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Communication

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An Arsenical–Maleimide for the Generation of New Targeted Biochemical Reagents

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Supporting Information

ABSTRACT: The finding that arsenic trioxide is an effective treatment for acute promyelocytic leukemia has renewed interest in the pharmacological uses of inorganic and organic arsenicals. Here we synthesized and characterized the reactivity of an arsenical–maleimide (As-Mal) that can be efficiently conjugated to exposed cysteine residues in peptides and proteins with the ultimate goal of directing these As(III) species to vicinal thiols in susceptible targets within cells and tissues. As-Mal conjugated to a surface cysteine in thioredoxin provides a more potent inhibitor for *Escherichia coli* thioredoxin reductase than comparable simple inorganic or organic arsenicals. As-Mal can be coupled to all of the eight cysteine residues of reduced unfolded ribonuclease A or to site-specific locations using appropriate cysteine mutations. We observed particularly strong binding to the two CxxC motifs of protein disulfide isomerase using a mutant RNase in which As-Mal was specifically incorporated at residues 26 and 110. As-Mal will serve as a facile reagent for the incorporation of As(III) species into a wide range of thiol-containing proteins, biomaterials, and surfaces.

Inorganic arsenicals, including the sulfur-containing minerals realgar and orpiment and the oxide, As_2O_3, have been used for millennia to treat a wide range of medical conditions, including leukemia, skin cancers, and solid tumors.[1,2] In the early 1900s, Ehrlich and Hata surveyed a range of organoarsenicals as antisyphilitics and introduced arsphenamine as the first rational chemotherapeutic. The related melarsen oxide (**1**) (Figure 1) and its derivatives have been widely used as antitrypanosomals.[1,2] While the medical applications of arsenicals declined in the 1940s with the development of antibiotics and other modern therapies, they are now the subject of renewed interest following the demonstration that As_2O_3 is remarkably effective as a treatment for acute promyelocytic leukemia.[3,4] In addition to inorganic arsenicals, several organoarsenicals are now in clinical trials for the treatment of leukemias and solid tumors.[2,5]

There is general agreement that the biological effects of As(III) species largely reflect their coordination to vicinal thiols.[1,6] Hence a continuing challenge is the efficient targeting of arsenicals while minimizing extraneous labeling and the toxicity associated with the many off-target thiol-containing proteins. For example, compound **2** (Figure 1) uses the tripeptide glutathione (GSH) as a vehicle for arsenical delivery via conjugation with 4-(2-bromoacetylamino)phenylarsonous acid.[2,7] However, the bromoacetyl function reacts rather slowly under conditions typically used in thiol bioconjugation reactions. Hence, we sought a simple arsenical reagent that could be introduced rapidly and stoichiometrically at cysteine residues on peptides and proteins. We chose maleimides because they exhibit high specificity for thiols, react several orders of magnitude more rapidly than bromoacetamide derivatives,[8,9] and have been widely used to conjugate cytotoxic agents to cysteine residues on monoclonal antibodies.[10−12] Here we show selective and efficient incorporation of an arsenical–maleimide reagent into peptides and proteins and demonstrate that these conjugates are more effective inhibitors than simple arsenical derivatives. This new arsenical–maleimide will also allow the facile preparation of designed arsenical materials, including resins for thiol-based chromatography.

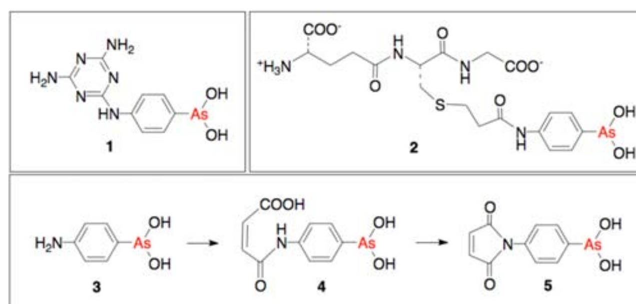

Figure 1. (top) Structures of melarsen oxide (**1**) and 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid (**2**). (bottom) Synthesis of the arsenical–maleimide 4-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)phenylarsonous acid (**5**) from **3**.

