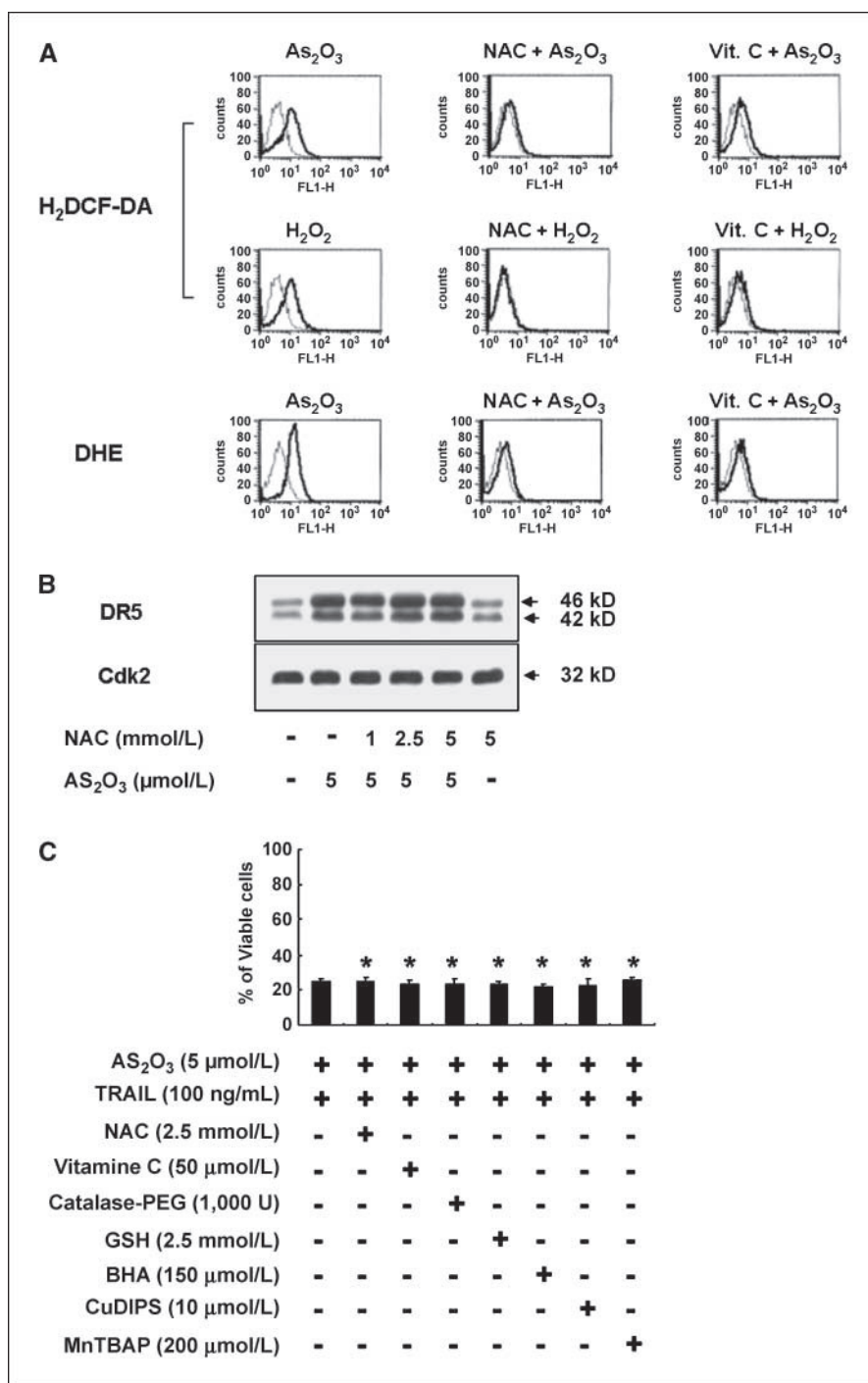


Figure 4. ROS generation is not critical for As₂O₃-induced DR5 up-regulation and the cell death induced by As₂O₃ plus TRAIL. **A**, generation of ROS by As₂O₃. U87MG cells were treated with 5 μmol/L As₂O₃ for 4 h and ROS levels were measured by FACS analysis after staining with H₂DCF-DA or dihydroethidium, as described in Materials and Methods. To further confirm the As₂O₃-induced ROS generation, U87MG cells were pretreated with the antioxidant *N*-acetylcysteine (2.5 mmol/L) or vitamin C (50 μmol/L) and further treated with 5 μmol/L As₂O₃ for 4 h, and ROS levels were measured. To compare the extent of oxidative stress induced by As₂O₃ with that induced by H₂O₂, same procedure was done using U87MG cells treated with 250 μmol/L H₂O₂ for 4 h. **B**, pretreatment with *N*-acetylcysteine does not block As₂O₃-induced increase in DR5 protein levels. U87MG cells pretreated with *N*-acetylcysteine at the indicated concentrations for 30 min were further treated with or without 5 μmol/L As₂O₃ for 12 h. Cell extracts were prepared for Western blotting of DR5. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. **C**, pretreatment with antioxidants does not block the cell death induced by As₂O₃ plus TRAIL. U87MG cells were pretreated with various antioxidants as indicated for 30 min and further treated with 5 μmol/L As₂O₃ plus 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. *Points*, average of three individual experiments; *bars*, SD. *Columns*, average of three independent experiments; *bars*, SE. *, *P* < 0.05 compared with cells treated with As₂O₃ plus TRAIL.

As₂O₃, in combination with other agents, may be a better option for treating malignant solid tumors. Here, we show that subtoxic doses of As₂O₃ in combination with TRAIL effectively induce apoptosis in various malignant glioma cells.

