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Dithiaarsanes Induce Oxidative Stress-Mediated Apoptosis in HL-60 Cells by Selectively Targeting Thioredoxin Reductase

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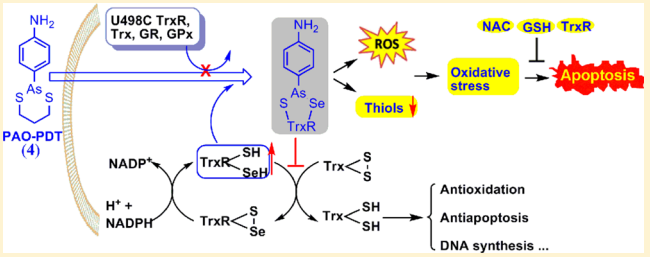
Supporting Information

ABSTRACT: The selenoprotein thioredoxin reductase (TrxR) plays a pivotal role in regulating cellular redox homeostasis and has attracted increasing attention as a promising anticancer drug target. We report here that 2-(4-aminophenyl)-1,3,2-dithiarsinane (PAO−PDT, **4**), a potent and highly selective small molecule inhibitor of TrxR, stoichiometrically binds to the C-terminal selenocysteine/cysteine pair in the enzyme in vitro and induces oxidative stress-mediated apoptosis in HL-60 cells. The molecular action of **4** in cells involves inhibition of TrxR, elevation of reactive oxygen species, depletion of cellular thiols, and activation of caspase-3. Knockdown of TrxR sensitizes the cells to **4** treatment, whereas overexpression of the functional enzyme alleviates the cytotoxicity, providing physiological relevance for targeting TrxR by **4** in cells. The simplicity of the structure and the presence of an easily manipulated amine group will facilitate the further development of **4** as a potential cancer chemotherapeutic agent.



1. INTRODUCTION

Arsenic is a semimetal or metalloid element widely distributed on earth. The majority of arsenic is found in minerals in conjunction with sulfur or metals. Arsenical-based molecules have been used as therapeutic agents for many centuries for various ailments such as psoriasis, syphilis, leukemia, and rheumatism.[1−3] However, concerns about their toxicity eventually led to their abandonment for the treatment of cancer before the clinical discovery of the favorable efficacy of arsenic trioxide (ATO, As_2O_3), which led to remission in patients with acute promyelocytic leukemia (APL) in the 1980s.[4] This old inorganic compound has achieved remarkable clinical success and has been validated as a front-line drug in the treatment of a number of malignant diseases, especially APL.[5] Organic arsenicals consist of an arsenic atom linked covalently to a carbon atom. The unique bonding property of carbon atom gives the diverse structure of organoarsenicals. Besides their structural diversity, organoarsenicals are often more stable and less toxic and have better bioavailability than inorganic arsenicals. Inspired by the success of ATO, many synthetic organoarsenicals or hybrid arsenics are currently under investigation for use in cancer treatment.[6−9]

The thioredoxin system, composed of thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH, is a highly conserved system and plays a crucial role in regulating a wide variety of redox signaling pathways involved in cell proliferation and death, transcription, DNA repair, angiogenesis, and embryogenesis.[10,11] Two major isoforms of TrxR/Trx are present in different intracellular organelles: TrxR1/Trx1 are predominantly localized in the cytosol and nucleus, and TrxR2/Trx2 are mainly localized within mitochondria. Despite the different localizations of the isoforms within cells, TrxR1 and TrxR2 have similar structures and share the same catalytic mechanism.[12] Compared to those from bacteria, mammalian TrxRs are large selenocysteine (Sec)-containing proteins with a uniform C-terminal sequence of -Gly-Cys-Sec-Gly, which is required for the activity of the enzyme.[13] The chemical property of Sec (selenol) resembles, but is generally more reactive than, that of Cys (thiol). Mammalian TrxR has broad substrate specificity, but it is the only known enzyme to reduce Trx in vivo. It was initially considered that TrxR maintains reduced Trx pools for ribonucleotide reductase in DNA synthesis[14] as well as for many antioxidant enzymes such as peroxiredoxins[15] and methionine sulfoxide reductases.[16,17] It is becoming increasingly clear that TrxR also plays critical roles in tumorigenesis, and accumulating studies suggest that TrxR might be a promising anticancer drug target.[18−20] Thus, expanding attention has been paid to the development and discovery of TrxR inhibitors as potential chemotherapeutic agents during recent years.[21−25]

Arsenic has two biologically important oxidation states, As(III) and As(V). Compared to the relatively inert property of As(V), As(III) easily reacts with closely spaced thiols, yielding stable cyclic dithioarsinite complexes in which both sulfur atoms are bound to arsenic. Most of the effects of arsenic compounds in cells have been through their ability to bind to thiols in cysteine residues.[26] Proteins whose function is dependent on one or both of the cysteine thiols are thus inactivated by the trivalent arsenicals. Also, many protein-labeling agents have been developed on the basis of the high affinity of trivalent

