Arsenic-Binding Proteins

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p-Azidophenylarsenoxide: An Arsenical "Bait" for the In Situ Capture and Identification of Cellular Arsenic-Binding Proteins

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Abstract: *Identification of arsenic-binding proteins is important for understanding arsenic health effects and for developing arsenic-based therapeutics. We report here a strategy for the capture and identification of arsenic-binding proteins in living cells. We designed an azide-labeled arsenical, p-azidophenylarsenoxide (PAzPAO), to serve bio-orthogonal functions: the trivalent arsenical group binds to cellular proteins in situ, and the azide group facilitates click chemistry with dibenzylcyclooctyne. The selective and efficient capture of arsenic-binding proteins enables subsequent enrichment and identification by shotgun proteomics. Applications of the technique are demonstrated using the A549 human lung carcinoma cells and two in vitro model systems. The technique enables the capture and identification of 48 arsenic-binding proteins in A549 cells incubated with PAzPAO. Among the identified proteins are a series of antioxidant proteins (e.g., thioredoxin, peroxiredoxin, peroxide reductase, glutathione reductase, and protein disulfide isomerase) and glyceraldehyde-3-phosphate dehydrogenase. Identification of these functional proteins, along with studies of arsenic binding and enzymatic inhibition, points to these proteins as potential molecular targets that play important roles in arsenic-induced health effects and in cancer treatment.*

Arsenic is widely distributed in the natural environment and living systems at trace concentrations. Chronic exposure to high levels of arsenic can lead to nerve and skin disorders, malignant tumors, and other adverse health effects.[1] Long before arsenic was recognized as a carcinogen, toxic arsenic compounds were used as effective drugs for certain diseases, e.g., the treatment of acute promyelocytic leukemia (APL).[2] Although the diverse biological effects of arsenic have been observed, the exact molecular mechanisms are not fully understood. One tangible mechanism is through the binding of trivalent arsenic to cellular proteins, such as redox-regulators, signaling and DNA repair proteins.[3] If arsenic binds to these proteins and inhibits their functions, a consequence could be elevated oxidative stress, cell damage and carcinogenesis.[4] Therefore, identification of arsenic-binding proteins is important for elucidating the biological effects of arsenic. Furthermore, in the clinical treatment of APL by arsenic, it has been shown that arsenic specifically kills APL cells by binding to the crucial PML-RARα oncoprotein which triggers the oligomerization, SUMOylation, ubiquitination, and finally proteasomal degradation of this oncoprotein.[5] The initial step of arsenic binding to this oncoprotein is key to the success of APL treatment. If similar specific arsenic-binding proteins can be identified from other cancers, these proteins could be the potential molecular targets for consideration of cancer treatment. Therefore, the identification of arsenic-binding proteins is important both for understanding the mechanisms of arsenic health effects and for discovering new molecular targets for arsenic-based cancer therapy.[6]

To identify arsenic-binding proteins, many efforts have been made to specifically capture these proteins. *p*-Aminophenylarsine oxide (PAmPAO) immobilized onto the stationary phase of a column was able to capture dozens to hundreds of arsenic-binding proteins in cell lysate.[7] However, this method suffers from several limitations, such as steric hindrance, nonspecific binding, and the requirement of lysing the cells first before capturing the proteins on the arsenical affinity column. Biarsenical FlAsH dye, which was initially developed by Tsien et al. for the imaging of targeted proteins genetically fused with a tetracysteine tag,[8] was also immobilized to beads or resins for the pulling down of tetracysteine-tagged proteins. The high binding affinity of FlAsH to tetracysteine enables the specific capture of targeted proteins.[9] Alternatively, an arsenic–biotin conjugate[10] has been used directly to treat living cells. The treated cells were then lysed and the streptavidin-coated beads were used to pull down the arsenic-binding proteins. However, the arsenic–biotin conjugate is relatively large, and it could interfere with the metabolic pathway of endogenous biotin in the cells.[11]

To overcome these limitations, we have designed an azide-labeled arsenic, *p*-azidophenylarsenoxide (PAzPAO), as a compact arsenical "bait" to enter the cells and capture arsenic-binding proteins in situ. The captured proteins can subsequently be characterized using the shotgun proteomics approach. The overall strategy is illustrated in Scheme 1. Unlike previous approaches that captured arsenic-binding proteins from cell lysate, we introduced the small arsenical molecule PAzPAO inside the living cells. PAzPAO is biocompatible, and the azide group in PAzPAO is metabolically stable.[12] The trivalent arsenical in PAzPAO is highly selective

