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Article

Anticancer Effects of the Trivalent Organoarsenical 2-Amino-4-(dihydroxyarsinoyl) Butanoate

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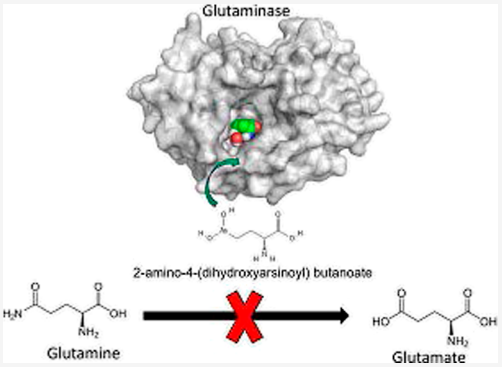
ABSTRACT: According to the National Cancer Institute, breast cancer is a leading cause of death in women. The lack of progesterone and estrogen receptors in triple-negative breast cancer (TNBC) cells results in a lack of response to hormonal, monoclonal, or tyrosine kinase inhibitor therapies. Despite intensive drug discovery, there is still no approved targeted treatment for TNBC. The metalloid arsenic has been used in herbal medicines, antibiotics, and chemotherapeutic drugs for centuries. This paper demonstrates that a trivalent arsenic-containing, nonproteogenic amino acid, R-AST−OH (2-amino-4-(dihydroxyarsinoyl) butanoate), inhibits kidney-type glutaminase (KGA), the enzyme that controls glutamine metabolism and is correlated with tumor malignancy. Cell-based assays using the TNBC MDA-MB-231 and HCC1569 cell lines showed that R-AST−OH kills TNBC cells and is not cytotoxic to a control cell line. The results of in silico molecular docking predictions indicate that R-AST−OH binds to the glutamine binding site and forms a covalent bond with an active site cysteine residue. We hypothesize that R-AST−OH is a single warhead for KGA that irreversibly binds to KGA through the formation of an As−S bond. We propose that R-AST−OH is a promising lead compound for the design of new drugs for the treatment of TNBC.



1. INTRODUCTION

Cancer is the second-leading cause of death in the United States, behind only heart disease, and breast cancer is the second-leading cause of death for women.[1] Breast cancer has historically been subclassified by three molecular markers: estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (EGFR2/Her2), all of which contribute to the prognosis and choice of treatment.[2] Breast cancers that are ER/PR positive are candidates for endocrine therapy, and Her2 positive breast cancers can be treated with anti-Her2 drugs, such as Herceptin. Cancer that is ER/PR/Her2 negative (or triple-negative breast cancer (TNBC)) is the most aggressive. Compared to other types of breast cancer, TNBC lacks targeted therapies and has a poorer prognosis.[2] 10−20% of breast cancers are TNBC, which spread beyond the breast and recur after treatment. Breast cancer diagnosis and treatment have improved significantly, but TNBC remains a challenge due to aggressive progression and lack of targeted therapies.[2]

Cancer cells have the ability to utilize glutamine as an energy source for rapid growth and proliferation. In addition, glutamine is used in many intracellular processes in cancer cells, including biosynthesis of proteins, lipids, nucleic acids, and generation of ATP.[3] Conversion of glutamine to glutamate by the mitochondrial enzyme glutaminase (GLS) is the first step in intracellular utilization,[3] and expression of glutaminase has been shown to be related to TNBC.[4] A new therapeutic approach to TNBC was developed using GLS inhibitors. There are two forms of GLS in human cells: glutaminase C and kidney-type glutaminase (KGA).[5] Oncogenes such as c-Myc, Raf, Ras, and Rho GTPases upregulate KGA expression in cancer cells, especially TNBC cells. Inhibition of the KGA isoform is a new direction for TNBC therapy.[5,6] The glutamate analogue DON (6-diazo-5-oxo-L-norlucine) inhibits glutaminase by attacking the side chain of Ser286 with a nucleophile. As a result of catalysis, the diazo (N2) group of DON is released, leaving 5-oxo-L-norleucine (ON) covalently bound to Ser286, inhibiting glutaminase activity.[7] Inhibition by DON is significantly better than other glutamate analogues.[8] However, DON's toxicity prevents it from progressing to clinical trials. The small molecule inhibitors BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide) and CB-839 inhibit KGA by binding to an allosteric site. Because of its poor solubility and bioavailability, BPTES is not a candidate for TNBC treatment. CB-839 is currently in phase-1 clinical trials for TNBC because of its selective inhibition of