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arsenicals with vicinal dithiols.[27−30] Because the C-terminal redox center, -Gly-Cys-Sec-Gly, is indispensable for the activity of TrxR and because ATO was reported to be a potent inhibitor of TrxR,[31] we reasoned that trivalent arsenicals might be potent inhibitors of TrxR and thus might be potential cancer chemotherapeutic agents. As part of our continuing interest in discovering and developing novel small molecule regulators of cellular redox systems,[21,32−36] we report herein the synthesis, biological evaluation, and mechanism of action of phenylarsenic oxide derivatives (Figure 1). 2-(4-Aminophenyl)-1,3,2-dithiarsinane (PAO−PDT, **4**) stoichiometrically binds to the C-terminal selenocysteine/cysteine pair in TrxR in vitro. The inhibition of TrxR by **4** is highly selective because other related enzymes, such as the Sec-to-Cys mutant of TrxR (U498C TrxR), glutathione reductase (GR), glutathione peroxidase (GPx), and Trx, are not affected by **4**. TrxR inhibited by **4** is still functional as a pro-oxidant NADPH oxidase for the constant generation of reactive oxygen species (ROS). Compound **4** displays its highest cytotoxicity toward human promyelocytic leukemia HL-60 cells among all tested cell lines. Further mechanistic study indicates that the cellular action of **4** involves the inhibition of TrxR, accumulation of ROS, depletion of cellular thiols, and induction of apoptosis. Overexpression of functional TrxR confers cyto-protection from **4**, whereas knockdown of the enzyme enhances the cytotoxicity, demonstrating the physiological significance of targeting TrxR by **4** in cells. We expect that **4** could be a novel cancer chemotherapeutic agent for further development.

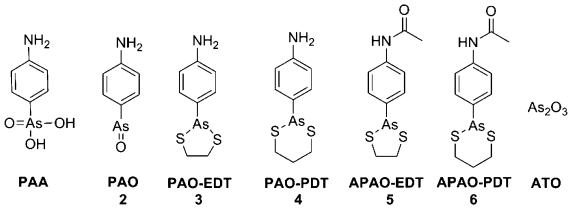
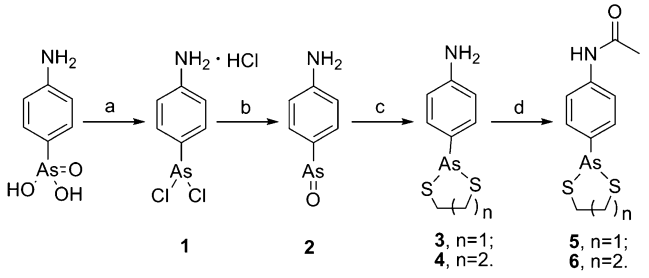


Figure 1. Chemical structures of organoarsenical compounds.

Scheme 1. Synthesis of Organoarsenical Compounds[a]

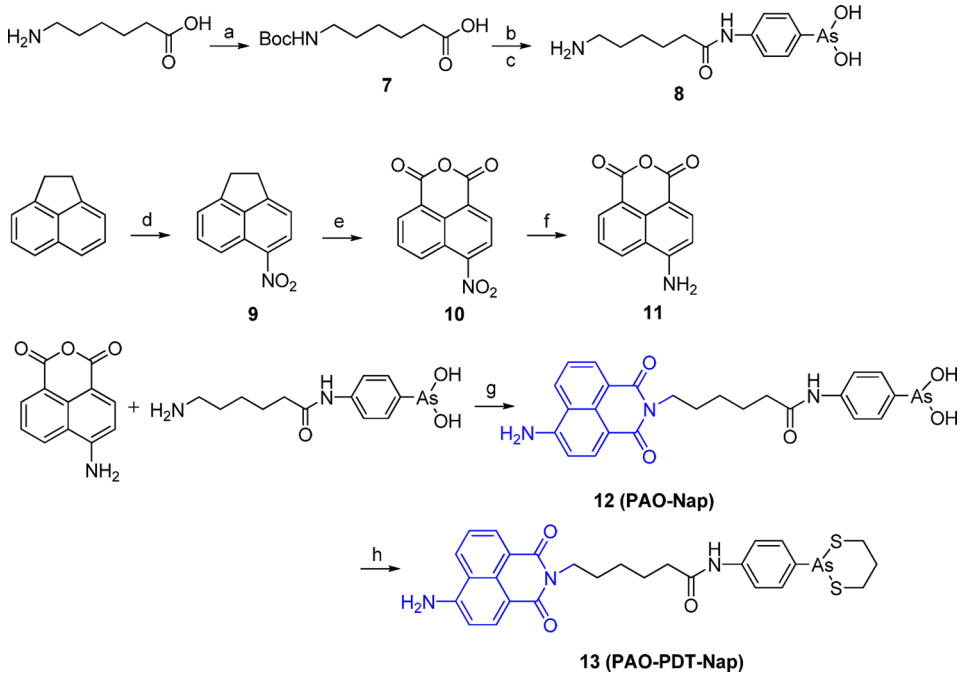


[a](a) MeOH, HCl, KI, SO_2, rt, 30 min; (b) 10% NH_4OH, rt, 56%; (c) MeOH, RSH, reflux, 70%; (d) DMF, CH_3COOH, EDCI, HOBT, 70%.