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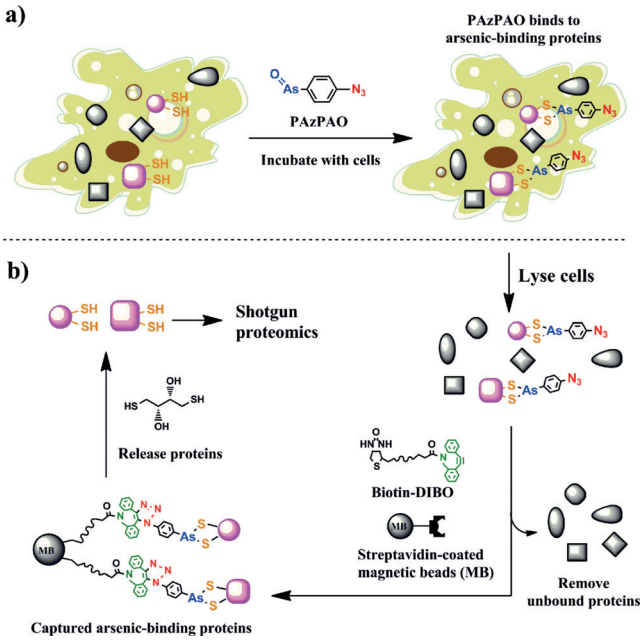

Scheme 1. a) Capture of arsenic-binding proteins inside cells and b) identification of the captured proteins. PAzPAO is incubated with cells and its trivalent arsenical binds to cellular proteins while its azide is available for the subsequent "click" chemistry. The "click" reaction between the azide and dibenzylcyclooctyne (biotin–DIBO) facilitates purification of the arsenic-bound proteins. These captured proteins are identified using the shotgun proteomics approach.

for the free thiol groups in proteins. Binding of arsenic to proteins leaves the azide "hook" freely available to "click" with a dibenzylcyclooctyne (DIBO) group. The copper-free "click" chemistry reaction between the azide group and the DIBO group, which is also termed the strain-promoted azide–alkyne cycloaddition (SPAAC) reaction, is highly efficient with no need for a catalyst.[13] Because we conjugate a biotin moiety to DIBO, the click reaction between biotin-DIBO and the azide group in the arsenic-binding proteins results in efficient labeling of the arsenic-binding proteins with biotin, which facilitates the subsequent purification using streptavidin-coated magnetic beads. Therefore, the reactive trivalent arsenical group and the small azide group in PAzPAO serve bio-orthogonal purposes.[14]

We designed and synthesized PAzPAO through two-step reactions from commercially available *p*-arsanilic acid (PASA) (Figure 1a). First, we synthesized PAmPAO by reducing the pentavalent arsenic group of PASA into trivalent arsenic.[15] Secondly, we converted the aromatic amine of PAmPAO into azide group using the one-pot synthesis method developed by Barral et al.[16] (see the Supporting Information (SI)). Chromatograms and ESIMS spectra of the synthesized PAmPAO and PAzPAO are shown in Figure S1b and S1c in the SI. PAzPAO was found to be very stable when kept in the dark, as confirmed by HPLC-UV analysis of a PAzPAO solution freshly prepared and the re-analysis of the same solution 30 days later (Figure S2). The excellent stability of this trivalent arsenical was probably because the benzyl ring could stabilize the trivalent arsenic through an electron resonance structure. The trivalent arsenical is reactive with

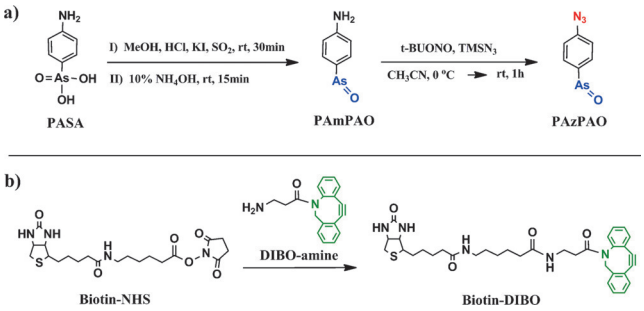

Figure 1. Chemical synthesis of a) PAzPAO and b) biotin–DIBO.

the free thiols in proteins. The extremely small azide group in PAzPAO is metabolically stable, and not naturally occurring in living systems.[12] We therefore expect that the bifunctional PAzPAO can act as an arsenical "bait" to efficiently interact with protein thiols in living cells.

