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Anticancer Activity of Small Molecule and Nanoparticulate Arsenic(III) Complexes

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Abstract

Starting in ancient China and Greece, arsenic-containing compounds have been used in the treatment of disease for over 3000 years. They were used for a variety of diseases in the 20th century, including parasitic and sexually transmitted illnesses. A resurgence of interest in the therapeutic application of arsenicals has been driven by the discovery that low doses of a 1% aqueous solution of arsenic trioxide (i.e. arsenous acid) leads to complete remission of certain types of leukemia. Since FDA approval of arsenic trioxide (As_2O_3) for treatment of acute promyelocytic leukemia (APL) in 2000, it has become a front line therapy in this indication. There are currently over 100 active clinical trials involving inorganic arsenic or organoarsenic compounds registered with the FDA for the treatment of cancers. New generations of inorganic and organometallic arsenic compounds with enhanced activity or targeted cytotoxicity are being developed to overcome some of the shortcomings of arsenic therapeutics, namely short plasma half-lives and narrow therapeutic window.

Arsenic has a rich history in medicine, but it has not always been administered with the intention of benefiting the recipient. Arsenic trioxide (As_2O_3, ATO), a white powder which dissolves readily in alkaline solution to form a stable, odorless, and tasteless solution of arsenous acid, was widely known as 'the king of poisons/the poison of kings'.[1] Arsenic poisoning mimics the hemorrhagic gastroenteritis symptomatic with cholera, allowed arsenic trioxide to be used as an acute poison. The well-known toxicity of As_2O_3 that led to applications as a rat poison in times of plague[2] did not prevent our predecessors from exploring other applications. Members of European royal courts used lower doses of arsenic to achieve the rosy lips and pale skin considered a sign of good breeding[1]. Mountaineers in the Austrian and Swiss Alps reportedly consumed small doses of arsenic on a regular basis in order to improve stamina and ward off disease.[3] While it seems a high risk for little benefit, the key to many profound lifesaving pharmacological applications of arsenic trioxide are perhaps rooted in the Renaissance dictum attributed to Paracelsus, namely that it is the dose that determines the poison. In the course of this Forum, we discuss the evidence of carefully controlled administration of a variety of forms of As^{III} for treatment of cancer.

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We focus first on the history and development of low dose treatments with the inorganic drug arsenous acid, and it subsequent development into Trisenox, into a front line, FDA-approved treatment for some types of blood cancers. The aqueous reaction chemistry and some of the physiological targets of this drug, which surprisingly can act to promote the reversion of a cancer cell to a normal phenotype, will also be discussed. Given the clear efficacy of arsenicals for specific cancer types, given that several of these agents clear rapidly and do not accumulate in the body, we argue that deeper investigation of the pharmacology of new complexes and nano-scale formulations that target the delivery and release of this metalloid are well warranted.

Some of the earliest records of direct applications of arsenic to treat disease are found in traditional Chinese medicine where it was used as a devitalizing agent prior to dental work. Hippocrates and other ancient Greek physicians used arsenic as an escharotic, a substance that destroys tissue and produces a thick black scab known as an eschar, to treat skin and breast cancers.[4] Two other common mineral forms of arsenic have also been major components in other traditional medicines in China for more than 1500 years: realgar, tetra-arsenic tetrasulfide (As_4S_4) and orpiment, diarsenic trisulfide (As_2S_3) (Figure 1). Following the success of arsenic trioxide, recently both As_4S_4 and As_2S_3 have been used in clinical trials in China for treatment of APL.[5] Arsenic became popular in western medicine in the 18th century, when Thomas Fowler patented a solution of potassium arsenite to be administered orally.[6] Known as Fowler's Solution, it saw a wide variety of uses for over 150 years including Hodgkin's disease, leukemia, asthma, pemphigus, psoriasis, and eczema.

Paul Ehrlich won the Nobel Prize in 1908 for his studies of immunity and the development of small molecule drugs targeting cellular receptors. This work lead to the development of his “magic bullet” hypothesis, namely that a properly designed molecule would bind to a receptor present on the syphilis-causing pathogen (the gram negative spirochete *T. pallidum*) and enable the specific delivery of a toxin to that organism. He first identified Salvarsan in 1907, but it was not commonly used until 1911 for the treatment of syphilis.[7] Prepared from the reduction of nitro-oxyphenyl arsonic acid, Salvarsan was used to treat syphilis and other diseases until penicillin became widely available during World War II. The structure of Salvarsan was the subject of some controversy for many years, since Elrich and others originally determined that Salvarsan had dimeric structure akin to azobenzene (Figure 2). Recent mass spectrometric data suggests that the compound is a mixture of the trimer (Figure 2) and pentamer (Figure 2) in solution.[8]

Acute Promyelocytic Leukemia (APL)

Aside from a few specific indications, arsenic had fallen out of wide use in western medicine by the mid-20th century due to the aforementioned toxicity at high doses. However, in 1971, a group of Chinese physicians tested the efficacy of a 1% solution of arsenic trioxide in patients with leukemia. The responses of the cohort were mixed; however, the group of patients that presented with Acute Promeylocytic Leukemia had drastically better response to arsenic trioxide than other patients[9]

Up to this point, the greatest clinical success of arsenic trioxide has been in the treatment of hematological cancers, most notably in acute promyelocytic leukemia (APL) which is a subtype of acute myeloid leukemia (AML).[10] APL was first identified as a distinct clinical entity in 1957 by a Norwegian physician, Hillestad, which he categorized as one of the most malignant forms of acute leukemia.[10] APL diagnosis is characterized by an accumulation of promyelocytes in the blood and bone marrow which can lead to severe bleeding tendencies with a rapid course of only a few weeks. On a molecular level, APL is characterized by a balanced reciprocal translocation between chromosomes 15 and 17, t(15;17)(q22;q21),

which results in a fusion of the promyelocytic leukemia (PML) gene with the retinoic acid receptor α (RARα) gene. The resulting PML-RARα fusion protein is the essential driver in ≥98% of APL patients and acts as a constitutive transcriptional repressor of normal RARα signaling. This results in a differentiation block for promyelocytes maturing to functional granulocytes.[10]

In the early 1970s it was shown that AML leukemic cells were particularly sensitive to a combination chemotherapy treatment of daunorubicin, a member of the anthracycline family that are thought to be DNA-intercalators, and cytarabine (Ara-C), a pyrimidine nucleoside analog that inhibits DNA polymerase during the synthesis phase of the cell cycle. While an early 1973 study showed this chemotherapy treatment resulted in 33% complete remission (CR) of APL patients,[11] more recent studies in the 1990s showed CR rates at 80-90%.[12] This chemotherapy regiment successfully inhibited the proliferation of leukemogenic cells but is not curative and the majority of patients eventually relapsed. Lacking better alternatives the regiment remained the standard of care for twenty years until the discovery and adoption of all-trans retinoic acid (ATRA) therapy. As a single agent ATRA has demonstrated effective but often short-lasting remission for the treatment of APL; however, when ATRA is administered in combination with anthracycline-based chemotherapy (most commonly daunorubicin or idarubicin) the complete remission rate stays equally high and the disease-free survival rate is drastically improved. Long-term results from a randomized trial run by the European APL group showed that ATRA in combination with chemotherapy (daunorubicin and cytarabine) resulted in 96% complete remission and a 10-year event-free survival rate of 76%.[13] The study also concluded that following complete remission, a prolonged maintenance treatment (up to two years) of intermittent ATRA and chemotherapy helped to reduce the rate of relapse.[13]