Figure 1 shows the synthesis of the arsenical–maleimide 4-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)phenylarsonous acid (As-Mal, **5**) in an overall yield of 76% via the cyclization of maleamic acid **4** [Figure S1 in the Supporting Information (SI)]. As-Mal shows two modes of reaction with thiols: one via Michael addition at the maleimide and the other through coordination at the As(III) center. A titration of As-Mal with GSH showed a decline in the absorbance at 320 nm (with a sharp end point at GSH/As-Mal = 1.0; Figure S2). These data are consistent with comparable experiments with *N*-phenylmaleimide (data not shown) and reflect rapid thiol–maleimide conjugation without undue interference from reversible binding to the arsenical moiety. The reaction between 100 μM As-Mal and equimolar GSH at pH 7.5 and 25 °C was essentially

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complete in 40 s (half complete in 2.2 s, comparable to the behavior of *N*-phenylmaleimide under these second-order conditions; Figure S3).

These experiments suggested that As-Mal should react rapidly with protein thiols provided that they are solvent-accessible. We first demonstrated placement of a single As-Mal group at the surface of a folded protein. Here, *Escherichia coli* thioredoxin, an oxidoreductase containing a redox-active pair of cysteine residues (C32 and C35), was mutated to leave the surface-accessible C32 available for conjugation. Treatment of this C35S mutant with 1 equiv of As-Mal rapidly generated a monolabeled derivative (at 100 μM concentrations, the reaction was half complete in <5 s; Figure S4). Monoalkylation was confirmed by the mass spectrum (Figure S5), the loss of the single 5,5′-dithiobis(2-nitrobenzoate) (DTNB)-reactive thiol group, and titration of the conjugated arsenical reagent with dithiothreitol (Figures S6 and S7).

Arsenical−peptide/protein conjugates might be expected to provide more specificity toward their targets than the parent arsenical reagent alone. The first confirmation of this was obtained using a thioredoxin (Trx) reductase from *E. coli*.[13] The flow of reducing equivalents from NADPH to Trx is schematically depicted in Figure 2A. After the CxxC motif on

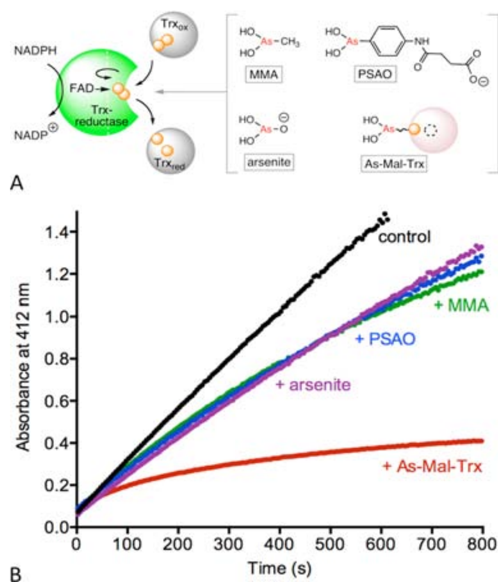

Figure 2. Inhibition of thioredoxin reductase by arsenicals. (A) Reduction of Trx by NADPH catalyzed by thioredoxin reductase, together with the arsenicals tested at 1 μM. (B) DTNB reoxidation of reduced Trx formed in the absence or presence of arsenicals (see the SI).

the reductase has received a pair of reducing equivalents from the FAD moiety, a conformational change leads to formation of a mixed disulfide with Trx docked against the reductase and then to the eventual release of reduced Trx.[13,14] Figure 2B shows that at a concentration of 1 μM, the simple arsenicals sodium arsenite, monomethylarsenous acid (MMA), and *p*-succinylamidophenylarsenoxide (PSAO) provided only modest inhibition of this bacterial thioredoxin reductase. However, at the same concentration, the As-Mal−Trx conjugate was considerably more potent, with a rapid and progressive loss of enzymatic activity.