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glutaminase.[9] However, not all TNBCs respond to CB-839 due to resistance.[9]

Over the last few centuries, metals such as platinum, gold, and metalloids, including arsenic- and boron-based compounds, have been developed as anticancer drugs.[10] Since 1865, Fowler's solution, which contains sodium arsenite as its active ingredient, was used to treat cancer and other diseases.[11] In 1910, Paul Ehrlich introduced the organoarsenical salvarsan to treat syphilis and trypanosomiasis.[12] In the modern era, arsenic trioxide (Trisenox) has been approved by the U.S. Federal Drug Administration as a chemotherapeutic drug for treatment of acute promyelocytic leukemia.[13] An advantage of trivalent arsenicals is that they easily form a covalent bond with the thiol group of cysteine residues in proteins and act as a covalent warhead.[13] Arsinothricin (2-amino-4-(hydroxymethylarsinoyl) butanoate (AST)) is a pentavalent organoarsenical and nonproteinogenic amino acid analogue of glutamine that is a potent inhibitor of glutamine synthetase. AST is an effective broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria.[14] The reduced trivalent organoarsenical R-AST−OH is the biosynthetic precursor of AST.[15] In addition, the pentavalent organoarsenical roxarsone (3-nitro-4-hydroxyphenylarsonic acid (Rox(V))) has been widely used in animal husbandry as an antimicrobial growth promoter.[16] Here, we show that the trivalent forms of R-AST−OH and roxarsone Rox(III) inhibit KGA activity at micromolar concentrations. Furthermore, using a TNBC cell line-based assay, we demonstrate the significant potential of R-AST−OH as a potent lead compound for treatment of TNBC.

2. RESULTS AND DISCUSSION

AST is a natural product produced by bacteria and an arsenic-containing nonproteinogenic amino acid that shows broad spectrum antibiotic activity.[4] In addition, AST has recently been shown to have antimalarial activity, preventing transmission of the eukaryotic parasite *Plasmodium falciparum*.[17] AST−OH is the biosynthetic precursor of AST.[15] The chemical structure of AST−OH is similar to the amino acid glutamine, and we hypothesize that AST−OH would inhibit the catalytic activity of the enzyme glutaminase and would have anticancer properties.

Inhibition of KGA catalytic activity was assayed spectrophotometrically using purified recombinant KGA with AST−OH, the structurally similar phosphonate AP4, or the well-known KGA inhibitor DON (Figure 1). The results showed that AST−OH and AP4 are better inhibitors than the competitive inhibitor DON, but none is particularly effective, with IC_{50} values in the submillimolar range (Figure 2A).

For that reason, we evaluated the effect of trivalent organoarsenicals, including R-AST−OH and the trivalent reduced form of the aromatic arsenical roxarsone (Figure 2B). For assaying inhibition of KGA by trivalent organoarsenicals, KGA was preincubated with R-AST−OH or trivalent Rox(III) for 15 min, and the enzymatic reaction was initiated by addition of the substrate, glutamine. The glutamate produced by KGA activity was assayed with a glutamine/glutamate-Glo kit (Promega), which is a luminescent assay for the detection of glutamate (Figure 2B). The calculated IC_{50} value of 2 μM shows that R-AST−OH is a 125-fold more effective inhibitor of KGA than pentavalent AST−OH and 4.5-fold more effective than Rox(III).