In many cases of refractory or relapsed APL the disease eventually becomes resistant to ATRA-therapy. It is in this salvage setting that arsenic trioxide was originally utilized for the treatment of refractory, ATRA-resistant APL.[12] As mentioned previously, arsenic has been utilized in traditional Chinese medicines for thousands of years, and in the early 1970s a group of physicians from Harbin Medical University in China identified arsenic as an active ingredient from a traditional Chinese therapy for leukemia leading them to use arsenic trioxide as a therapy for APL.[14] Results from the first moderately sized trials of arsenic trioxide for the treatment of APL came out of Harbin Medical University and the Shanghai Institute of Hematology in the mid-1990s and reported impressive rates of complete remission and overall survival for both newly diagnosed and relapsed APL.[14] The success of arsenic trioxide in China led to these trials being repeated worldwide with much success.[15] While arsenic was originally reserved for refractory APL, in 2010 the North American Leukemia Intergroup published the results of a trial testing arsenic trioxide as consolidation treatment for APL patients in their first remission. In this study adding two five-week consolidation courses of arsenic trioxide at 0.15 mg/kg/day via intravenous infusion for five days a week increased the three-year disease-free survival rate to 90% while the control group not treated with arsenic trioxide had a rate of 70% disease-free survival.[16] Additional studies have shown that arsenic trioxide is the single most effective agent for treating APL. One study using single-agent arsenic trioxide therapy as a front-line treatment reported in 2011 that of 197 patients with newly-diagnosed APL, 85.8% achieved complete remission with a 5-year disease-free survival rate of 66.7%.[17]

Every study mentioned so far has used an intravenous formulation of As_2O_3 that, while very effective and generally well-tolerated, requires daily infusions, often for ten weeks, and has resulted in some reports of cardiotoxicity with QTc prolongation measured by electrocardiogram. Given the demonstrated benefit of ATO in the treatment of APL, a group in Hong Kong has developed an oral formulation of ATO that can be self-administered in

the outpatient setting.[18] Several studies by Au et al. have found that orally administered ATO is well absorbed with bioavailability of up to 95% of an equivalent dose of intravenous ATO. The slower oral absorption of ATO also led to a reduction in peak plasma arsenic levels which was associated with lower levels of cardiotoxicity.[18, 19] The success of arsenic as an effective, economical, and easily-administered therapy for APL has warranted testing in a number of other cancers which will be discussed later in this Forum.

Toxicity and Reaction Chemistry of Arsenic(III)

The toxicity of arsenic has been widely observed and is likely better known in popular culture than the rich therapeutic history of arsenic. Acute arsenic poisoning has symptoms similar to many other diseases, including vomiting, diarrhea, bloody urine, and eventual death. These common disease-like symptoms led to the wide use of arsenic as a poison. During the rush to World War I arsenic was developed into a chemical weapon. Methyldichloroarsine was used to great effect by the German army, and the United States developed, but never deployed, 2-chloroethenylarsonous dichloride, also known as lewisite. [20]

The salts of arsenous acid, such as sodium arsenite ($NaAsO_2$), potassium arsenite ($KAsO_2$), calcium arsenite ($CaHAsO_3$), copper arsenite ($CuHAsO_3$, Scheele's green), copper acetoarsenite ($3Cu(AsO_2)_2{\cdot}Cu(O_2CCH_3)_2$, Paris green), and lead arsenite ($PbHAsO_3$), and the salts of arsenic acid, such as calcium arsenate ($Ca_3(AsO_4)_2$), and lead arsenate ($Pb_3(AsO_4)_2$) are also poisonous. They have been used as anticancer agents (sodium arsenite and potassium arsenite), in preserving hides, in the manufacturer of soap and antiseptics, and in viticulture as insecticides, weed killers, germicide and rodenticides.[21, 22]

Aside from its more infamous uses as an acute poison, arsenic is sometimes present in high concentrations in ground water, resulting in the exposure of entire populations, depending upon the local geologic strata.[23] Natural groundwater contamination is the most common source of chronic arsenic exposure though it may also be encountered through dietary sources as well as anthropogenic sources such as mining, smelting, and agricultural runoff.[24] The most common side effect of chronic arsenic exposure is hyperpigmentation of the skin which, according to the US EPA, typically begins after 6 months to 3 years of high contamination levels of 0.04 mg/kg/day or 5 to 15 years of low contamination levels of 0.01 mg/kg/day.[25] More serious side effects of chronic exposure include hyperkeratosis of the palms and feet which often leads to skin lesions that are thought to increase incidence of squamous cell skin cancer. Bladder, lung, liver, kidney, and prostate cancers have also been linked to chronic arsenic exposure as well as leukopenia, peripheral vascular diseases (Blackfoot disease), anemia, neuropathy, encephalopathy, and diabetes. While many of these diseases have been linked to chronic arsenic exposure, researchers have had difficulty determining dose dependence due to complications involved with estimating total arsenic exposure from drinking water for various populations.[26] Arsenic also may be acutely toxic at much larger doses and is most often observed with severe gastrointestinal distress (vomiting, abdominal cramps, and diarrhea), heart dysrhythmias, headaches, and kidney and/or liver failure.[27]

The introduction of arsenic trioxide in the treatment of APL and other cancers naturally led to concerns about its acute and chronic toxicities. This was addressed by the development of a very low dose administered daily over a month long course of treatment. A common dosing schedule for arsenic trioxide therapy for APL is two courses of 0.15 mg ATO per kilogram of patient bodyweight daily for five days a week for five weeks with a two week rest between courses.[16] With this dosing, ATO is generally well-tolerated with commonly reported side effects of differentiation syndrome (APL-specific effect associated with

elevated white blood cell counts), headache, elevated liver enzymes, and cardiac QTc prolongation and arrhythmias. Most of these side effects are low-grade and reversible after halting lowering or halting ATO therapy.[15] In a follow-up study of APL patients in remission after receiving ATO therapy, urine analysis for arsenic showed that urine arsenic concentrations in patients who were off ATO treatment for at least 24 months were only slightly above that of healthy controls and were well below safety limit recommendations by the US Agency for Toxic Substances and Disease Registry and the Ministry of Health of China.[28] A second follow-up study of APL patients who had received ATO therapy at least 24 months prior confirmed these findings of low arsenic retention through the analysis of hair and nails of patients.[29]

As mentioned elsewhere in this article, arsenic trioxide is the most effective single agent therapy for APL. The recent release of 10-year follow-up studies on APL patients receiving ATO therapy has shown that ATO increases the rate of event free survival and also has little/no long term side effects.[28] The use of ATO in induction/consolidation therapy of APL may allow for the reduction of anthracycline chemotherapy which is known to have much more serious and longer lasting side effects. Currently orally administered formulations of arsenic trioxide are being studied and seem to have a similar efficacy as IV-administered ATO while reducing acute toxicities (especially QTc prolongation) from the lower peak plasma arsenic levels encountered with slower oral absorption of ATO.[18]