To enable the capture of arsenic-binding proteins with efficient click chemistry and the subsequent biotin–streptavidin affinity procedures, we conjugated biotin to DIBO (Figure 1b) through an amide reaction between biotin-NHS and DIBO-amine in DMSO. The synthesized biotin–DIBO was purified with HPLC and confirmed by ESIMS (Figure S1e).

Our strategy involved non-enzymatic click chemistry to capture arsenic-binding proteins in situ. We first investigated the efficiency of the click reaction (Figure 2a). We mixed 0.5 mM PAzPAO and 1 mM biotin–DIBO in Tris-HCl buffer (100 mM, pH 7.4), and monitored the reaction process with HPLC-UV (254 nm) (Figure 2b) and ESIMS (Figure S1f). The reaction product biotin–PAO (Figure 2b, peak 3 at 13.3 min) is detectable after only 5 min of reaction between PAzPAO (peak 1 at 12.1 min) and biotin–DIBO (peak 2 at

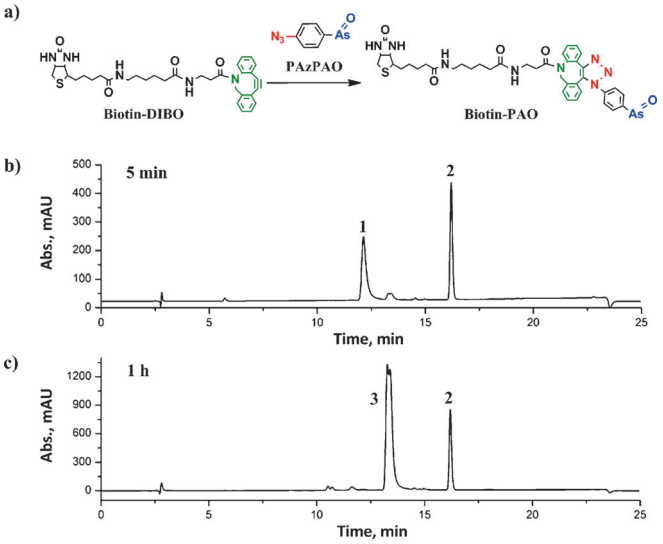

Figure 2. a) "Click" reaction between biotin–DIBO and PAzPAO. HPLC-UV (254 nm) chromatograms from the analyses of reaction mixture at 5 min (b) and 1 h (c) after the click reaction between biotin–DIBO (peak 2) and PAzPAO (peak 1), forming biotin–PAO (peak 3).

16.1 min). After 1 h reaction (Figure 2c), all the PAzPAO is reacted to form biotin–PAO (peak 3). These results show that the click reaction between biotin–DIBO and PAzPAO is fast and efficient.

Having synthesized PAzPAO and confirmed its efficient click reaction with biotin–DIBO, we further demonstrated applications of our approach to capturing arsenic-binding peptides and arsenic-binding proteins. We used three examples: glutathione, peptides from tryptic digest of bovine serum albumin (BSA), and cellular proteins in A549 human lung carcinoma cells. We first examined whether biotin–PAO could capture the reduced glutathione (GSH) (Figure 3a). When