2. RESULTS AND DISCUSSION

2.1. Chemical Synthesis. The dithiaarsanes were synthesized as previously described[37,38] via coupling the appropriate dithiols with 4-aminophenylarsenoxide (PAO, **2**), which was obtained by the reduction of 4-aminophenylarsonic acid (PAA).[38,39] The dithiol ligands, ethane-1,2-dithiol (EDT) and propane-1,3-dithiol (PDT), react with **2**, affording 2-(4-aminophenyl)-1,3,2-dithiarsolane (PAO−EDT, **3**) and **4**, respectively. Further acetylation of **3** and **4** furnishes the corresponding acetylated compounds, 2-(4-acetamidophenyl)-1,3,2-dithiarsolane (APAO−EDT, **5**) and 2-(4-acetamidophenyl)-1,3,2-dithiarsinane (APAO−PDT, **6**). The synthetic route is illustrated in Scheme 1. The structures formed between trivalent arsenicals and dithiols are markedly more stable than that of the noncyclic products formed with monothiols. Indeed, our attempt to prepare acyclic dithiol arsenites from **2** and monothiols failed

Scheme 2. Synthesis of PAO−Nap (12) and PAO−PDT−Nap (13)[a]



[a](a) Boc_2O/1,4-diaxane, rt, 90%; (b) $Et_3N/ClCO_2Et/CH_3CN/NH_4OH$, rt, 50%; (c) TFA, rt, 60%; (d) $AcOH/HNO_3$, rt, 63%; (e) $Na_2Cr_2O_7$/AcOH, reflux, 76%; (f) $SnCl_2{\cdot}2H_2O$/HCl/EtOH, reflux, 79%; (g) DMF, 100°C, 33%; (h) PDT/EtOH, reflux, 86%.

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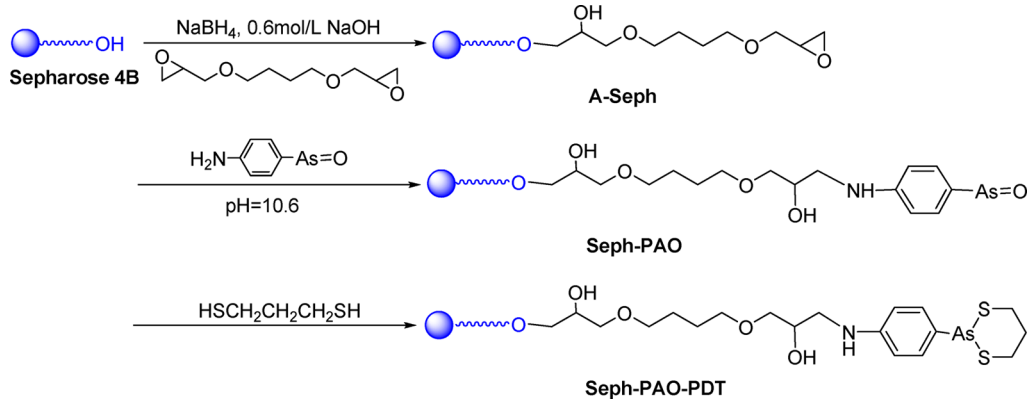
Scheme 3. Preparation of Immobilized Organoarsenical Compounds



because the acyclic compounds were unstable and spontaneously decomposed to the starting materials.[37] The fluorescence-labeled organoarsenical compounds were constructed by attaching a naphthalimide fluorophore using aminocaproic acid as a linker (Scheme 2).[29] The immobilized **4** was prepared by conjugation of the target molecule to sepharose 4B using 1,4-butanediol diglycidyl ether as a linker (Scheme 3).[40] The detailed synthetic procedures and characterization of **2** and its various derivatives are presented in the Supporting Information. The purity of the synthesized compounds was determined by HPLC. Except for the two labeling reagents, **12** (95.9% purity) and **13** (93.6% purity), all other compounds have purities >98% (Table S1).

2.2. Cytotoxicity Screening. Initial cytotoxicity screening is summarized in Table 1. PAA, a pentavalent arsenic compound, shows the least toxicity, whereas other trivalent arsenicals display high potency. This result agrees with previous results that indicated that pentavalent arsenicals are usually less toxic.[41,42] In addition, derivatization of **2** with dithiol ligands to cyclic dithiaarsanes **3** and **4** further improves the potency. Acetylation of the amino group, yielding **5** and **6**, does not significantly alter the activity. It appears that the six-membered dithiaarsanes (**4** and **6**) are more active than the five-membered ones (**3** and **5**). The most potent dithiaarsane, **4**, had a concentration causing half inhibition of cell proliferation (IC_{50}) of 0.7 and 0.6 μM after treatment of HL-60 cells for 48 and 72 h, respectively. Furthermore, **4** shows less toxicity toward two normal cell lines, L02 and HEK 293T cells, with IC_{50} values of 10.2 and 2.7 μM, respectively (Figure S1). Collectively, the results demonstrate that **4** displays selective cytotoxicity toward HL-60 cells with high potency. Thus, follow-up studies were mainly focused on compound **4**.

Table 1. Sensitivity of Different Types of Cancer Cell Lines to Arsenic Compounds[a]

	cell lines				
	HL-60		7721	HeLa	HepG2
compounds	48 h	72 h	48 h	48 h	48 h
PAA	>10	>10	>10	>10	>10
2	6.2 ± 1.5	5.1 ± 1.1	9.4 ± 0.2	7.7 ± 0.5	>10
3	2.5 ± 0.6	1.7 ± 0.4	9.9 ± 1.9	6.1 ± 1.5	>10
4	0.7 ± 0.1	0.6 ± 0.1	2.8 ± 1.0	3.4 ± 0.2	>10
5	2.5 ± 0.3	2.0 ± 0.2	9.0 ± 1.3	>10	>10
6	0.8 ± 0.4	0.7 ± 0.2	3.4 ± 0.9	4.6 ± 0.9	>10
ATO	6.5 ± 0.8	5.7 ± 1.3	>10	>10	>10

[a]The data (IC_{50}) were obtained by the MTT assay and are expressed as the mean ± SD of three independent experiments.