Arsenic trioxide, when dissolved in water at neutral pH forms arsenous acid, $As(OH)_3$ (Figure 2). This species is neutral at physiological pH (pKa_1 = 9.3, pKa_2 = 13.5, pKa_3 = 14.0).[30] The aqueous form of arsenic(V) oxide is arsenic acid, H_3AsO_4 (Figure 2), and in physiological conditions, it exists as hydrogen arsenate and dihydrogen arsenate (pKa_1 = 2.19, pKa_2 = 6.94, pKa_3=11.5).[31]

In all cases, exposure to trivalent arsenic (As^{III}) is more toxic than exposure to its pentavalent form (As^{V}). It is thought that the increased toxicity of As^{III} compared to As^{V} is due to the increased affinity of As^{III} for sulfur ligands.[32] Arsenic in groundwater is typically inorganic arsenite forms of As^{III} while dietary arsenic tends to be As^{V} derivatives such as dimethylarsinic acid or arsenobetaine (major component of dietary arsenic found in seafood). The greatest contribution to total urinary arsenic levels is dimethylarsinic acid.[33]

Organic arsenic compounds have long been used for treating various disease, with their greatest drawbacks being toxicity. Melarsoprol (Figure 2) was developed in the late 1940s and is still used for standard-of-care treatment of African Trypanosomiasis, commonly known as sleeping sickness.[34] It is used for more advanced stages of the disease, due to fatal reactive encephalopathy in 5-10% of patients treated with melarsoprol. Other organoarsenicals, such as salvarsan, display dose-limiting acute toxicity as well as chronic toxicities, and have fallen out of use for safer alternatives.

Arsenate as a phosphate analog

Arsenate has long been known to participate in similar reactions as phosphates. Recently there was a controversial report in the literature that arsenate had been shown to replace phosphate along the DNA backbone in an extremophile bacterial strain. Subsequent analyses in other laboratories have demonstrated that there is no detectable arsenic in the DNA of these bacteria,[35] and that these organisms require phosphate as an essential nutrient for growth.[36]

Arsenate (AsO_4^{3-}) is known to participate in certain cellular processes in place of phosphate (PO_4^{3-}), particularly phosphorylation reactions.[37] When arsenate is substituted for phosphate during glucose phosphorylation, it results in the rapid decomposition of glucose-1-

arsenate.[38] Arsenate and other transition metal oxoanions, such as vanadate, are potent inhibitors of many phosphatase enzymes.[39] This action is thought to be the result of the increased stability of a metalloester intermediate compared to the native phosphoester intermediate. Other enzymes are not inhibited by arsenate or vanadate, and can perform these reactions on substrates that have arsenate or vanadate in place of the normal phosphate.[40, 41]

Despite the potential disruption of these metabolic pathways, there is little evidence that arsenate is responsible for the majority of the symptoms of acute arsenic toxicity. First, the intracellular concentrations required to inhibit these enzymes is high (10^{-4} M)[39] and not achievable in patients without acute arsenic toxicity. Second, the phosphate concentration in the cell (10-100 mM)[42] is high enough that even a toxic dose of arsenic to the cell would not be enough to displace phosphate from these essential metabolic pathways.

Arsenic-thiol Thermodynamics

Arsenic exerts its effect in cells by interacting with proteins and peptides, typically through the sulfhydryl groups of cysteine residues. These interactions are energetically favored but have heats of reaction that make the interactions rapid and reversible. One important ligand that arsenic binds is glutathione. Glutathione (GSH) is a tripeptide molecule (γ-L-glutamyl-L-cysteinyl glycine) that is responsible for protecting the cell from reactive oxygen species generated by normal cellular metabolism.[43]

The interaction of arsenous acid with glutathione occurs in a stepwise manner, where each hydroxyl ligand is displaced by the nucleophilic sulfur ligand.[44] The interaction of the arsenic center with a single sulfur ligand has the smallest heat of reaction, with each additional interaction more exothermic (ΔH_1=-2.5 kcal mol^{-1}, ΔH_2= -3.1 kcal mol^{-1}, ΔH_3= -33.1 kcal mol^{-1}). This is likely due to the increased electronic degeneracy of these interactions. The formation of the *tris*-glutathione arsenite, $As(GS)_3$, species is spontaneous at pH 7.4 at 37°C (ΔG = -8.8 kcal mol^{-1}).[44] Scott et al. isolated and characterized an arsenite complex of glutathione as $As(GS)_3$ by mass spectrometry.[45] Delnomdedieu et al. found that glutathione reduces arsenate to arsenite and forms a $As(GS)_3$ complex (Figure 3).[46]

The formation constants of the cysteine (Cys) complexes $[As(Cys)_3]$, logK = 29.84(6), and $[As(Cys)(OH)_2]^-$, logK = 12.01(9), and of the glutathione complexes $[As(GS)_3]^{3-}$, logK = 32.0(6), and $[As(GS)(OH)_2]^{2-}$, logK = 10(3) were calculated from potentiometric and spectroscopic data.[47] Spectroscopic data clearly indicate that the arsenite binding site is S-atom of cysteine. At pH 7.0-7.5 with arsenic and glutathione concentrations of 5 mM and 15 mM, respectively, approximately 70% of the arsenic species present are $[As(GS)_3]^{3-}$.[47] The other major species in this range are $As(OH)_3$ and $[As(GS)(OH)_2]^{2-}$.

Methylated arsenic also interacts with sulfur ligands, albeit with higher affinity than inorganic arsenous acid.[44, 48] The formation of the bis-glutathione methyl arsenite species is slightly more favored than the *tris*-glutathione arsenite (ΔG = -10.1 kcal mol^{-1}). The increased reactivity of methylarsenous acid with sulfur ligands is thought to be the basis for the increased toxicity of this species.[49] The thermodynamics of arsenic-protein interactions are less well understood, however, examining the interactions of arsenites with thiol ligands provides some insight into the strengths of these interactions. Much like the monothiol case of glutathione, these interactions are energetically favored; however, dithiol interactions are more energetically stable than monothiol interactions due to the chelate effect.[44]

Cellular Mechanisms for Arsenic Transport and Detoxification

At elevated temperatures and in membranes with large amounts of cholesterol, $As(OH)_3$ is able to rapidly cross lipid membranes, likely due to the increased fluidity of the membranes.[50] It is likely that arsenic acid enters cells by co-opting a phosphate transporter.[51] Arsenous acid is also able to cross cell membranes by utilizing the neutral species transporter aquaglyceroporin 9 (AQP9).[51, 52] Aquaglyceroporins are members of the major intrinsic protein (MIP) superfamily and are responsible for the transport of glycerol and other neutral solutes into the cells. Another member of this superfamily is aquaporin which regulates water transport in cells.[53] The transport of neutral molecules into and out of cells by aquaglyceroporins is typically a regulated response to metabolic cues.[54] Arsenic has been shown to utilize AQP9 and AQP7 channels as a pathway for cellular uptake.[52] In leukemia cells, the presence of these transporters, particularly AQP9, confers sensitivity to arsenous acid as well as potassium antimonyl tartarate, another metalloid compound.[55, 56] The expression of AQP9 in *Xenopus laevis* oocytes increases arsenous acid uptake 40 fold over 90 seconds.[52] AQP7, in contrast, only increases arsenous acid uptake by 10 fold in the same assay. [52]