Previous studies showed that As₂O₃ induces apoptosis of hematopoietic cancer cells via various mechanisms, including opening of the mitochondrial permeability transition pores, as well as activation of Bax, p53, and caspases (9, 35). One of the most widely studied apoptotic effects of As₂O₃ is induction of oxidative stress via generation of H₂O₂ and ROS (36–38). In the present study, we found that As₂O₃ treatment of glioma cells leads to ROS

generation, as shown using the fluorescent dyes, H₂DCF-DA, and dihydroethidium. Notably, however, the generated ROS was not required for As₂O₃-induced DR5 up-regulation or for apoptosis induced by As₂O₃/TRAIL cotreatment. Recently, Liu et al. reported that combined treatment with As₂O₃ and TRAIL synergistically induces apoptosis in myeloma cells, accompanied by an increase in the surface expression of the TRAIL death receptors DR4 and DR5 (39). In addition, Szegezdi et al. reported that As₂O₃ sensitizes leukemic cells, but not several types of solid tumor cells (including PC3 prostate, HeLa cervical, and Colo205 colon cancer cells), to TRAIL-mediated apoptosis (28). The same study also showed that

As₂O₃-sensitized TRAIL-induced leukemic cell apoptosis was associated with Akt inhibition, down-regulation of c-FLIPs, and increased cell surface expression of DR5. In the present study, Akt inhibition or c-FLIPs down-regulation was not commonly observed in As₂O₃-treated glioma cells, demonstrating the differences between the As₂O₃-induced signaling pathways in leukemic and glioma cells. However, we did observe significant up-regulation of DR5, not only at its cell surface expression but also at its mRNA and total protein levels, in various glioma cells. Given that TRAIL is known to trigger apoptosis through binding to DR4 and DR5 (4), the expression levels of these death receptors may play a critical role in determining the intensity and/or duration of death receptor-mediated apoptotic signaling in response to TRAIL. In the present study, we found that transfection with siRNA against DR5 efficiently reduced the cell death induced by the combined

treatment, demonstrating that DR5 plays a critical role in As₂O₃-sensitized TRAIL-induced apoptosis in glioma cells.