We next wanted to test arsenical−protein conjugates as potential inhibitors of protein disulfide isomerase (PDI). PDI has recently been suggested as a target for antiviral,[15] antithrombotic,[16] and anticancer therapies.[17] Human PDI is composed of four thioredoxin domains, with the **a** and **a′** domains carrying CxxC motifs that are responsible for the varied oxidoreductase and isomerase activities of the enzyme.[18,19] PDI proteins have multiple intracellular roles in eukaryotes. However, they are also found at the cell surface, where they can modulate extracellular redox poise[20−22] and are involved in platelet activation,[20,23,24] enhancing metastasis,[25,26] and viral fusion[22,27] and where they might be targeted by protein-based inhibitors and antibodies. Typically, the substrates of PDI family members are proteins that retain disordered or conformationally mobile regions.[18,19] A widely employed substrate of PDI is reduced, or disulfide-mispaired, pancreatic ribonuclease A (RNase), and hence, we used this protein as an initial vehicle for arsenical conjugation. Reduction of the four native disulfides of RNase leads to a tractable unfolded protein that can be labeled with eight As-Mal moieties (Figure S8). However, here we prepared a limited series of site-directed mutants, including those in which only cysteines 1 and 4, 4 and 8, or 1 and 8 were retained in the sequence. These were subsequently labeled with As-Mal (Figure 3A; also see the SI and Figures S9 and S10).

The widely used insulin reductase assay was chosen to assess these inhibitors.[28,29] Here PDI catalyzes the reduction of insulin disulfides driven by the water-soluble phosphine TCEP, and the accumulation of the isolated B chain is followed by light scattering. In the absence of an As-Mal-RNase derivative, the onset of turbidity occurred at ∼230 s, whereas it was strongly suppressed by the 1,8-bis-As-Mal-RNase and to a lesser extent with 1,4- and 4,8-bis-As-Mal-RNase (Figure 3E). For perspective, the monoarsenical 4-As-Mal-RNase was considerably less effective than 1,8-bis-As-Mal RNase, and the 1,4,8-tris-As-Mal RNase was roughly comparable to the 1,8 derivative (Figure 3E). To confirm that inhibition reflected the conjugation of arsenicals to RNase, we evaluated the effect of the parent unconjugated cysteine RNase mutant proteins and found no significant inhibition of insulin reduction (Figure S11). Furthermore, the simple monoarsenical PSAO failed to delay the onset of turbidity significantly at a concentration of 10 μM (Figure 3E). Thus, the placement and spacing of arsenicals along a protein chain can be exploited to modulate the inhibitory potency.

Figure 3C shows a spectrophotometric titration of 1,8 bis-As-Mal-RNase with reduced PDI, in which the increase in absorbance at 300 nm accompanying thiol coordination to hydrated As(III) species was followed. The data were fit to a K_d of 22 ± 7 nM with a stoichiometry of 1.21 ± 0.16 RNase per PDI. These data are consistent with the complexation of both the **a** and **a′** CxxC motifs of reduced PDI with 1,8-bis-As-Mal-RNase (Figure 3B). For comparison, a direct assessment of the binding of PSAO to reduced PDI showed much weaker binding with a K_d of 1.1 μM,[29] consistent with failure to impact the insulin reductase assay significantly. Finally, since the levels of GSH in the extracellular matrix reach ∼10 μM,[30,31] we included GSH in the assay at this concentration and found that it did not significantly attenuate the inhibition observed with 1,8-bis-As-Mal-RNase. In contrast, intracellular concentrations of GSH are in the 0.5−10 mM range,[32] and we found that inclusion of 5 mM GSH provides insignificant inhibition of PDI by these arsenicals (data not shown).