The expression of AQP9 has been demonstrated to confer arsenic sensitivity to leukemia cell lines *in vitro*.[56] When AQP9 expression is induced by DNA transfection, the arsenic concentration required for 50% cell growth inhibition (IC_{50}) is reduced by 3.8 fold.[56] The effect is similar when AQP9 expression is induced pharmacologically using Vitamin D pretreatment; the cells are rendered 2.5 fold more sensitive to arsenic treatment.[57] This effect is shown in a panel of 11 leukemia cell lines, where AQP9 expression correlates with directly with arsenic uptake and sensitivity. Interestingly, AQP9 is most highly expressed in acute promeylocytic leukemia (APL). [57]

Arsenic can be present in groundwater to widely varying levels depending on local mineral strata. Organisms exposed to moderate arsenic levels have developed specialized systems for detoxifying and removing arsenic from cells.[51] In bacteria, a specialized system called the *Ars* operon is controlled by the ArsR protein in response to arsenic exposure and begins exporting arsenic from the cell by first reducing arsenates to arsenites and then utilizing a specialized arsenite transporter.[51]

In mammalian cells, the metabolism of arsenic involves export from the cell and metabolic detoxification of the arsenic. Metabolic processing of inorganic arsenic(III) is carried out by a series of methylation reactions. These reactions involve the reaction of arsenous acid with arsenic(III) methyltransferase (AS3MT).[58-61] AS3MT uses S-adenosyl methionine as a methyl donor in this reaction.[58] This protein catalyzes both the methylation of arsenous acid as well as methylarsonous acid in the presence of adequate reducing and methyl equivalents.[60, 61] It is not entirely clear if the product of methylation is methylarsonous acid or methylarsonic acid, however, examination of a bacterial S-adenosyl methionine methyltransferase, ArsM, indicates that the arsenic center remains arsenic(III) during the reaction.[62] There are three cysteines in the protein that are important for catalytic activity, two likely participate directly in arsenic binding, and the third is involved in the methylation of arsenous acid,[60] but not methylarsonous acid.[62] The position of the arsenic center is such that the arsenic lone electron pair is positioned towards the methyl group of S-adensosyl methionine, facilitating methylation *via* an S_N2 reaction.[62] Further crystallographic, EXAFS, or other physical examination of the active site is required to produce a more precise mechanism of this enzyme.

Glutathione also plays a role in the direct export of arsenous acid from the cell.[63] Glutathione complexes with metal ions are substrates for the multi-drug resistance export

proteins (MRP1-10) that are found in cells.[64] These proteins are responsible for exporting a diverse range of ionic substrates. Arsenic-glutathione complexes (Figure 3) are substrates for MRP1,[63, 64] and high levels of expression of MRP1 is associated with arsenic resistance in cancer cells.[65] Glutathione-S-transferase pi (GST-pi) is responsible for catalyzing the reaction of arsenic with glutathione and enabling the subsequent export of the arsenic-glutathione complex.[65] Depleting intracellular glutathione by inhibiting its synthesis sensitizes cells to arsenous acid and other arsenic compounds.[66-68] High expression levels of GST-pi results in resistance to arsenous acid.[65]

Targets of Arsenous Acid Activity

Due to the large concentration of thiols in the cell, with the majority of them in the reduced state, arsenic has a wide array of targets. The majority of the proteins that bind arsenic have spatially close cysteine resides that bind the arsenic through the sulfhydryl side chain. In cancer cells, there are a more limited set of interactions that are thought to exert the anti-tumor effect of arsenous acid.

Metabolic proteins

Arsenous acid acts to increase the levels of reactive oxygen species in cells; however, there is little evidence that oxidizing equivalents originate from the reduction of arsenous acid to metallic arsenic or arsine. Arsenic is known, however, to interact with a mitochondrial membrane protein complex, called the mitochondrial membrane transition pore complex.[69] As arsenic binds to this pore complex, it initiates the collapse of the mitochondrial membrane potential and the release of cytochrome c from the mitochondria into the cytoplasm of the cell. [70, 71] Other researchers have demonstrated that arsenous acid will bind to enzymes involved in glucose metabolism.[72]

Proteins regulating intracellular redox potentials

Another class of proteins affected by intracellular arsenic is involved in redox balance and antioxidant pathways.[6, 73] These proteins have redox active sulfhydryl groups that detoxify reactive oxygen species produced by normal mitochondrial function. Arsenic acts on these proteins by binding to the thiol groups and removing their ability to bind other nucleophiles, such as superoxide, hydroxide radicals, or oxide radicals.

Thioredoxin is an oxidoreductase that contains a dithiol active site. It performs a number of signaling and homeostasis activities related to cellular oxidation state and can also modulate the levels of adventitious reactive oxygen species.[74] The dithiol active site is maintained in the reduced state by another enzyme, thioredoxin reductase. Thioredoxin reductase is a NADPH-dependent flavoenzyme that utilizes an active site composed of a redox-active cysteine pair and a redox-active selenocysteine-cysteine pair.[75] Arsenic containing compounds interact strongly with selenocysteine residues in proteins.[76] Arsenous acid and methylarsonous acid inhibits enzyme activity which prevents the reduction of the disulfide form of thioredoxin and may result in the accumulation of oxidized proteins within the cell.[77] Mass spectroscopy of peptide fragments of thioredoxin reductase reveal that arsenic binds to both redox-active sites within the active site, preventing normal oxidation and reduction of the disulfide and the selenosulfide bonds.[78]

The effect of arsenic on the cell cycle is thought to be mediated through the stabilization of microtubules.[79-82] In cells treated with arsenic, a portion of cells halt normal cell cycling in the mitosis phase due to the stabilization of tubulin required for progression through the normal cell cycle.[82] This effect is similar to paclitaxel; however, in combination the two drugs behave antagonistically which suggests that arsenic may be a useful agent in patients with taxane-resistant tumors. [79]

Arsenic interactions with mutant proteins in Leukemia: PML-RARα

The key driver to leukemogenesis in APL is the PML-RARα fusion protein resulting from the reciprocal t(15;17) translocation. This translocation recruits histone deacetylases and other transcription repressors that inhibit normal RARα signaling which regulates myeloid differentiation. In APL, the disrupted RARα signaling prevents promyelocytes from differentiating to mature granulocytes. While the normal function of PML has not been fully elucidated, it appears to function as a tumor suppressor protein and has been tightly associated with p53 regulation of important cell processes like differentiation, senescence, and apoptosis.[83] PML is also fascinating in its localization to highly dynamic yet discrete nuclear structures known as PML nuclear bodies (NBs) which appear to play a role in stress response by regulating post-translational modification of a number of proteins. The PML-RARα fusion in APL disrupts the formation of NBs in the nucleus though treatment with arsenic trioxide reverses this abnormality. [84] Arsenic trioxide has been shown to induce apoptosis at high concentrations (1-2 μM) and promote cell differentiation at low concentrations (0.25-0.5 μM).[14]