2.3. Selective Inhibition of TrxR in Vitro. Trivalent arsenicals easily react with closely spaced protein thiols. As the arsenic atom in **4** is already bound with PDT, we speculated that its cytotoxicity might be caused by the replacement of PDT by more reactive ligands present in the cells. In this sense, TrxR might be a candidate for interaction with **4** for the following reasons: (1) TrxR contains a highly reactive selenol group, (2) the enzyme has a C-terminal sequence of -Gly-Cys-Sec-Gly, providing a closely spaced selenol/thiol pair, and (3) this selenol/thiol pair is accessible because it is exposed on the surface of the enzyme. Indeed, **4** efficiently inhibits TrxR (Figure 2A). Next, we compared the inhibition potency of **4**

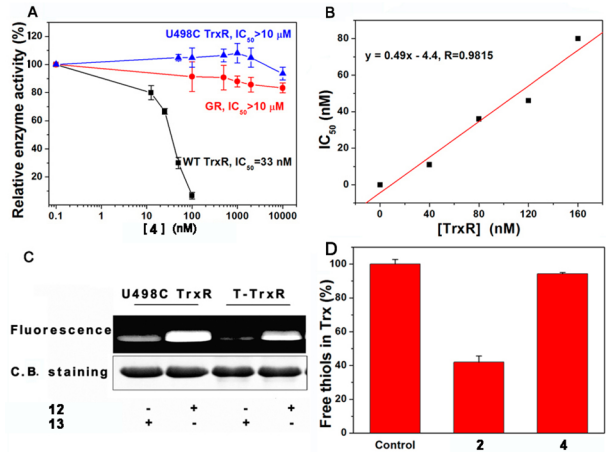


Figure 2. Selectively targeting TrxR by **4** in vitro. (A) Inhibition of WT TrxR, U498C TrxR, and GR by **4**. NADPH-reduced recombinant rat TrxR (80 nM), U498C TrxR1 (1.0 μM), and GR (0.25 U/mL) were incubated with the indicated concentrations of **4** for 30 min at room temperature, and the enzyme's activity was determined. All activity is expressed as the percentage of the control. Data are expressed as the mean ± SE of three independent experiments. (B) Correlation of the IC_{50} values of **4** and the TrxR concentrations. NADPH-reduced recombinant rat TrxR (40, 80, 120, and 160 nM) was incubated with different concentrations of **4** for 30 min at room temperature, and the enzyme activity was determined by the DTNB assay. (C) Labeling of different proteins by **12** and **13**. The reduced proteins were incubated with **12** or **13** for 30 min at room temperature. The samples were separated by SDS-PAGE. Top: Fluorescence imaging of the gel; Bottom: Commassie Blue (C. B.) staining of the gel. (D) Loss of free thiol groups in Trx after treatment with **4**. The remaining free -SH groups in the reduced Trx after treatment with **2** or **4** for 0.5 h at 37 °C were determined by DTNB titration. Data are expressed as the mean ± SE of three independent experiments.

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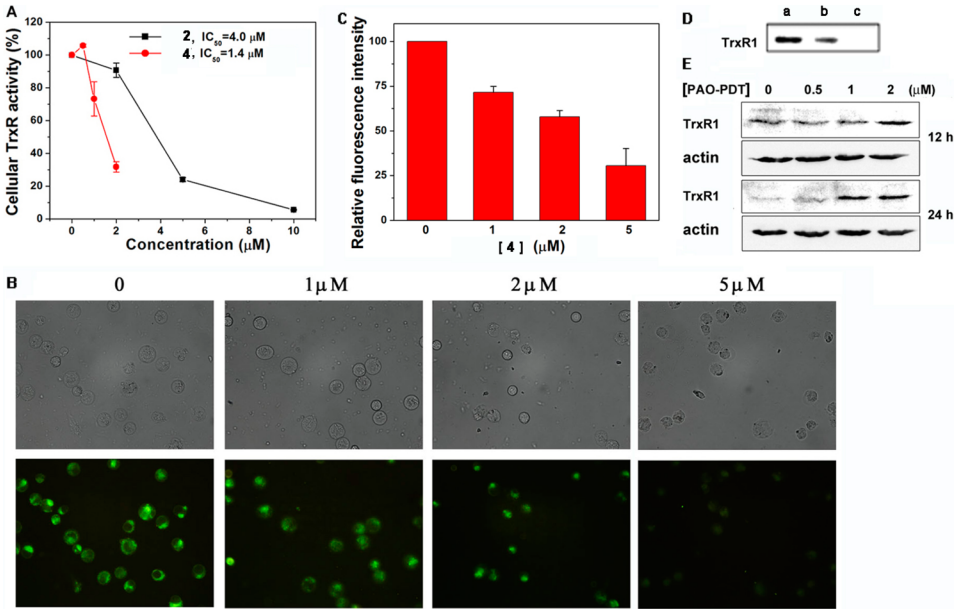