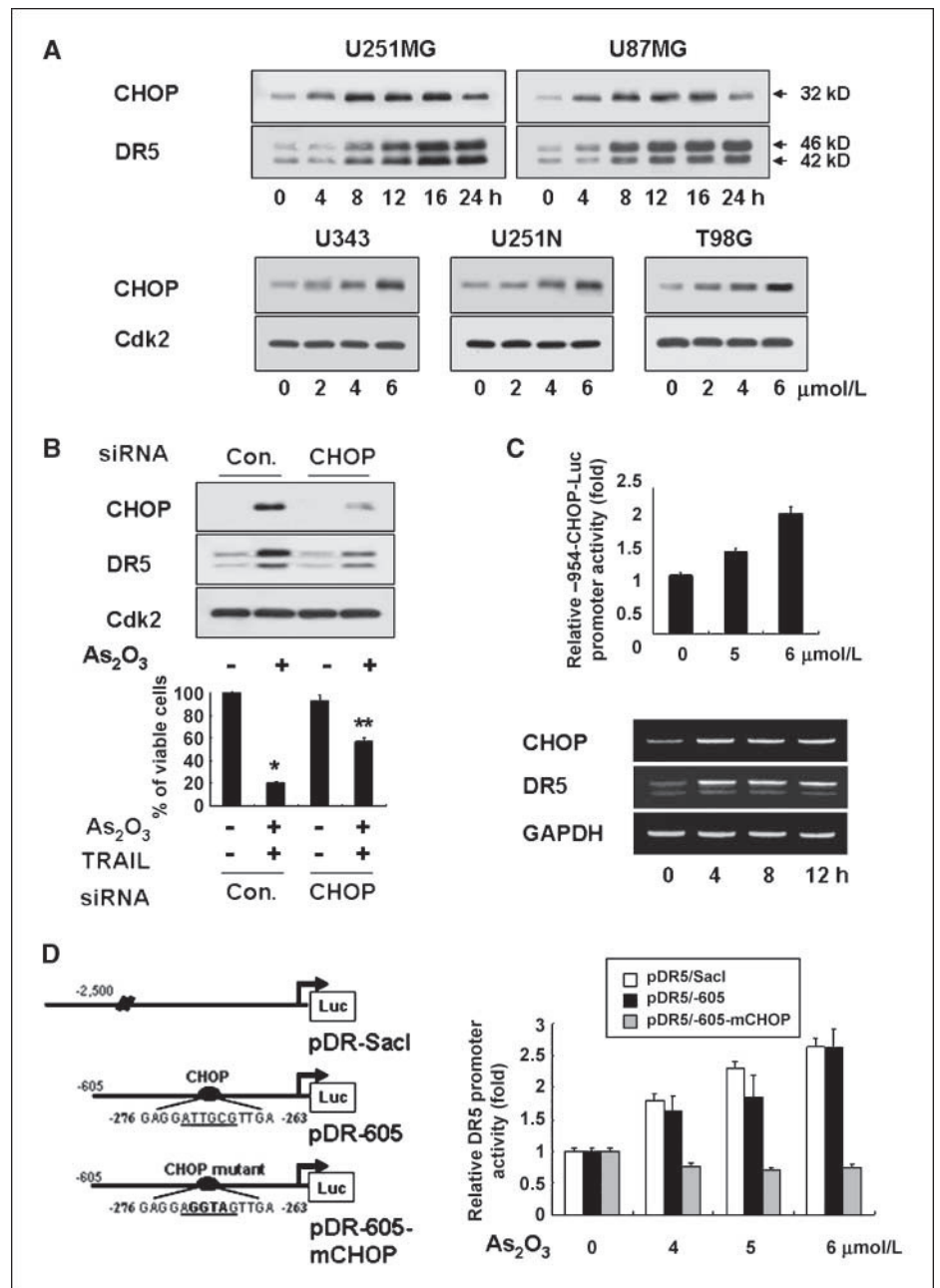
Notably, we also found that the CHOP transcription factor is critical for As₂O₃-induced DR5 up-regulation, as indicated by three lines of evidence: (a) As₂O₃-induced DR5 up-regulation is preceded by up-regulation of CHOP at the mRNA and protein levels; (b) As₂O₃-induced activation of DR5 promoter activity is abrogated by a mutation in the CHOP binding site of the DR5 promoter; and (c) siRNA-mediated CHOP knockdown significantly blocks As₂O₃-induced DR5 up-regulation and As₂O₃/TRAIL-mediated cell death. CHOP, which is also known as GADD153, is induced by DNA damage-inducing stimuli (40) and is one of the most highly induced genes during endoplasmic reticulum stress (41). However, in the present study, we did not detect DNA damage responses, such as phosphorylation of γ -H2AX (42) or induction of GADD34