The real time binding affinity of R-AST−OH and Rox(III) with purified KGA was determined by using isothermal

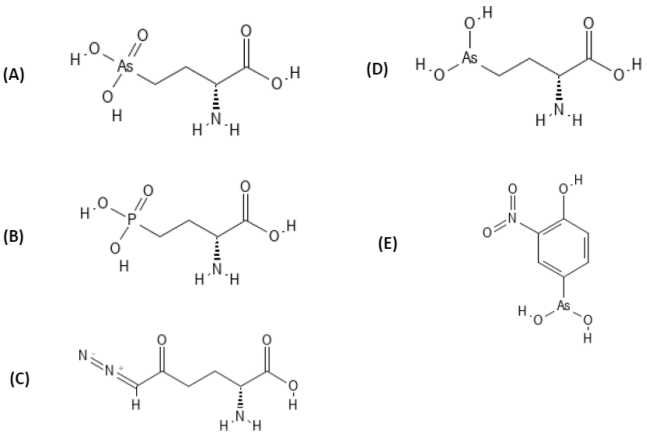

Figure 1. Chemical structures: (A) hydroxyarsinothricin (AST−OH), (B) 2-amino-4-phosphonobutyric acid (AP4), (C) 6-diazo-5-oxo-L-norleucine (DON), (D) trivalent hydroxyarsinothricn (R-AST−OH), and (E) trivalent roxarsone (Rox(III)).

titration calorimetry (ITC). The apparent binding affinity (K_d) was 40 and 30 μM for R-AST−OH and Rox(III), respectively (Figure 3). Although this appears to be high compared with the IC_{50} values, it is likely because trivalent arsenicals bind slowly to the enzyme by forming an irreversible covalent bond, which is the optimum type of inhibition.

To examine whether either R-AST−OH or Rox(III) inhibits growth of cancer cells, cell viability assays were conducted with TNBC cell lines MDA-MB-231 (basal B subtype) and HCC1569 (basal A subtype). The effect of the trivalent organoarsenicals was compared with the FDA approved anticancer drug, Trisenox (ATO), an inorganic trivalent arsenical (Figure 4). The cytotoxicity of those compounds was also examined using normal breast cells (nontumorigenic human breast epithelial cells) MCF12A. At 10 μM R-AST−OH, approximately 64% of MDA-MB-231 cells and 47% of HCC1569 cells lost viability (Figure 4). The IC_{50} for R-AST−OH can be inferred to be 3 and 5 μM for HCC1569 and MDA-MB-231 cells, respectively. The IC_{50} for Rox(III) is 9 and 10 μM for HCC1569 and MDA-MB-231 cells, respectively, and for ATO, it is 6 and 4 μM for HCC1569 and MDA-MB-231 cells, respectively. IC_{50} values for R-AST−OH, ATO, and Rox(III) with MCF12A cells were 75.0, 13.6, and 5.6 μM, respectively, indicating that R-AST−OH has the least toxicity to normal cells among tested compounds. Overall R-ASTOH has more effective anticancer activity than Rox(III) in both cancer cell lines. Cell permeability assays with MDA-MB-231 cells showed that ATO and Rox(III) have better permeability than R-AST−OH (Figure 4D), so even with lower uptake, R-AST−OH is still more effective than the other trivalent arsenicals.

The binding mode of R-ASTOH and Rox(III) with KGA was analyzed by in silico docking using AUTODOCK4. Both inhibitors fit well into the catalytic site. The arsenical moiety of R-AST−OH is surrounded by KGA residues Asn388, Glu381, and Cys418 (Figure 5A). The distance between the arsenic and the sulfur atoms of Cys418 is predicted to be 3.9 Å. The carboxylate group of R-AST−OH interacts with the catalytic residue Ser286. Similarly, the arsenical moiety of Rox(III) is surrounded by Glu381 and Cys418. The nitro group of Rox(III) interacts with Ser286. The arsenic atom of Rox(III) is close to sulfur atom of Cys418, at a distance of 3.6 Å. In general, trivalent arsenic and sulfur have high affinity for each