Figure 3. Interaction of **4** and TrxR in cells. (A) Inhibition of TrxR activity in HL-60 cells by **2** and **4**. Data are expressed as the mean ± SE of three experiments. (B) Imaging TrxR activity in living HL-60 cells. HL-60 cells were treated with the indicated concentrations of **4** for 8 h followed by further treatment with TRFS-green (10 μM) for 4 h. Phase-contrast (top) and fluorescence (bottom) images were acquired by fluorescence microscopy (Leica DMI4000). (C) Quantification of relative fluorescence intensity in individual cells. Ten cells were selected randomly, and the relative fluorescence intensity in individual cells in panel B was quantified using the Leica Qwin software accompanied by the Leica microscope. Data are expressed as the mean ± SE. (D) Compound **4** binds to cellular TrxR1. The HL-60 lysate was incubated with Seph−PAO−PDT or A-Seph on a rotator mixer for 30 min at room temperature. After centrifugation, the solution was removed, and the sepharose was washed and eluted with DMPS. The eluent was analyzed by western blotting (lane b). Lane a: positive control using HL-60 cell lysate for western blotting. Lane c: control using A-Seph instead of Seph−PAO−PDT. (E) Upregulation of TrxR1 protein expression in HL-60 cells. Cells were treated with the indicated concentrations of **4** for 12 and 24 h, and the cell extracts were prepared and analyzed by western blotting with an antibody against TrxR1. Actin was used as a loading control.

toward TrxR, GR, and U498C TrxR, where Sec498 was replaced by Cys (Figure 2A). Compound **4** effectively inhibits TrxR with an IC_{50} value around 36 nM, whereas it exhibits very weak inhibition of GR ($IC_{50} \gg 10$ μM) and even less of an effect on U498C TrxR. The interaction of **4** with GPx, a Sec-containing enzyme, was also investigated. No apparent inhibition of GPx was observed, even in the presence of 10 μM **4** (Figure S2). Analysis of the IC_{50} value of **4** (~36 nM) and the TrxR concentration (80 nM) suggest the binding of **4** to the enzyme in a 1:1 ration. We further determined the IC_{50} values of **4** toward varying concentrations of TrxR (Figure 2B). Fitting the IC_{50} values versus the enzyme concentrations gives a straight line ($r^2 = 0.9815$) with a slope of 0.49, supporting the idea that **4** stoichiometrically binds to TrxR. The selective inhibition of WT TrxR but not U498C TrxR or GR demonstrates that the Sec residue is crucial for the action of **4**. To further confirm the importance of the vicinal dithiol in binding **4**, we generated the truncated TrxR (T-TrxR) by deleting the C-terminal -Sec-Gly dipeptide. After preparing fluorophore-labeled **2** (**12**) and fluorophore-labeled **4** (**13**, Scheme 2), we performed a protein-labeling assay (Figure 2C). Both U498C TrxR and T-TrxR could be efficiently labeled by **12** (lanes 2 and 4 in Figure 2C). However, **13** could bind only selectively to U498C TrxR but not to T-TrxR (lane 1 in Figure 2C), a protein having no accessible vicinal dithiol. This observation, together with the potent inhibition of TrxR by **4**, indicates that **4** specifically interacts with the C-terminal redox center of TrxR. In addition, no apparent inhibition of Trx by **4**, but remarkable inhibition by **2**, was observed (Figure 2D). Coincubation of 1 mM GSH with 80 nM TrxR could not prevent inhibition of TrxR by 40 nM **4** (data not shown). Taken together, **4** selectively inhibits TrxR in vitro, and this inhibition is heavily relies on the Sec residue and the vicinal dithiol/selenol motif in the enzyme.

2.4. Interaction of 4 with TrxR in Cells. Treatment of HL-60 cells with **4** causes a remarkable decrease of cellular TrxR activity, with an IC_{50} value of around 1.4 μM, which is 3-fold lower than the value for **2** (4.0 μM, Figure 3A). **4** is the most potent TrxR inhibitor in HL-60 cells among all of the tested compounds (Figure S3), consistent with its highest cytotoxicity being toward HL-60 cells. Using our recently developed TrxR probe, TRFS-green,[32] inhibition of TrxR in living HL-60 cells was confirmed (Figure 3B,C). To further determine targeting TrxR by **4** in a cellular context, we performed a pulldown assay. After incubating the cell lysate with immobilized **4** followed by DMPS elution, the TrxR1 protein was unambiguously detected (Figure 3D, lane b). Incubation of the lysate with activated sepharose (A-Seph, Scheme 3) followed by the same treatment does not give any signal (Figure 3D, lane c). The cell lysate without any treatment was loaded as a positive control (Figure 3D, lane a). Interestingly, exposure of HL-60 cells to 2 μM **4** for 12 h or 1 μM **4** for 24 h significantly increased TrxR1 protein expression (Figure 3E), but the total TrxR activity in the cells remained inhibited to less than 30% of that of the control (data not shown). The elevation of the loss-of-function enzyme (i.e., **4**-modified TrxR) might contribute to the ROS generation in cells (vide infra).