Figure 5. CHOP mediates DR5 up-regulation at the transcriptional level in human glioma cells.

A. As₂O₃ up-regulates CHOP in various glioma cells. Cell extracts were prepared from U251MG or U87MG cells treated with 5 μ mol/L As₂O₃ for the indicated time points. Western blotting of CHOP and DR5 was performed. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. To examine the effect of As₂O₃ in other glioma cells, U343, U251N, and T98G cells were treated with As₂O₃ at the indicated concentrations for 12 h. Western blotting of CHOP was performed, and Cdk2 was used for a loading control of Western blotting.

B. suppression of CHOP expression by siRNA reduces As₂O₃-induced DR5 up-regulation and As₂O₃-stimulated TRAIL-induced apoptosis in U87MG cells. U87MG cells were transfected with scrambled negative control RNA or siRNA duplexes against CHOP, incubated for 24 h, and further treated with 5 μ mol/L As₂O₃ alone for 12 h. First, Western blotting of CHOP was performed to confirm the down-regulation of CHOP by siRNA transfection (top). Western blotting of DR5 was also performed to examine the knockdown effect of CHOP on As₂O₃-induced DR5 up-regulation. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. To examine the effect of CHOP down-regulation on As₂O₃-sensitized TRAIL-induced apoptosis, transfected cells with scrambled negative control RNA or CHOP siRNA were treated with 5 μ mol/L As₂O₃ plus 100 ng/mL TRAIL for 24 h. Cellular viability was determined using calcein-AM and EthD-1 (bottom). Columns, average of three independent experiments; bars, SE. *, $P < 0.005$ compared with untreated cells; **, $P < 0.05$ compared with cells treated with As₂O₃ plus TRAIL.

C. As₂O₃ enhances the promoter activity and mRNA levels of CHOP. U87MG cells were transfected with the luciferase construct containing the CHOP promoter region (-954-CHOP-Luc) and then treated with As₂O₃ at the indicated concentrations for 8 h, lysed, and assayed for luciferase activity. Columns, mean of at least three independent experiments; bars, SD (top). Total RNA was isolated from U87MG treated with 5 μ mol/L As₂O₃ for the indicated time points, and RT-PCR analysis of DR5, CHOP, and GAPDH was performed (bottom). **D.** effect of As₂O₃ on DR5 promoter activity. Schematic structures of the DR5 promoter constructs used for the luciferase activity assays (left). U87MG cells were transfected with pDR5/SacI, pDR5/-605, or CHOP-mutated pDR5/-605 and then treated with varying concentrations of As₂O₃, lysed, and assayed for luciferase activity (right). Columns, mean of at least three independent experiments; bars, SD.