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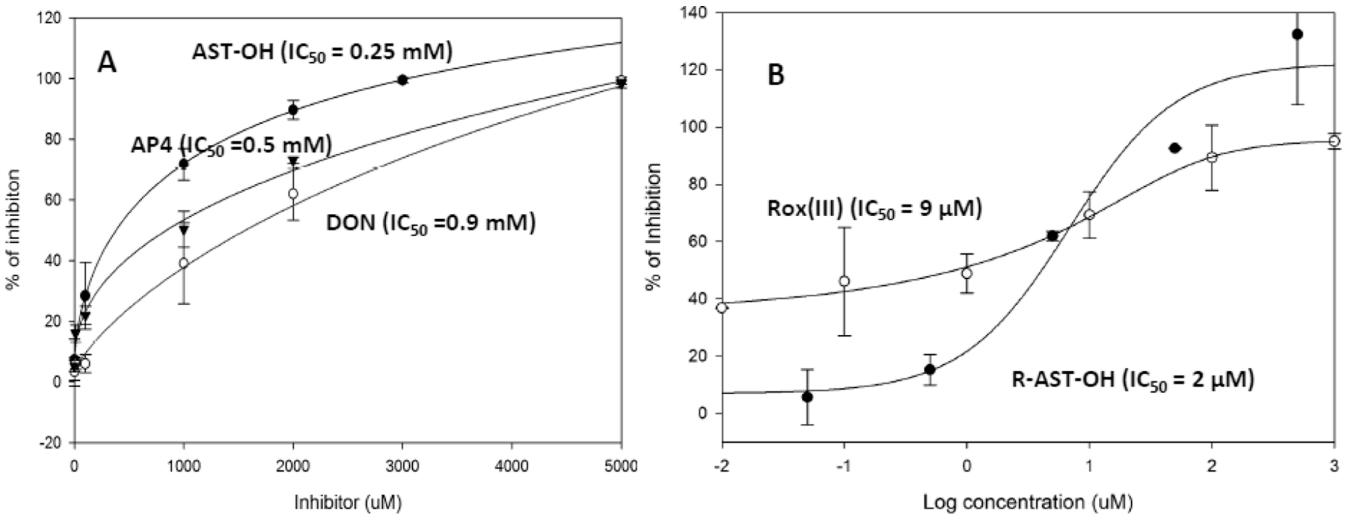

Figure 2. Assays of KGA inhibition: (A) spectroscopic assay with AST−OH, AP4, and DON. (B) Luminescent assay with R-AST−OH and Rox(III).