The PML protein contains the RBCC motif characterized by an N-terminal RING finger motif, which is a cysteine rich type of zinc finger domain, two cysteine rich B box zinc finger domains, and a coiled coil domain which mediates homodimer formation.[85] The abundance of cysteine-containing zinc-binding sites in PML led researchers to hypothesize that arsenic may directly bind to the PML protein. Using a biotin-arsenic pull-down affinity assay fluorescent organic arsenic compounds, several groups have shown that arsenic co-localizes and directly binds to both wild-type PML and the PML- RARα fusion proteins.[86, 87] Zhang et al. used the purified PML RING peptide containing C3HC4 (aa 57-91) zinc finger for arsenic-binding analysis and with MALDI-TOF reported that one PML RING molecule was capable of binding two arsenic atoms. Near-ultraviolet absorbance spectroscopy showed the formation of arsenic-sulfur bonds while X-ray absorption spectra showed the local structures of the trivalent arsenics which were each coordinated by three cysteines in ZF1 and ZF2 of PML RING.[87] Upon binding to arsenic, PML RING is thought to undergo a conformational change that results in oligomerization, likely due to intermolecular As-S bonds. Arsenic-binding also promotes sumoylation by UBC9 which then recruits the 11S proteasome for degradation.[88] While these reports of *in vitro* arsenic activity strongly suggest a mechanism for arsenic-induced PML redistribution and degradation, it is unclear what effects the strongly reducing nature of the nucleus would have on these As-S and S-S interactions inside the cell.

Ongoing Clinical Trials

This Forum article is not intended to be an exhaustive review on the clinical use of arsenic in modern medicine, for that the reader should turn to a number of thorough reviews referenced here, especially those of Tallman,[12, 89] Chen,[14, 88] de Thé,[9] and Dilda.[90]Table 1 below summarizes much of the available data that has been published from the 129 clinical trials registered with the FDA.

There are currently 129 clinical trials registered with the FDA at www.clinicaltrials.gov that deal with arsenic. Of these 129 clinical trials, 107 trials incorporate arsenic trioxide or arsenic-based drugs as therapeutics for the treatment of cancer. Of particular interest are ZIO-101 (Darinaparsin) and GSAO (Figure 2), two novel organoarsenical drugs that are under evaluation for the treatment of solid tumors.[91] The cancer-related trials can subdivided into three groups: leukemias (75 trials), lymphomas (11 trials), and other cancers, mostly solid tumors (25 trials). There were several trials that dealt with more than one of these subgroups. Clearly hematological malignancies have been the major focus of clinical arsenic research with the largest number of trials involving acute promyelocytic

leukemia APL (18), multiple myeloma (16), myelodysplastic syndromes (13), non-APL acute myeloid leukemia (8), and chronic myeloid leukemia (6). In solid tumors, arsenic is has been used in glioblastoma (5) mostly as a radiosensitizer for radiation therapy.

The majority of the remaining trials (19 of 22) focused on chronic arsenic exposure, typically from groundwater contamination or occupational hazards while one ongoing trial is testing arsenic trioxide for the treatment of lupus.

Shortcomings of Arsenic Trioxide as an Anti-cancer Agent

Despite the strong efficacy of arsenic in hematological malignancies, free arsenic trioxide has a number of serious limitations. The primary limitation to wide use of arsenic trioxide as an anti-cancer agent is the systemic toxicity associated with large amounts of arsenic in the blood. Low doses of arsenic trioxide work well for treating hematological malignancies; however, solid tumors remain a treatment challenge. In the animal model, there have been numerous studies performed in breast, ovarian, and other cancers with positive results.[90] Unfortunately these successes have not translated into clinical success in human patients. This is likely due to the rapid renal clearance of arsenic metabolites which limits the concentration of arsenic that can be delivered to a tumor site.

Nanoparticulate Arsenic Compounds Designed to Improve Arsenic Delivery to Tumors

Novel arsenic sulfide preparations for the treatment of cancer

Recently, researchers have begun investigating arsenic sulfide compounds for their anti-tumor properties.[119, 120] In addition to new clinical trials taking place in China,[5] new compounds and formulations are also being developed. Wang and coworkers reported the first synthesis of realgar (As_4S_4) quantum dots produced from bulk realgar, and produced a stabilized colloidal formulation of these nanoparticles using ethylenediamine and acetic acid.[121] Further work has produced a hydrogel comprised of these realgar nanoparticles (Figure 6). The hydrogel formulation is pH responsive and has improved anti-tumor cell activity compared to the stabilized realgar nanoparticles.[122]

Arsenic nanobins show improved anti-tumor efficacy compared to arsenous acid

In order to extend the clinical utility of arsenic trioxide, our laboratory has developed a novel method for stably encapsulating arsenic trioxide inside nano-scale liposomes called nanobins.[123-129] The nanobins utilize a metal-ion gradient loading mechanism that relies on the reaction of arsenous acid with a divalent metal ion such as nickel(II), cobalt(II)[123] or platinum(II).[125]

The preparation of the nanobins proceeds by first dissolving lipids and cholesterol in chloroform. The lipid formulation is intended to be stable in the plasma and long-circulating, and so poly(ethylene glycol) and cholesterol are included. The lipids are dissolved at a molar ratio of 0.51/0.45/0.04 1,2-distearoyl-sn-glycero-3-phosphocholine/cholesterol/ 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]. The chloroform is then removed by rotary evaporation to form a lipid film. After overnight drying under high vacuum, the lipids are hydrated in a metal acetate, $M(OAc)_2$ solution, typically nickel(II) acetate or *cis*-diaqua-diammine platinum(II) acetate. The lipid solution is then subjected to high-pressure extrusion to size the nanobins, after which any external metal solution is removed and replaced by buffer, using either size-exclusion chromatography or tangential flow filtration. Arsenic trioxide solution is then added to the exterior of the nanobins, and is allowed to load for up to 12 hours at elevated temperature.

Arsenic loading is driven by the complexation and precipitation of arsenic with the metal ions inside the nanobins, and the efflux of protonated acetic acid from the nanobin (Figure 7). The complexation of arsenic with metal salts is required for stability, otherwise encapsulated arsenic will leak rapidly from the liposome.[130]

This loading mechanism is capable of concentrating arsenic inside the liposome to > 270mM as a solid, crystalline payload. If this amount of arsenic were to be dissolved in the volume of a mammalian cell, the resulting concentration would be on the micromolar scale, very likely resulting in cell death. The presence of a lipid bilayer surrounding the arsenic payload greatly attenuates the toxicity of the arsenic, reducing the toxicity 3-4 fold in cell culture experiments compared to arsenic trioxide.[124-126]

The nanobins have been shown to improve the anti-tumor efficacy of arsenic trioxide in a mouse model of breast cancer.[126] In this model, triple-negative breast cancer cells were implanted in the 4th mammary fat pad of nude mice and allowed to grow for two weeks. The animals were then treated with equivalent amounts of arsenic trioxide or nickel-arsenic nanobins [NB(Ni,As)]. The nanobin treatment group showed durable reduction in tumor growth compared to the arsenic trioxide group, which showed no statistically significant difference from the untreated control groups. This improved efficacy was due to the increased tumor accumulation of arsenic where the nanobins were able to deliver 2-3 times more arsenic to the tumors. The nanobins also reduced the arsenic clearance rate from the plasma by over 300-fold, greatly increasing the exposure of the tumor to the drug.