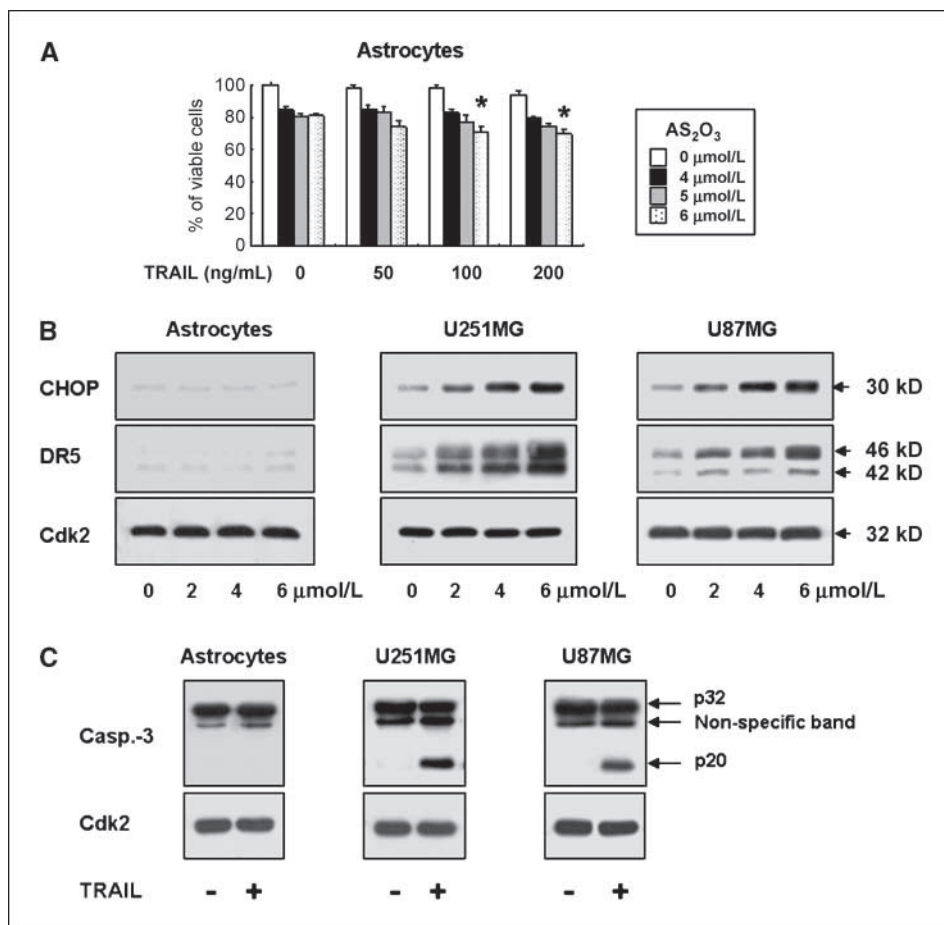


Figure 6. Neither As₂O₃-mediated CHOP and DR5 up-regulation nor TRAIL-mediated partial proteolytic processing of caspase-3 is induced in human astrocytes, which are resistant to the combined treatment with As₂O₃ and TRAIL. *A*, human astrocytes are resistant to the combined treatment with As₂O₃ and TRAIL. Human astrocytes were treated with As₂O₃ for 30 min and further treated with TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1. *Columns*, average of three independent experiments; *bars*, SE. *, *P* < 0.005 compared with untreated cells. *B*, astrocytes, U251MG and U87MG cells were treated with As₂O₃ at the indicated concentrations for 12 h, and cell extracts were prepared for Western blotting of CHOP and DR5. Equal loading of the protein samples was confirmed by Western blotting of Cdk2. *C*, TRAIL-induced partial proteolytic processing of caspase-3 does not occur in astrocytes. Astrocytes, U251MG, and U87MG cells were treated with 100 ng/mL TRAIL for 24 h, and cell extracts were prepared for Western blotting of caspase-3. Western blotting of Cdk2 served as a loading control of the samples.