2.5. ROS Production in Vitro and in Cells. Several TrxR inhibitors are known to modify the enzyme to shift it from being an antioxidant to a source of ROS;[34,36,43,44] thus, we

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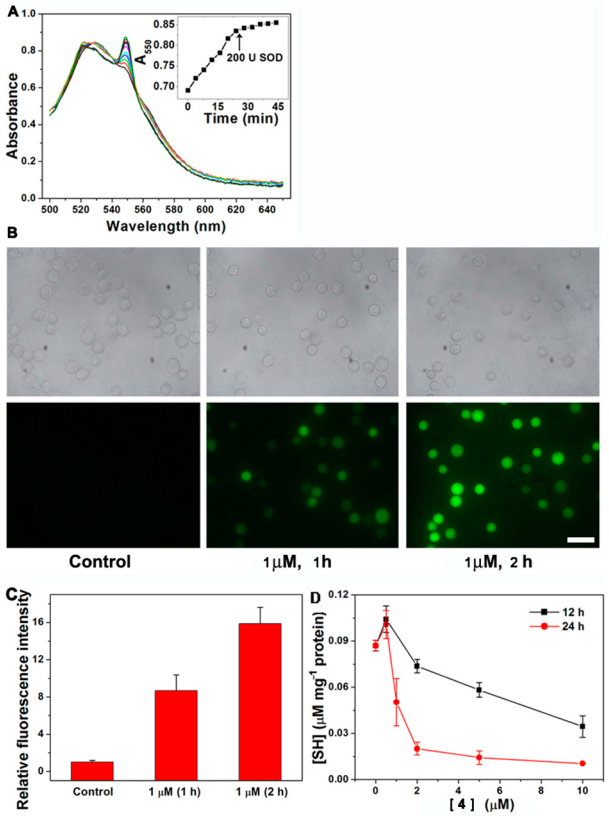

Figure 4. Elicitation of oxidative stress by **4**. (A) Induction of superoxide anions production by **4**-modified TrxR in vitro. **4**-modified TrxR was incubated with NADPH, and cytochrome c was added to monitor the production of superoxide anions. The inset shows the change of absorbance at 550 nm after addition of cytochrome c and superoxide dismutase (SOD). (B) Accumulation of ROS in the cells. HL-60 cells were treated with **4** for 1 or 2 h followed by incubation with DCFH-DA (10 μM) for an additional 30 min. Phase-contrast (top) and fluorescence (bottom) images were acquired by fluorescence microscopy (Leica DMI4000). Scale bar: 30 μm. (C) Quantification of fluorescence intensity in individual cells in panel B. The relative fluorescence intensity in individual cells was quantified using the Leica Qwin software accompanied by the Leica microscope. (D) Depletion of intracellular thiols by **4**. After the HL-60 cells were treated with **4**, total cellular thiols were quantified by DTNB titration. Data are expressed as the mean ± SE of three experiments.

determined if **4** has a similar effect. As shown in Figure 4A, **4**-modified TrxR displayed steady cytochrome c reduction activity in the presence of NADPH, and this cytochrome c reduction activity was inhibited by addition of SOD, indicating the production of superoxide anions by such a process. To extend this in vitro discovery, we also demonstrated the time-dependent generation of ROS in HL-60 cells after **4** stimulation (Figure 4B) by staining the cells with a ROS probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA).[33,36] The relative ROS production is quantified in Figure 4C. Compound **4** not only inhibits the physiological function of TrxR but also upregulats its expression in cells (Figure 3). As we demonstrated earlier, **4**-modified TrxR is still functional as a pro-oxidant to oxidize NADPH and constantly generate ROS in vitro; the elevation of the modified TrxR contributes, at least in part, to the ROS accumulation in HL-60 cells. Cellular thiols are endogenous antioxidants to neutralize ROS. As a consequence of TrxR inhibition and ROS accumulation, a drastic loss of cellular thiols was observed after treatment with **4** (Figure 4D).

2.6. Physiological Significance of Targeting TrxR by 4.

As we have demonstrated the selective interaction of **4** with TrxR in vitro, we further asked the physiological relevance of targeting TrxR by **4**. Pretreatment of HL-60 cells with NAC, a thiol antioxidant and GSH synthesis precursor in cells, confers cytoprotection against **4**-induced cell death, and higher concentrations of NAC almost completely rescue the cells (Figure 5A). **4**-induced cell death was blocked by NAC, but

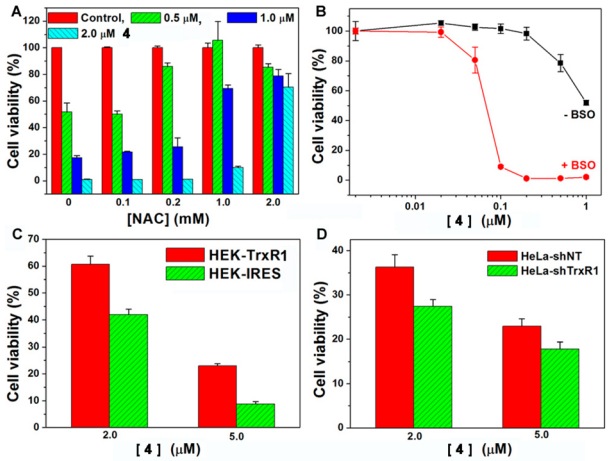

Figure 5. Physiological significance of targeting TrxR by **4**. (A) Protection of HL-60 cells by *N*-acetylcysteine (NAC). HL-60 cells (2×10^4 cells) were incubated with the indicated concentrations of NAC and **4** for 48 h. Cell viability was determined by the trypan blue exclusion assay. (B) Augmentation of the cytotoxicity by inhibition of GSH synthesis. HL-60 cells (1×10^4 cells) were treated with 100 μM buthionine sulphoximine (BSO) for 24 h to lower the intracellular GSH level, followed by **4** treatment for an additional 48 h. The viability was determined by the MTT assay. (C) Cytotoxicity of **4** on HEK-IRES and HEK-TrxR1 cells. The cells (1×10^4 cells) were treated with the indicated concentrations of **4** for 48 h, and the cell viability was determined by the MTT assay. (D) Cytotoxicity of **4** on HeLa-shNT and HeLa-shTrxR1 cells. The cells (1×10^4 cells) were treated with the indicated concentrations of **4** for 48 h, and the cell viability was determined by the MTT assay. All data are expressed as the mean ± SE of three experiments.