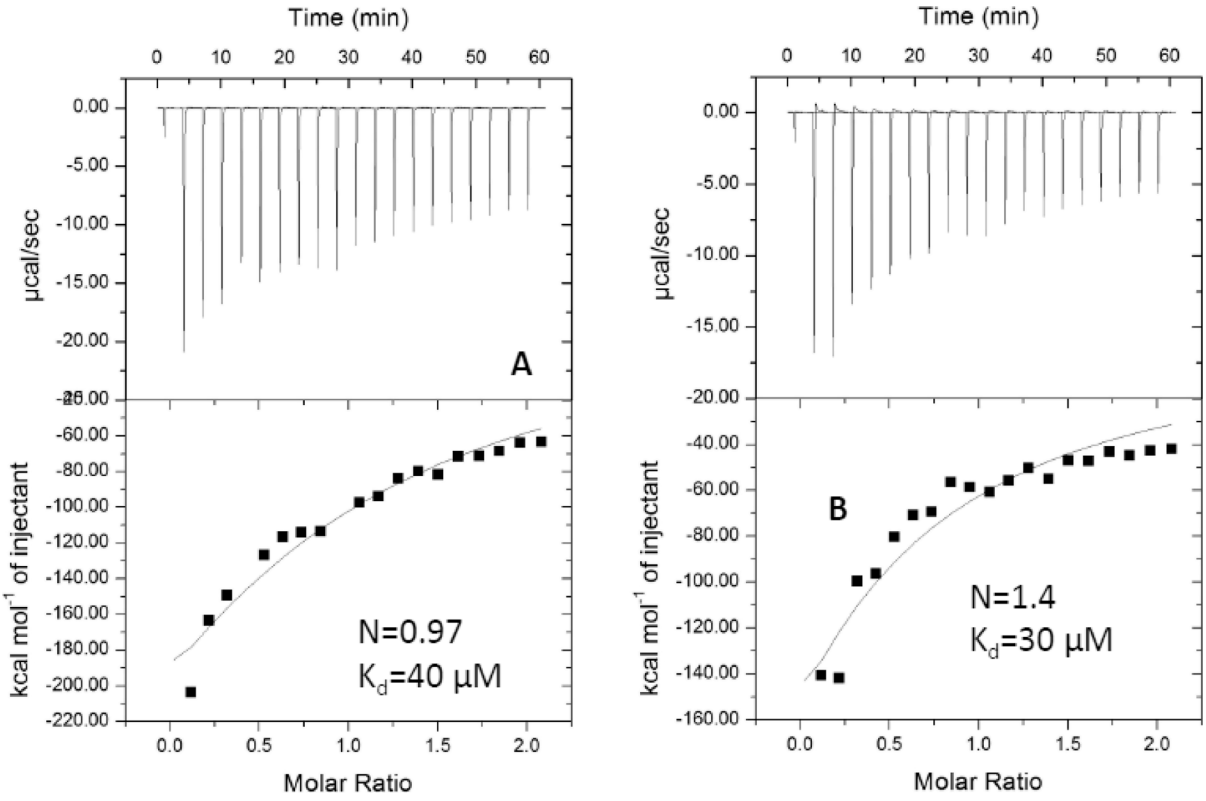

Figure 3. Isothermal calorimetry (ITC) analysis of (A) R-AST−OH and (B) Rox(III) binding to KGA.

other and will form a covalent bond at this distance, indicating that R-AST−OH and Rox(III) should be irreversible covalent inhibitors of KGA.

Over the last few decades, there has been much interest in metals and metalloids as potential anticancer therapeutics. The results of our study show that the trivalent arsenicals R-AST−OH and Rox(III) exhibit a stronger inhibitory effect on glutaminase than the well-characterized competitive inhibitor DON.[7] The binding affinity of R-AST−OH and Rox(III) measured by ITC is higher than the IC_{50} value of those compounds determined spectrophotometrically. We believe that the spectrophotometric assay reflects slow binding kinetics, while the ITC results reflect the thermodynamics, which predominate in irreversible inhibition. The results of the docking analysis indicate that the distance between the arsenic atom and the sulfur atom of cysteine is short enough to create a covalent bond between them. Covalent bond formation would essentially result in irreversible inhibition, consistent with the higher binding affinity found by ITC.

Both R-AST−OH and Rox(III) produced significant loss of viability in both TNBC cell lines MDA-MB-231 and HCC1569, suggesting that both would have anticancer effects. Comparing R-AST−OH and Rox(III) with the well-known anticancer drug ATO, both were more cytotoxic to growth of