(43) after As₂O₃ treatment (data not shown), possibly due to the use of subtoxic doses of As₂O₃. In addition, we did not detect endoplasmic reticulum stress response-associated changes (44), such as up-regulation of GRP78, GRP94, or activating transcription factor-4, phosphorylation of eIF2α, or splicing of XBP-1 in As₂O₃-treated glioma cells, contrastingly with those in thapsigargin-treated cells (Supplementary Fig. S2). These results collectively suggest that the As₂O₃-induced up-regulation of CHOP and subsequent induction of DR5 do not involve DNA damage or endoplasmic reticulum stress response.

One of the critical factors determining the success of cancer therapy is the ability to selectively kill malignant cancer cells while sparing normal cells. In addition, successful chemotherapy relies on the proper choice and combination of anticancer drugs. Thus, it is necessary to identify combined treatments that yield maximal synergistic death-inducing effects in cancer cells and only minimal side effects in normal cells. Here, we found that normal astrocytes were very resistant to the combined treatment with As₂O₃ and TRAIL, whereas glioma cells underwent rapid apoptosis in the presence of these drugs. Although further preclinical and animal studies will be required to support the clinical application of As₂O₃/TRAIL cotreatment, our results suggest that this combined treatment may offer a safe and attractive strategy for treating malignant gliomas.

Notably, As₂O₃ treatment results in a CHOP-mediated DR5 up-regulation in glioma cells but not normal astrocytes, potentially forming the mechanistic basis for the glioma-selective sensitizing

effect of As₂O₃ on TRAIL-mediated apoptosis. Consistent with this hypothesis, Song et al. have recently reported that the resistance of human astrocytes to TRAIL-induced apoptosis may be due to their low expression levels of DR4 and DR5 (45). Similarly, we found that the basal expression levels of DR5 were much higher in various glioma cells compared with normal astrocytes (Supplementary Fig. S3). However, TRAIL resistance is also seen in a variety of glioma cell types, suggesting that the TRAIL resistance of glioma cells is likely to involve other intracellular signaling molecules.

In various glioma cells, we observed that the 32-kDa procaspase-3 was partially cleaved to a 20-kDa intermediate after TRAIL treatment. However, further cleavage into the active p17 subunit did not occur as shown in Fig. 1C, suggesting that TRAIL can prime but not complete the proteolytic processing of procaspase-3 in these cells. We did not detect proteolytic processing of other caspases in TRAIL-treated glioma cells, suggesting a failure in the subsequent activation of the caspase cascade. The protein levels of c-FLIP, which is also known to confer TRAIL-resistance to certain types of cancer (46), varied across the tested cell lines (Supplementary Fig. S3). However, the protein levels of XIAP and survivin were significantly elevated in all tested glioma cells compared with astrocytes. Therefore, consistent with our previous proposition (8), our results suggest that elevated levels of inhibitors of apoptosis, such as XIAP or survivin, may help block processing of the caspase-3 p20 intermediate form to the p17 subunit in TRAIL-treated glioma cells.

Whereas TRAIL may act to prime the proteolytic processing of procaspase-3, As₂O₃ triggers up-regulation of DR5, leading to

stimulation of the death receptor-mediated apoptotic signaling pathway and subsequent completion of procaspase-3 processing. Thus, TRAIL-induced priming of procaspase-3 processing may sensitize glioma cells to As₂O₃ by reducing the apoptotic threshold, hence overcoming the TRAIL-resistance of some glioma cells. In contrast, normal astrocytes are doubly protected from TRAIL-mediated apoptosis, both by the lack of CHOP-mediated DR5 up-regulation by As₂O₃ and by the lack of TRAIL-mediated priming of procaspase-3 processing.

In summary, we herein show that glioma cells may be preferentially sensitized to TRAIL-induced apoptosis via DR5 up-regulation.

TRAIL-based therapy using DR5-up-regulating sensitizers, such as As₂O₃, may offer a promising new strategy for selectively killing malignant glioma cells while sparing normal astrocytes.

Acknowledgments

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