other general antioxidants, including vitamin C and vitamin E, did not prevent this process (data not shown), suggesting that the induction of cell death by **4** might be related to GSH depletion. Thus, we determined the effect of GSH on the cytotoxicity of **4**. Consistent with the protection by NAC, inhibition of cellular GSH synthesis by BSO remarkably enhances the cytotoxicity of **4** (Figure 5B). Under our experimental conditions, pretreatment of HL-60 cells with 100 μM BSO for 24 h downregulates the intracellular GSH level to less than 20% of that of the control (data not shown). GSH is a pivotal component of the glutathione network, which is another redox regulation system in cells besides the thioredoxin system, and also acts as a backup of the thioredoxin system.[45] Sensitizing the cells to **4** by the depletion of GSH supports the involvement of the thioredoxin system in the biological action of **4**.

To further disclose the involvement of TrxR in the cytotoxicity of **4**, we compared the sensitivity of HEK cells stably overexpressing TrxR1 (HEK-TrxR1) and control cells that were stably transfected with a vector (HEK-IRES) upon **4** treatment.[33,46] As shown in Figure 5C, **4** displays significantly higher cytotoxicity toward HEK-IRES cells than toward HEK-TrxR1 cells. To further address the physiological relevance of

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TrxR-mediated **4** cytotoxicity, we generated a cell line stably knocking down TrxR1 expression by transfection of shRNA specifically targeting the enzyme. Because of the low transfection efficiency of HL-60 cells, we chose HeLa cells for the knockdown experiments.[33,46] Importantly, **4** shows elevated cytotoxicity toward HeLa-shTrxR1 cells compared to that of the control cells (HeLa-shNT) (Figure 5D). The efficiency of TrxR expression as well as the enzyme activity in TrxR-overexpressing and knockdown cells is validated in Figure S5. Collectively, our results strongly support the idea that the biological action of **4** in cells is related to its interaction with TrxR.

2.7. Induction of Apoptosis in HL-60 Cells. Abrogation of apoptotic pathways is frequently found in malignant cells, which arises from a complex interplay of genetic aberrations and misregulated death pathways.[47] Arsenic compounds are known to activate apoptotic signaling in numerous types of cells.[48−51] Herein, we also demonstrated that **4** kills HL-60 cells predominantly through the induction of apoptosis. The results from the Annexin-V−FITC/PI double staining (Figure 6A,B) indicate that **4** triggers apoptotic cell death in a manner that is both dose- and time-dependent. Under all circumstances in our experiments, the population of necrotic cells (FITC-negative and PI-positive cells) was always as low as that of the control. Caspase-3 is a crucial component of the apoptotic machinery in different cell types, and the activation of caspase-3 is a central event in the process of apoptosis.[52] Thus, we determined the cellular activity of caspase-3 after cells were treated with **4**. Compound **4** treatment significantly increased caspase-3 activity in HL-60 cells (Figure 6C). When HL-60 cells were incubated with **4** followed by Hoechst staining, the majority of the cells displayed condensed and highly fluorescent nuclei, a characteristic morphology of cells undergoing apoptosis (Figure 6D). Taken together, our data reveal that **4** mainly induces apoptotic cell death in HL-60 cells.

The biological importance of inhibition of TrxR is at least 3-fold. First, the inhibition of TrxR reduces the availability of reduced Trx, leading to a decrease in the activity of many antioxidant enzyme systems that rely on Trx as an electron donor, which eventually results in an accumulation of ROS and the collapse of the cellular redox balance.[53] Second, reduced Trx directly interacts with various apoptosis-related enzymes, such as ASK1,[54] procaspase 3,[55] and NF-κB,[56] to suppress apoptosis; thus, inhibition of TrxR would be expected to promote apoptosis. Third, TrxR inhibition could result in the formation of selenium compromised thioredoxin reductase-derived apoptotic proteins (SecTRAPs),[57,58] in which the Sec residue in the active site is modified by electrophiles. These SecTRAPs lose their physiological function (i.e., the ability to reduce oxidized Trx), but they maintain NADPH oxidase activity for the generation of ROS, which finally contributes to increased intracellular oxidative stress. The elevation of loss-of-function TrxR1 by **4** might lead to more SecTRAPs in HL-60 cells. Taken together, inhibition of TrxR by **4** thus leads to oxidative stress and promotes apoptosis.