In summary, this work provides a simple way to conjugate arsenicals to cysteine-bearing peptides and proteins and

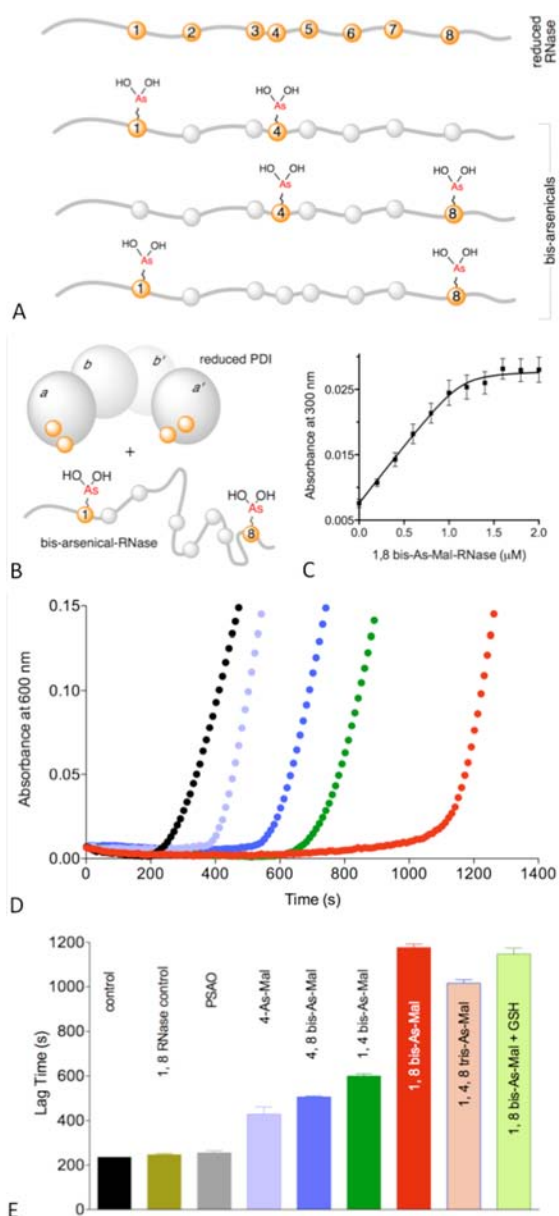

Figure 3. RNase-based arsenicals. (A) The 124-residue native chain of reduced RNase, showing cysteine residues at positions 26, 40, 58, 65, 72, 84, 95, and 110 (labeled, for clarity, as cysteines 1−8). Below the native chain are shown sextuple cysteine-to-serine mutants of RNase, allowing arsenicals to be installed with a range of spacings (here 1−4, 4−8, and 1−8). (B) Domain structure of reduced PDI with CxxC motifs in the **a** and **a**′ domains and the structure of 1,8-bis-As-Mal-RNase. (C) Representative data for titration of 1,8-bis-As-Mal-RNase with reduced PDI. (D) Comparison between mono- and bis-As-Mal-RNase derivatives in the insulin reductase assay using the same colors as shown in the bar graph of lag times in (E). The inhibitor concentrations were chosen to reflect a total of 10 μM arsenic in the assay.

demonstrates that the resulting adducts are more potent inhibitors than simple monoarsenical derivatives. Arsenicals might be delivered intracellularly via cell-penetrating peptides[33] or thiol-bearing dendrimers[34] for targeting species that bind arsenicals avidly in competition with GSH. While maleimide−thiol conjugates can be subject to reverse Michael reactions over extended times in physiological media, this is not necessarily a disadvantage because it provides the potential for targeted, controlled release of cytotoxic species.[35] Finally, the facility of the conjugation chemistry described here could allow multiple peptidic and protein scaffolds to be screened prior to manipulation of linker stability.[11,36]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and Methods, synthesis of As-Mal, and characterization of As-Mal-derivatized proteins. 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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