The nanobin formulation is analogous to liposomal doxorubicin, sold as Doxil (Janssen) or Lipodox (Sun Pharma Global FZE) in the United States, wherein doxorubicin is stably encapsulated inside a liposome using a pH gradient created by encapsulated ammonium sulfate. Our laboratory uses similar processing technology to manufacture the nanobins, which ensures consistent and homogeneous particle size and drug loading

These nanobins are stable, with a greater than six month shelf-life, likely due to the presence of a direct arsenic-metal bond. Previous attempts to encapsulate arsenous acid had very short shelf lives, because the arsenic would easily leak out of the vesicles.[131] In the case of the diaqua-cisplatin arsenic nanobins, the arsenic reacts directly with the platinum center, resulting in a platinum-arsenic bond length of 2.32 Å.[125] This structure was elucidated by EXAFS, and has spurred further research in our group into these arsenic-platinum bonds.

Coordination Chemistry of Arsenous Acid and Design of New Pt-As Anticancer Agents

Platinum compounds with arsenous acid were not known in the literature until above mentioned nanobins were formulated. The coordination chemistry of arsenous acid is not well understood since only two complexes of arsenous acid, one with nickel and one with palladium, have been isolated and characterized by X-ray so far. The complex of arsenous acid with nickel(II) is a heterometallic supramolecular adduct with cucurbit[6]uril (cuc), $[W_3(Ni(As(OH)_3)S_4(H_2O)_8Cl])\cdot cuc\cdot 13H_2O$, in which arsenous acid is coordinated through arsenic(III) acting as a Lewis base (Figure 8).[132, 133] In this complex, the arsenic center has trigonal pyramidal geometry with the Ni^{II}-As^{III} bond length of 2.225(4) Å. In the arsenous acid complex with palladium, $[Mo_3(Pd(As(OH)_3)S_4(H_2O)_6Cl_3]_2Cl_2\cdot C_{36}H_{36}N_{24}O_{12}\cdot 19H_2O$, the geometry around the arsenic center is more tetrahedral with O-As-Pd bond angle of 113.8° and O-As-O bond angle of 104.7°.[134] The observed Pd-As bond length is 2.368(3) Å.

A new synthetic approach to combine platinum and aqueous form of arsenic trioxide in one molecular compound has been recently discovered.[135] The new complexes were synthesized

from cisplatin and arsenic trioxide in water-nitrile mixture. In arsenoplatin-1 (1) (Figure 9) platinum forms expected square planar geometry, but arsenic(III) forms trigonal bipyramidal geometry with arsenic(III) acting unexpectedly as a Lewis base and as a Lewis acid simultaneously.[135] Multiple interactions of arsenic(III) are partly responsible for the very short Pt^{II}-As^{III} bonds in these complexes which are in 2.2687(4)- 2.2732 (3) Å range. The shortest bond obtained is just 0.0012 Å longer than the shortest Pt^{II}-As^{III} bond found in one organoarsenical compound according the CSD. The Pt^{II}-As^{III} bond in nanobins is 2.3 Å on the basis of EXAFS. In arsenoplatins this bond is even shorter reflecting the stability of Pt^{II}-As^{III} core in arsenoplatins.

Stability of the Pt^{II}-As^{III} core is confirmed in substitution reactions in aqueous solution. Substitution of chloride ion in arsenoplatin-1 with thiocyanate ion in water occurs immediately at room temperature, and the addition of $AgNO_3$ to facilitate substitution is not necessary (Scheme 1). [135]

The rapid substitution is likely driven by the strong *trans* effect of the arsenic moiety. Obtained SCN^-derivate undergoes isomerization in DMSO solution, and the equilibrium mixture contains 64 ± 1.2% of S-isomer and 36 ± 1.5% of N isomer. In these substitution/ isomerization reactions the Pt^{II}-As^{III} core remains intact, confirmed on the basis of a single crystal X-ray structure of the SCN^- derivate. The stability of the core is caused by arsenic(III) forming multiple interactions; with platinum(II) acting as a Lewis base, and with oxygen atoms from acetylamido moieties acting as a Lewis acid, which leads to the formation of two chelate rings in the molecular structure. Arsenoplatins are stable in saline solutions. These complexes are soluble in DMSO, methanol, and partially in water.[135]

The idea behind the synthesis of arsenoplatins was to design a new type of compounds containing both arsenic(III) and platinum(II), which would have potential to overcome resistance obstacles.[136-138] When the cytotoxicity of arsenoplatins was investigated against various cancer cell lines, we found that in some cell lines arsenoplatin compounds were more cytotoxic than cisplatin. In the case of ovarian cisplatin resistant $A2780^{CP}$ cell line arsenoplatin-1 was two times more cytotoxic than cisplatin with the resistant factor (RF) of 1.1, where RF < 2 denotes non cross- resistance.[139-142]

The anticancer efficacy of arsenoplatins may be the result of the strong *trans* effect of arsenic moiety combined with the *trans* stereochemistry of N-atoms at the platinum center. The new coordination mode of arsenous acid discovered in our lab will trace a path for the syntheses of new small molecular compounds, derivates of the parent compounds, with different structural motifs and with different modes of action. Investigation of arsenoplatin compounds *in vivo* is an ongoing project.

Outlook for Arsenic in Therapeutic Agents

The complex nature of arsenic's mechanism of action suggests that there will be many uses of arsenic in medicine in the future. The development of delivery vehicles or molecules that will prevent systemic toxicity while delivering efficacious doses of arsenic to disease sites is a promising method that may help expand the medical use of this imporant metalloid. Previously, numerous organic arsenic compounds were developed to reduce toxicity and improve efficacy, with mixed results. In light of these new inorganic arsenic-metal compounds, perhaps arsenoplatins and other coordination compounds represent a new class of arsenic drugs that will prove useful for arsenic therapy in the future.