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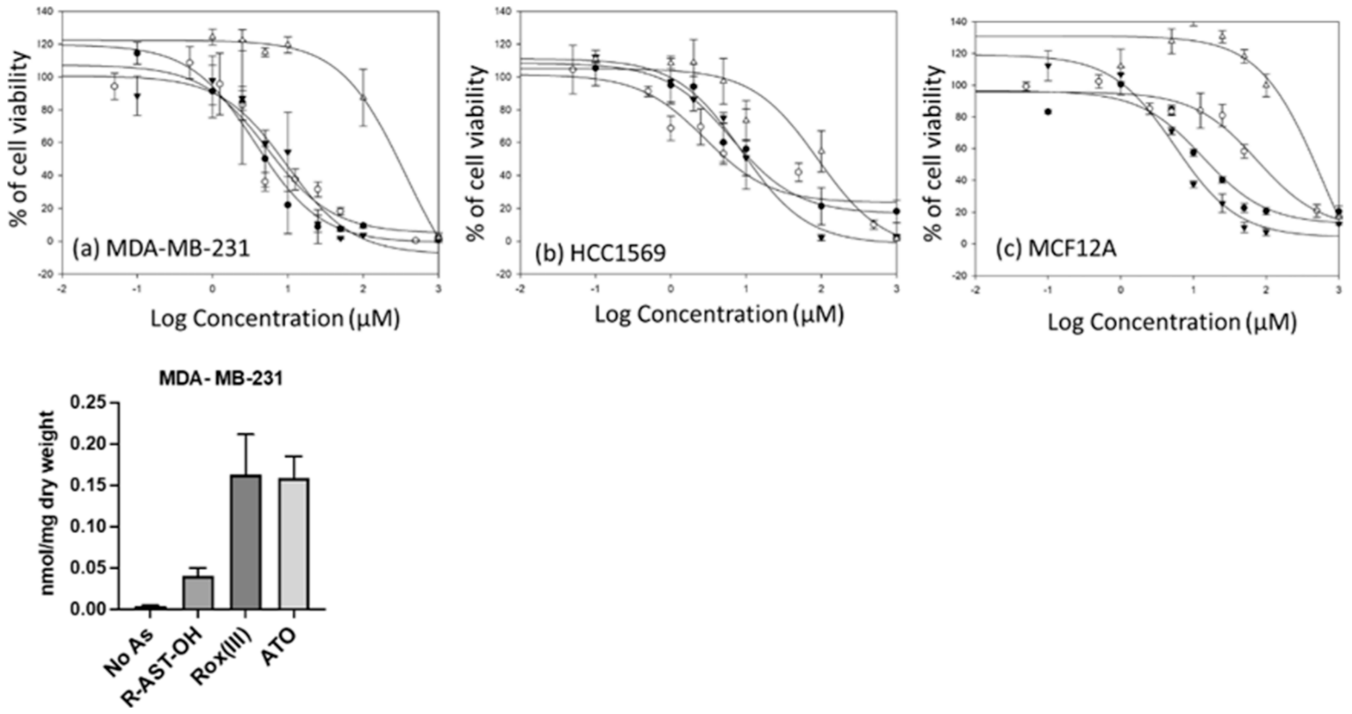

Figure 4. Antiproliferative activity in TNBC cell lines (A) MDA-MB-231, (B) HCC1569, and (C) the normal breast epithelial cell line MCF12A with R-AST−OH (○), Rox(III) (▼), ATO (●), or reducing agent alone (△). (D) Permeability of arsenic compounds was measured using MDA-MB-231 cells. The significance of uptake is analyzed by one-way ANOVA test: $*p < 0.05$ ($N = 3$). Shown is the average, with SEM as error bars.

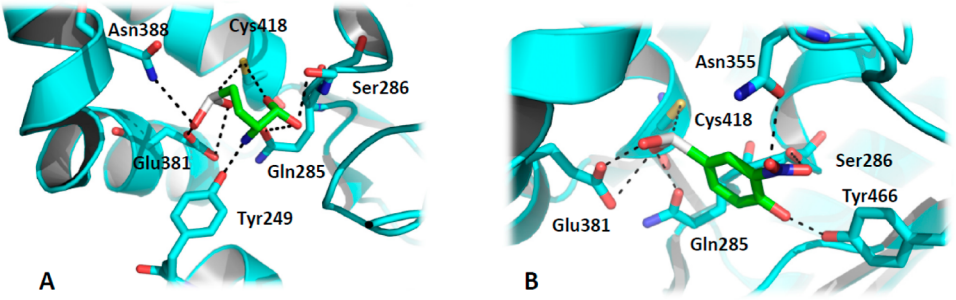

Figure 5. In silico docking of (A) R-AST−OH and (B) Rox(III) with KGA. Arsenicals are shown as green sticks.

MDA_MB-231 and HCC1569 cells than ATO, supporting our suggestion that R-AST−OH could be more effective therapeutic agents than ATO. In addition, viability assays with the normal breast cell line MCF12A cells demonstrated that R-AST−OH exhibits the lowest toxicity, indicating that it could be a good lead compound for treatment of breast cancer.