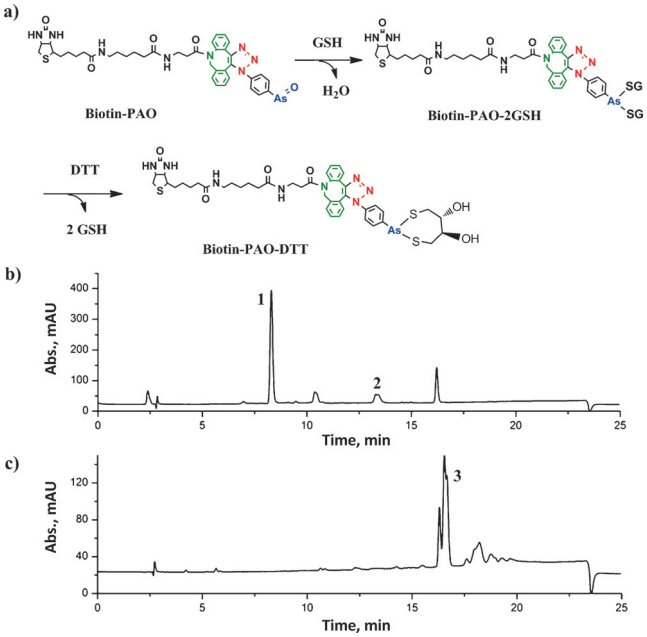

Figure 3. a) Reactions illustrating GSH captured by biotin–PAO and replaced by DTT. HPLC-UV (254 nm) chromatograms from the analyses of a reaction mixture of biotin–PAO and GSH (b) and further addition of DTT (c). Peaks 1, 2 and 3 correspond to biotin–PAO–2GSH, biotin–PAO, and biotin–PAO–DTT, respectively.

we incubated 0.5 mM biotin–PAO with 1 mM GSH for 20 min, most of the biotin–PAO reacted with GSH to form biotin–PAO–2GSH (peak 1 in Figure 3b). Mass spectrometry analysis (Figure S3a) showed that this compound is the complex of biotin–PAO with two GSH after the elimination of one water molecule. The capture efficiency was estimated to be 90% according to the ratio of peak areas of biotin–PAO–2GSH to biotin–PAO, demonstrating the ability of biotin-PAO to efficiently capture GSH. To examine whether the captured GSH can be released from biotin–PAO, we added 1 mM DTT into the above solution, and found that the biotin–PAO–2GSH complex completely disappeared and a new peak appeared at 18.8 min (Figure 3c). This new peak is due to biotin–PAO–DTT, which is confirmed by its ESIMS spectra (Figure S3b). These results indicate that DTT, which contains two vicinal thiols spaced by four carbons, can form a more stable seven-membered ring with trivalent arsenic and therefore replace GSH from biotin–PAO.[10b,17]

The results from the small thiol model peptide, involving a thiol–arsenic complex and competitive displacement with dithiols, provide support for the feasibility of our strategy.

To further explore the practicability of the overall technique, we tested selective capture of peptides from the tryptic digest of BSA. As shown in Figure S4, 31 peptides from the tryptic digest of BSA contain no cysteine, 17 peptides contain one cysteine, and 9 peptides contain two vicinal cysteines. To generate peptides containing cysteine residues with free thiol, we reduced BSA with an efficient thiol-free reductant, tris(2-carboxyethyl)phosphine (TCEP), and digested BSA immediately using trypsin without the alkylation step. We also produced peptides, whose thiol groups were alkylated by iodoacetamide, as negative controls. Then we analyzed the peptides using HPLC-ESIMS, and monitored the molecular ion of each BSA peptide in selected ion chromatograms. As shown in Figure 4, before the specific

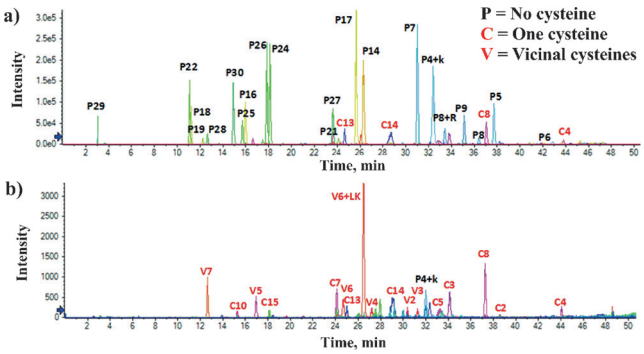

Figure 4. Selected ion chromatograms from the HPLC-ESIMS monitoring of the molecular ions of BSA tryptic peptides before (a) and after (b) the selective arsenic capture procedures. The peptides contain no cysteine (P), one cysteine (C), or two vicinal cysteines (V).

capture procedures, only four peptides with one cysteine (C4, C8, C13 and C14) were observed, and most of the peptides detected contained no cysteine (Figure 4a). In contrast, after the capture procedures, the vast majority of the peptides without cysteine were removed. Peptides with one cysteine, and especially peptides with two vicinal cysteines were preferentially detected (Figure 4b). No peptide from the negative control sample was detected (data not shown) after the capture procedures, thus implying the specificity of arsenical "bait" to the thiol groups of peptides. These results demonstrate the practicability of the overall selective capture procedures for the analysis of tryptic digest of proteins.