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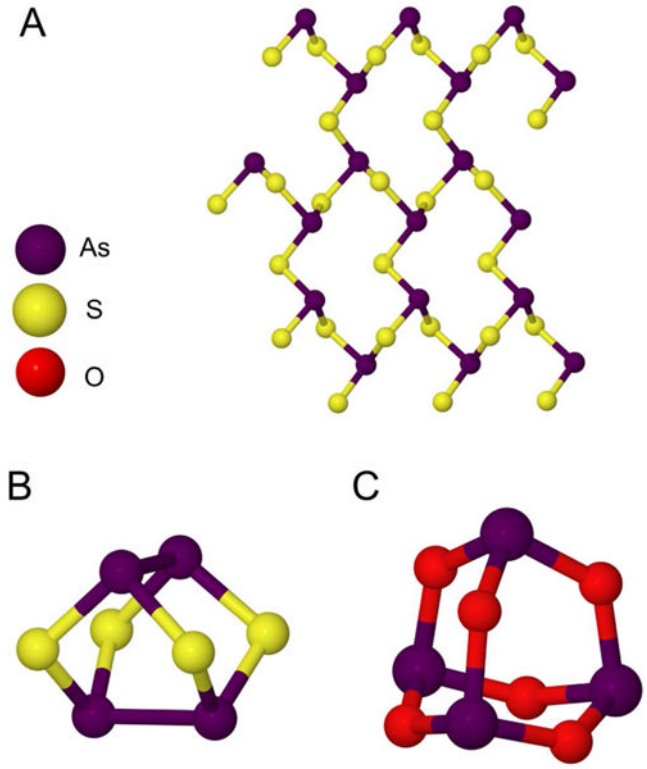


Figure 1.
Structure of major arsenic ores. A) Orpiment. B) Realgar. C) Arsenic trioxide or “white arsenic”. [143, 144]

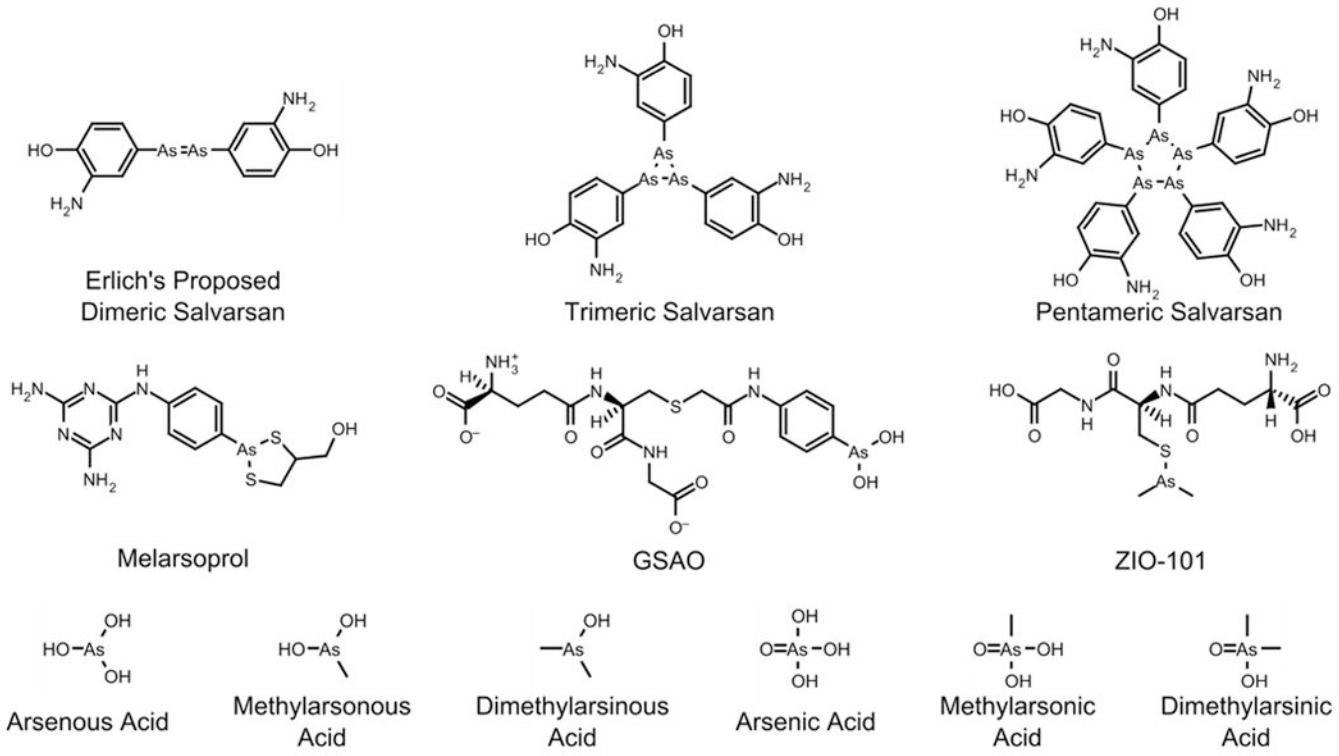


Figure 2.
Structures of relevant arsenic compounds.

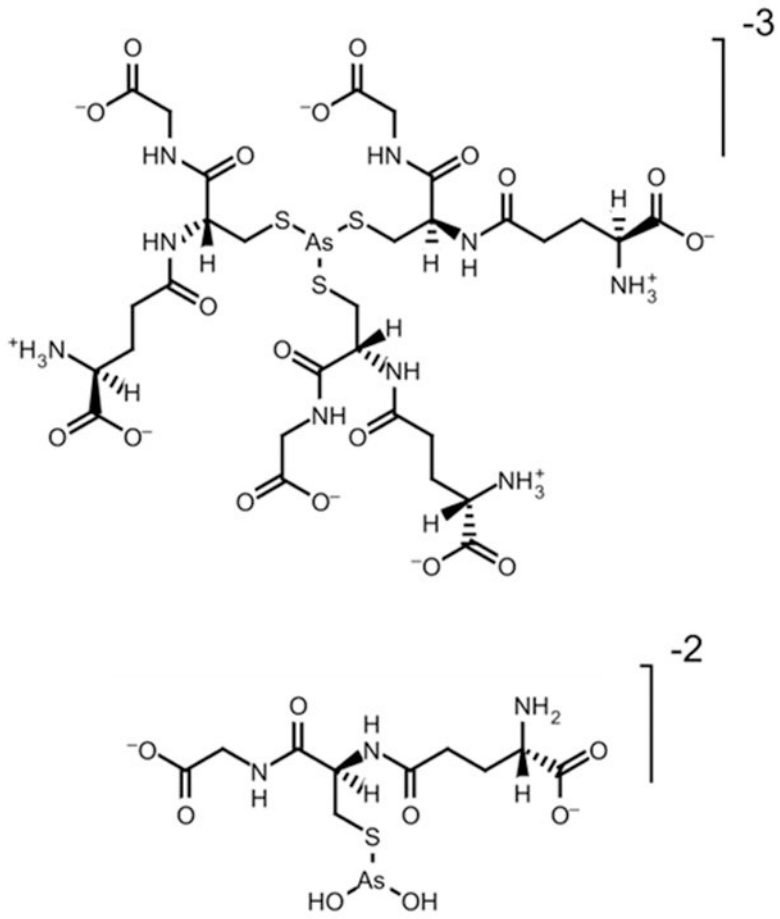


Figure 3.
Proposed structure of arsenic-glutathione complexes.[46]