We propose that the mechanism of R-AST−OH effectiveness is its ability to inhibit KGA, an essential enzyme in breast cancer cells. In MDA_MB-231 cells, KGA expression levels are 62.6 (transcripts per million or nTBM), while in HCC1569 cells, the levels are 135 (nTPM) (The Human Protein Atlas).[18] Thus, the effect of R-AST−OH on cell viability is not directly proportional to the level of KGA expression, suggesting that R-AST−OH may have other targets in addition to KGA. In addition, the differential uptake of arsenicals should be considered. In MDA-MB-231 cells, ATO has the highest permeability, and R-AST−OH has the lowest permeability. We predict that the R-AST−OH would show significantly more potent anticancer efficacy through chemical modification to produce more permeable derivatives. In summary, the novel organoarsenical R-AST−OH is more cytotoxic toward breast cancer cells than the inorganic arsenical ATO, which is a proven drug for treatment of soft tissue malignancies. Our results suggest that R-AST−OH may be an effective chemotherapeutic agent for breast cancer. The glutamine analogs DON, azaserine, and acivicin bind to the active site of KGA and competitively inhibit. In clinical studies, however, these compounds have been shown to have limited antitumor activity and significant side effects.[19] R-AST-OH is also an effective competitive inhibitor of KGA compared with those compounds. In contrast, BPTES and CB-839 are allosteric inhibitors.[20] The frequency of resistance to competitive inhibitors is lower than that to allosteric inhibitors since mutations in substrate-binding sites are more likely to inactivate enzymes than mutations in allosteric sites. Thus, R-AST−OH should be more effective than allosteric inhibitors BPTES and CB-968.

3. MATERIALS AND METHODS

3.1. Reagents. All chemicals and enzymes were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA), unless otherwise stated. AST−OH was chemically synthesized as described pre-

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viously.[15] Chemically synthesized AST−OH is a racemic mixture, and we consider only the L-form in all studies mentioned in this paper. AST−OH is authenticated by high pressure liquid chromatography (series 2000, PerkinElmer) (HPCL) coupled to inductively coupled plasma mass spectrometry (ELAN DRC-e; PerkinElmer, Waltham, MA, USA) (ICP-MS) before use. Trivalent R-AST−OH and Rox(III) were prepared by chemical reduction of pentavalent AST−OH and Rox(V).[15]

3.2. Purification of Recombinant KGA. The kidney isoform of human glutaminase (KGA) consists of 669 amino acid residues. The gene for KGA without the mitochondrial localization sequence (residues 72−598) was chemically synthesized by GenScript USA Inc. (New Jersey) with codon optimization for expression in *Escherichia coli*. The synthetic gene was inserted into the vector plasmid pET28a(+). The resulting plasmid contained the KGA gene with the sequence for a six-histidine tag at the 3′-end and under control of the T7 promoter. The plasmid was transformed into *E. coli* Rosetta (DE3) cells for protein expression. Cells of *E. coli* Rosetta (DE3) bearing pET28a(+)-KGA were grown in lysogeny broth (LB) medium[21] with shaking at 37 °C. At an $A_{600\ nm}$ of 0.5−0.6, 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added and growth continued at 16 °C for 20 h. The cells were harvested by centrifugation and suspended in buffer A (50 mM MOPS, 20 mM imidazole, pH 7.5, 0.5 M NaCl, and 20% glycerol). The cells were lysed by a one-time passage through a French pressure cell at 20,000 psi and immediately mixed with 2.5 μL/g of wet cells of the protease inhibitor diisopropylfluorophosphate. The cell lysate was centrifuged at 35,000 rpm using a T865 rotor (Thermo Fisher Scientific, Waltham, MA, USA) for 60 min at 4 °C. The supernatant solution was applied onto a Ni-NTA column (QIAGEN Sciences, Hilden, Germany) at a flow rate of 1.5 mL/min and washed with 20 column volumes (100 mL) of buffer A. Bound protein was eluted with buffer A containing 0.2 M imidazole. Fractions containing the protein were pooled and concentrated using a 30 kDa Amicon Ultra centrifugal filter (EMD Millipore, Billerica, MA, USA). The concentrated protein was rapidly frozen and stored at −80 °C until use.