We further implemented our approach to capture arsenic-binding proteins from A549 human lung carcinoma cells. We chose to incubate A549 cells with 5 μM PAzPAO and PAmPAO for 30 min, on the basis of toxicity (Supporting Information)[18] (Figure S5) and uptake (Figure S6) results. We further used a fluorescent dye carrying a DIBO group (Alexa Fluor 647 DIBO alkyne) to label and image the PAzPAO-treated cells. Strong fluorescence was observed in the PAzPAO-treated cells (Figure S7c), compared with the negligible fluorescence observed in the PAmPAO-treated cells (Figure S7b), which demonstrates that the azide group of

PAzPAO was stable inside the cells and it could be specifically labeled by the DIBO-containing probe.

From the A549 cells that were incubated with the PAzPAO, 48 arsenic-binding proteins were identified, ranked according to the Mascot scores (Table S1). No arsenic-binding protein was detected in the A549 cells incubated with PAmPAO. PAmPAO served as a negative control because it does not have the azide group for the "click" chemistry. These results further demonstrate the selectivity and bio-orthogonality of the arsenical "bait" technique.

Among the 48 identified arsenic-binding proteins in A549 cells (Table S1), the two most abundant proteins are the important antioxidant proteins thioredoxin and peroxiredoxin-1. Their exponentially modified protein abundance index (emPAI) values are 10.28 and 7.02, which are proportional to protein content, as described by Ishihama et al.[19] Using Equation (S1a) and (S1b),[19] we calculated the molar and weight percentages of these two proteins to be 16% and 11%, and 8.4% and 10%, ranking at the top of the 48 identified arsenic-binding proteins. A series of other antioxidant proteins, including peroxiredoxin-5, peroxiredoxin-6, thioredoxin-dependent peroxide reductase, glutathione reductase, and protein disulfide isomerase A3, were also identified as arsenic-binding proteins. These proteins share a similar dithiol–disulfide active site,[20] indicating the high binding affinity of PAzPAO to proteins containing vicinal cysteines inside living cells, even in the presence of high GSH content.[21] Strong binding of arsenic to these important antioxidant proteins could alter their normal functions, and subsequently result in elevated oxidative stress and cell damage.[22] Arsenic-induced oxidative stress has been reported.[23]

We also identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an arsenic-binding protein. GAPDH is an important glycolytic protein, over-expressed in human lung cancer tissues.[24] As a housekeeping protein, GAPDH catalyzes the sixth step of glycolysis, which is also the first energy yielding step. The over-expression of GAPDH in cancer cells suggests a molecular basis of elevated glycolysis and an important role of energy-creating reaction in cancer cell growth.[21] To further confirm its arsenic-binding nature, we incubated GAPDH with PAzPAO, and analyzed the mixture with ESIMS (Figure 5a). The ESIMS peaks of different isomers of GAPDH were entirely shifted to the higher molecular weight after incubation of GAPDH with PAzPAO. The mass spectral results are consistent with the binding of PAzPAO to two cysteine residues, resulting in the elimination of a water molecule (Figure 5b). Analysis of the structural nature of GAPDH suggests that it is possible for the C152 and C156 at the active site to bind with PAzPAO (Figure 5c).[25] Free conversion between the thiol group and the thioester intermediate of C152 at the active site of GAPDH is critical for performing its normal functions. We therefore hypothesized that the binding of arsenic to cysteine at the active site of GAPDH could inhibit its glycolytic activity. To test this hypothesis, we determined the in vitro and cellular effects of PAzPAO on the activity of GAPDH. Slope differences in Figure 5d show that 100 μM PAzPAO inhibited