Because of the high affinity of transition metals with thiol/selenol, a vast amount of transition metal complexes have been developed as TrxR inhibitors.[21−23,59−64] These complexes usually display high potency in the inhibition of TrxR in vitro. The small organic molecule **4** quantitatively binds to TrxR, yielding comparable or superior potency to that of those metal complexes. Despite the toxicity, arsenicals remain of high interest in the treatment of various diseases.[6,8,37] Arsenoxide is the active form of most trivalent arsenicals. However, the high polarity and easy

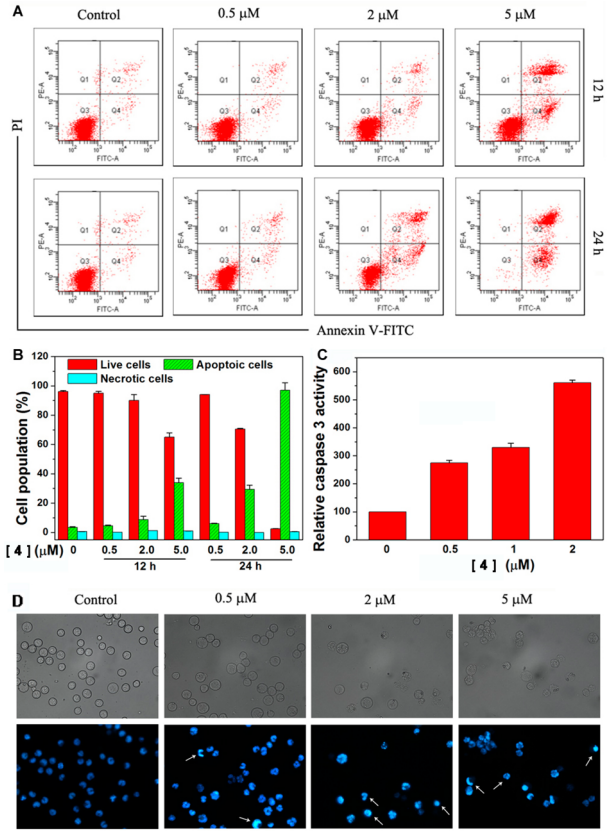

Figure 6. Induction of apoptosis in HL-60 cells. (A) Analysis of apoptosis by Annexin-V/PI double-staining assay. The cells show four different populations marked as follows: double-negative (unstained) cells are live cells (lower left, Q3), Annexin-V-positive and PI-negative cells are in early apoptosis (lower right, Q4), Annexin-V/PI double-stained cells are in late apoptosis (upper right, Q2), and PI-positive and Annexin-V-negative cells are necrotic (upper left, Q1). (B) Quantification of apoptosis by Annexin-V/PI double-staining assay. Cells in Q1 and Q3 were considered necrotic and live, respectively. The cells in Q2 and Q4 were considered apoptotic. (C) Activation of caspase-3 by **4**. HL-60 cells were incubated with the indicated concentrations of **4** for 24 h, and cellular caspase-3 activity was determined by a colorimetric assay. All data are expressed as the mean ± SE of three experiments. (D) Analysis of apoptosis by nuclear condensation. Hoechst 33342 staining showed typical apoptotic morphology changes after treatment with **4**. HL-60 cells were incubated with different concentrations of **4** for 24 h followed by Hoechst 33342 staining. Phase-contrast (top panel) and fluorescence (bottom panel) images were acquired by fluorescence microscopy.

oxidability hinder its bioavailability. Introducing the dithiol moiety might be an efficient way to improve these drawbacks.[29,37,65] Furthermore, the dithiol ligands also contribute to increasing the specificity of the drugs, as **2** nonselectively binds to vicinal dithiols or thiol/selenols, whereas **4** appears to be higher in affinity than **2**. The cytotoxicity of PAO−dithiol complexes is also dependent on dithiol ligands: the six-membered dithiaarsane (**4**) gives stronger potency than the five-membered one (**3**). Collectively, our preliminary structure−activity relationship results may shed light on modifying melarsoprol and GSAO, two organic arsenicals currently under clinical trials, to achieve better efficacy. The observation that dithiol compounds at low concentrations increase arsenite toxicity[66] could also be explained by our discoveries: dithiols complex with arsenites to improve the bioavailability and the specificity, thus enhancing the toxicity.

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Although ATO has demonstrated great achievements in the treatment of APL, clinical outcomes of this inorganic drug in other tumors have been poor, mainly due to limited bioavailability and severe side effects of the drug.[2,8,67] We demonstrated that **4** shows better potency than ATO in the present study and clarified the mechanism underlying the anticancer activity of **4**. Furthermore, **4** is stable in cell culture medium or phosphate buffered saline, and no significant loss of **4** was observed after 12 h (Figures S6 and S7). We expect that **4** could be a novel cancer chemotherapeutic agent for further development.

3. CONCLUSIONS

We discovered and fully elucidated the anticancer mechanism of organoarsenic compound **4**. **4** potently and selectively inhibits TrxR via stoichiometrically binding to the C-terminal Sec/Cys pair in the enzyme in vitro. The molecular mechanism of **4** in cells involves upregulation but inhibition of TrxR, elevation of ROS, depletion of cellular thiols, and eventual induction of oxidative stress-mediated apoptosis. Knockdown of TrxR enhances the cytotoxicity of **4**, whereas overexpression of the functional enzyme confers cytoprotection, providing physiological significance to the interaction of TrxR with **4** in cells. The novel targeting mechanism and the simplicity of the structure with an easily manipulated amine group make **4** a promising scaffold for further development in discovering potential cancer chemotherapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and characterization of organoarsenical compounds, experimental procedures for recombinant proteins preparation, cytotoxicity evaluation, enzyme activity assays, pull-down assay, western blotting, ROS detection, cellular thiols measurement, and apoptosis assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

APL, acute promyelocytic leukemia; BSO, buthionine sulphoximine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; GPx, glutathione peroxidase; GR, glutathione reductase; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; Sec, selenocysteine; SecTRAPs, selenium compromised thioredoxin reductase-derived apoptotic proteins; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase

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