3.3. Assay of KGA Activity. Glutaminase activity and inhibition by AST−OH, AP4, and DON were assayed using published procedures.[8] Briefly, the catalytic activity of KGA was determined by measuring the production of NADH from the increase in absorption at 340 nm at 37 °C for 30 min in a buffer containing 50 mM tris-acetate (pH 8.6), 150 mM K_2HPO_4, 0.1 mg/mL bovine serum albumin, 0.25 mM EDTA, 1 mM DTT, 4 mM NAD^+, 5 mM glutamine, and 1 unit of glutamine dehydrogenase. The reaction was initiated by addition of glutamine. The reducing agent used for the preparation of R-AST−OH and Rox(III) did not affect the assay. R-AST−OH and Rox(III) inhibition of KGA was determined using glutamine/glutamate-GLO Assay Kit (Promega, Madison, WI, USA). For assaying inhibition of KGA by trivalent arsenicals, KGA was preincubated with the arsenicals for 15 min, and the enzymatic reaction was initiated by addition of the substrate glutamine. The effect of those inhibitors was compared to the half-maximal inhibitory concentration (IC_{50}) of each compound. IC_{50} values were calculated by fitted regression using a Sigma Plot (Inpixon, Palo Alto, CA).

3.4. ITC. The parameters of inhibitor binding with purified KGA were determined by ITC. Binding assays were carried out using a MicroCal iTC200 (GE Healthcare Bio Sciences, Piscataway, NJ) with protein concentrations between 50 and 100 μM and inhibitor concentrations from 0.5 to 1 mM. Data were collected at 20 °C with 20 injections at 10 min intervals with a stirring speed of 1000 rpm. Spectra were analyzed using Origin 7.0 software (TA Instruments, New Castle, DE), and stoichiometry (n), binding constants (K_d) and enthalpy (ΔH (kcal/mol)) were calculated with a one site binding model.

3.5. Cell Culture. TNBC cell lines MDA-MB-231 (CRM-HTB-26), HCC1569 (CRL-2330), and the nontumorigenic mammary epithelial cell line MCF12A (CRL-10782) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and used to test anticancer effect/cell toxicity of arsenic compounds. All cell lines were cultured in the respective media suggested by the vendors in a 5% CO_2 humidified incubator at 37 °C.

3.6. Anticancer Effect/Cell Toxicity Assay. All cell lines were seeded at a density of 3.0×10^4 cells/well in 96-well plates. After 24 h, the cells were further cultured in the presence or absence of the indicated concentrations of arsenic compounds for another 72 h, following which viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay (Promega).

3.7. Arsenic Uptake Assay. MDA-MB-231 cells were seeded at a density of 1.0×10^5 cells/well in 6-well plates. After 24 h, the cells were further cultured with or without 5 μM arsenic compounds for another 72 h. Cells were rinsed with PBS four times, followed by collecting pellets. For measurement of cellular uptake, the dried cells were digested with 70% nitric acid (≥99.999% trace metals basis) at 70 °C for 60 min, allowed to cool to room temperature, and diluted to a final concentration of 2% nitric acid with HPLC-grade water, and the total arsenic content of each sample was quantified using ICP-MS.

3.8. In silico Docking of KGA with R-AST−OH and Rox(III). The 3D structure of R-AST−OH and Rox(III) was generated using the Molview server. Both molecules were docked with the crystal structure of KGA (PDB ID: 4O7D)[8] using AutoDock4[22] in AutoDockTools.[22] The grid center was positioned on the DON binding site of KGA with a size of 40 × 40 × 40 Å. The other docking parameters were set as the default values. The top ranked confirmation was selected for further analysis. The binding energies of the complex of KGA-R-AST−OH and KGA-Rox(III) are −4.27 and −4.89 kCal/mol, respectively. The molecular graphics were performed using PyMol.[23]

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

As(III), arsenite; As(V), arsenate; MAs(III), methylarsenite; MAs(V), methylarsenate; DMAs(V), dimethylarsenate; DMAs(III), dimethylarsenite; TMAs(V)O, trimethylarsine oxide; arsinothricin, AST; AST−OH, pentavalent hydroxyarsinothricin; R-AST−OH, trivalent hydroxyarsinothricin; AsS,

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arsenosugar; SAM, *S*-adenosylmethionine; HPLC, high pressure liquid chromatography; ICP-MS, inductively coupled plasma mass spectroscopy

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