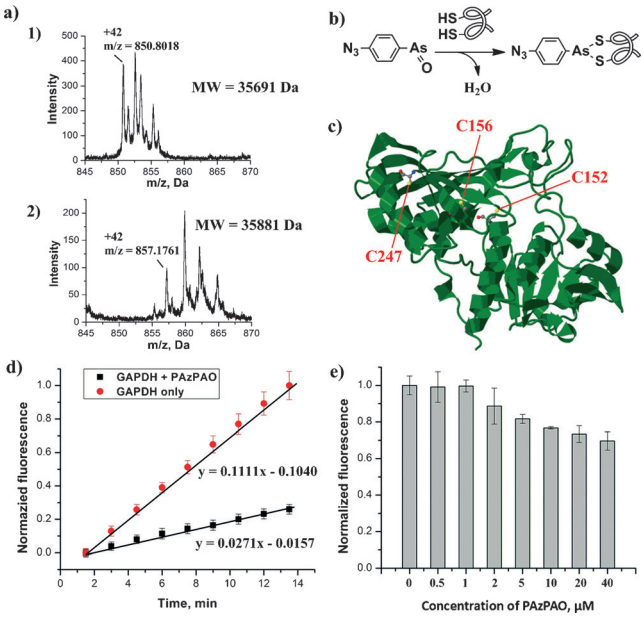

Figure 5. a) Mass spectra from the ESIMS analyses of GAPDH isomers before (1) and after (2) binding with PAzPAO. The deconvoluted mass (molecular weight) from the measured *m/z* spectrum at charge state 42 is 35691 Da for the GAPDH protein. This is in good agreement with the theoretical molecular weight of GAPDH (35691 Da). Binding of GAPDH to PAzPAO results in a shift of the spectrum to a higher deconvoluted mass (35881 Da). This corresponds to the addition of PAzPAO (209 Da) and a loss of a water molecule. b) Schematic showing PAzPAO binding to two cysteines in a protein, resulting in the elimination of a water molecule. c) Ribbon diagram of GAPDH monomer (PDB code IU8F). Sulfur atoms in the side chains of three cysteines (C152, C156 and C247) are indicated as yellow balls. C152 is at the active site of GAPDH. C152 and C156 are the two possible vicinal thiol groups that bind to PAzPAO. This figure was prepared using Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/. d) Fluorometric analysis of the activity of GAPDH in the presence or absence of 100 μM PAzPAO. e) Concentration-dependent inhibition of GAPDH in A549 cells by PAzPAO (incubation for 30 min).

the activity of the GAPDH protein by 76%. The activity of GAPDH in A549 cells was reduced by 30% when the cells were treated with 20 μM PAzPAO for 30 min (Figure 5e). These results demonstrate that PAzPAO can bind to GAPDH and inhibit its enzymatic activity.

Previous work has shown that arsenic binding to PML-RARα oncoprotein in APL cells is a molecular basis for the treatment of APL using trivalent arsenic.[5] Whether the strategy of successful treatment of APL by arsenic can be extended to the treatment of other cancers depends on a number of factors, one of which is the specific molecular target that binds to arsenic. It is well known that most cancer cells rely on high rate of aerobic glycolysis for generating energy, a phenomenon termed "the Warburg effect".[26] Our results on arsenic binding to GAPDH suggest that GAPDH could be a potential molecular target for arsenic-based cancer therapy, by blocking the hyper-active glycolytic pathway of cancer cells.[27]

In conclusion, we have developed an effective approach to capture the cellular arsenic-binding proteins in situ using

PAzPAO. The main advantages include the biocompatibility and bio-orthogonality of the small molecule PAzPAO, the in situ binding of PAzPAO to proteins in living cells, the efficient click reaction between PAzPAO and DIBO, and the selective capture and identification of arsenic-binding proteins and peptides. The identified proteins can be candidates for further studies on their binding to arsenic and the consequent effects. Binding of arsenic to GAPDH and the inhibition of its activity demonstrate an example of a potential molecular target. Among the 48 arsenic-binding proteins identified in A549 cells, the two most abundant proteins are anti-oxidant proteins. These anti-oxidant proteins have cysteines at their active sites, and their binding to arsenic could reduce their activities, which could contribute to the arsenic-induced oxidative stress. These examples demonstrate that our technique is useful for identifying proteins as potential therapeutic target(s) and proteins that play important roles in arsenic health effects.

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