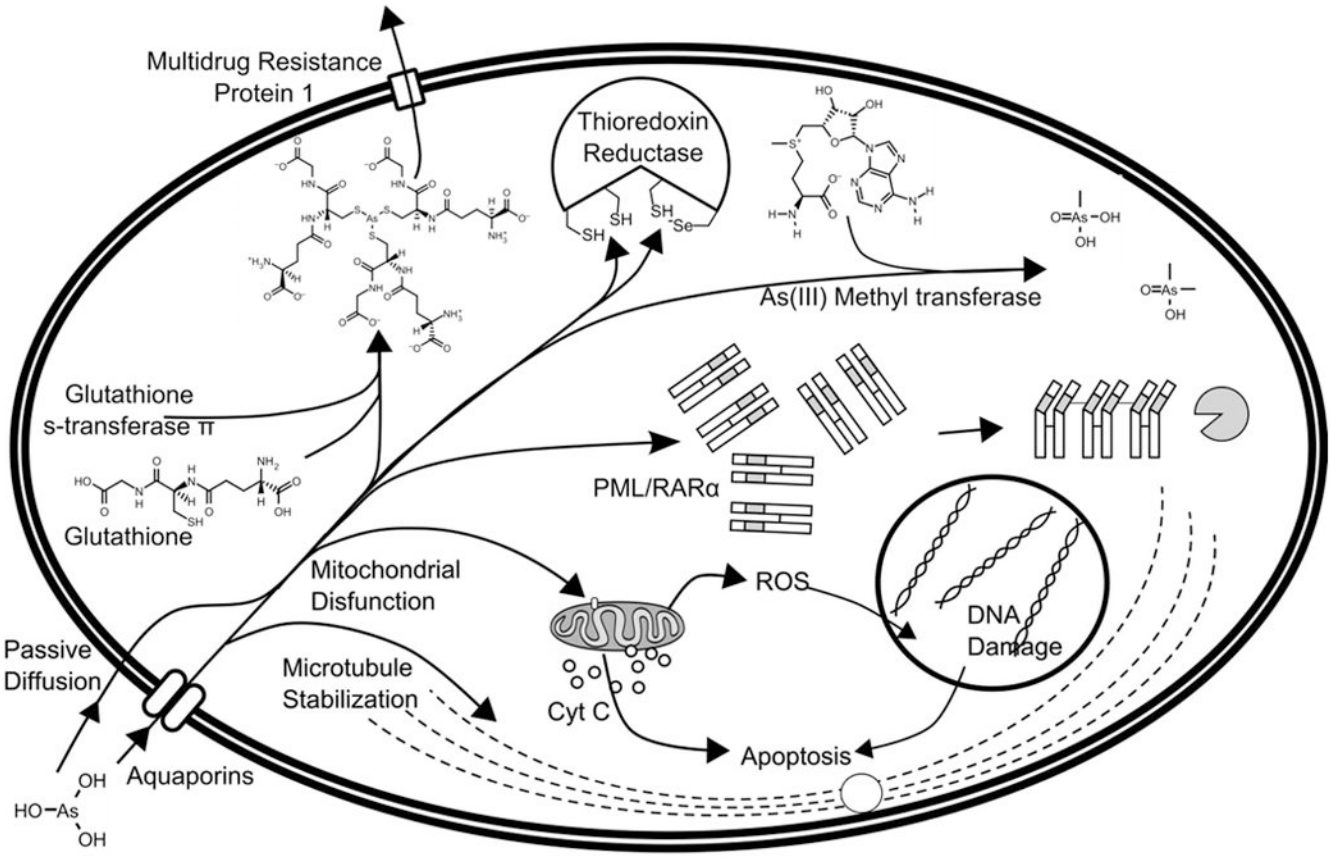


Figure 4.

Proposed mechanisms of action for arsenic trioxide. Arsenous acid enters mammalian cells either by passive diffusion, or by the neutral solute transporter proteins in the aquaporin family.[51, 52, 55, 56] It then binds to the numerous thiol moieties present in the cell, particularly glutathione, the interaction of which is catalyzed by glutathione s-transferase.[65] Arsenous acid can bind to microtubules, preventing depolymerization and halting the cell cycle.[79] Arsenic also binds to the active site of thioredoxin reductase, which in turn causes the buildup of oxidized, mis-folded proteins in the cell.[78] In acute promyelocytic leukemia, arsenic binds to a zinc finger fusion protein, PML/RARα, which causes aggregation and degradation of the protein.[86, 87] Arsenic can be metabolized into methylarsonic acid or dimethylarsinic acid.[62] Arsenic interacts with mitochondrial proteins to collapse the mitochondrial membrane potential, produce reactive oxygen species (ROS), release cytochrome c into the cytoplasm and initiate apoptosis.[73]

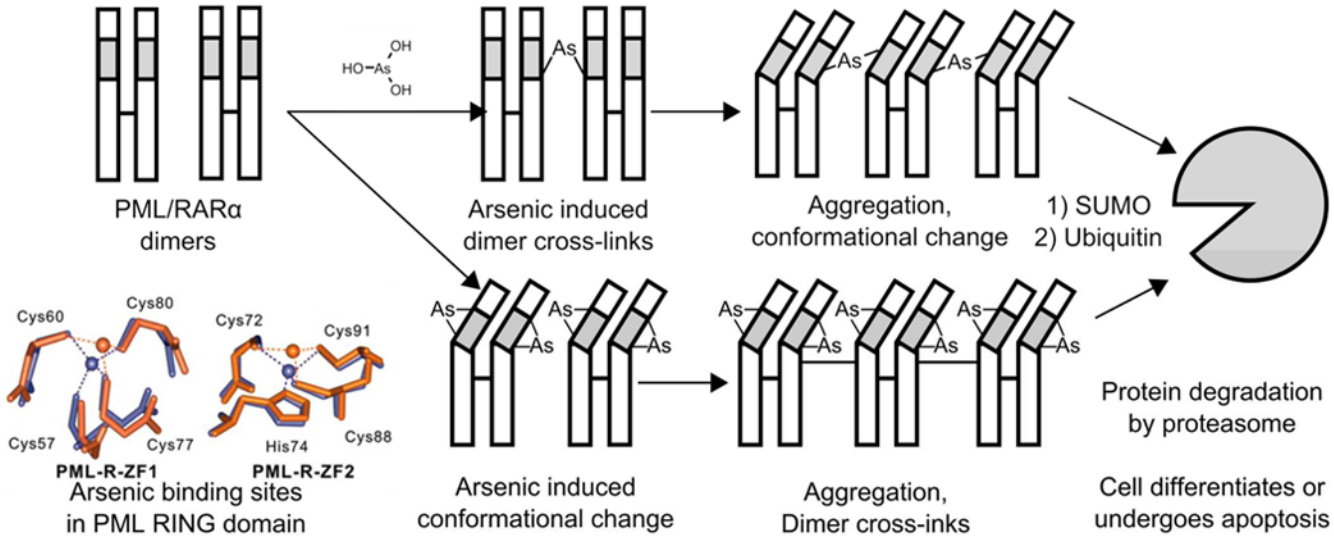


Figure 5.

Mechanism of PML/RARα degradation. It is unclear in which order these processes occur, however, arsenous acid may induce crosslinking between PML/RARα dimers which results in a conformational change, or it may induce a conformational change in PML/RARα dimers that causes aggregation. The fate of the aggregated PML/RARα is degradation in the proteasome, which results in cell death or differentiation. Inset, the change of coordination geometry when arsenic is bound to the RING domain of PML (orange) compared to the zinc binding (blue).[84, 86, 87]

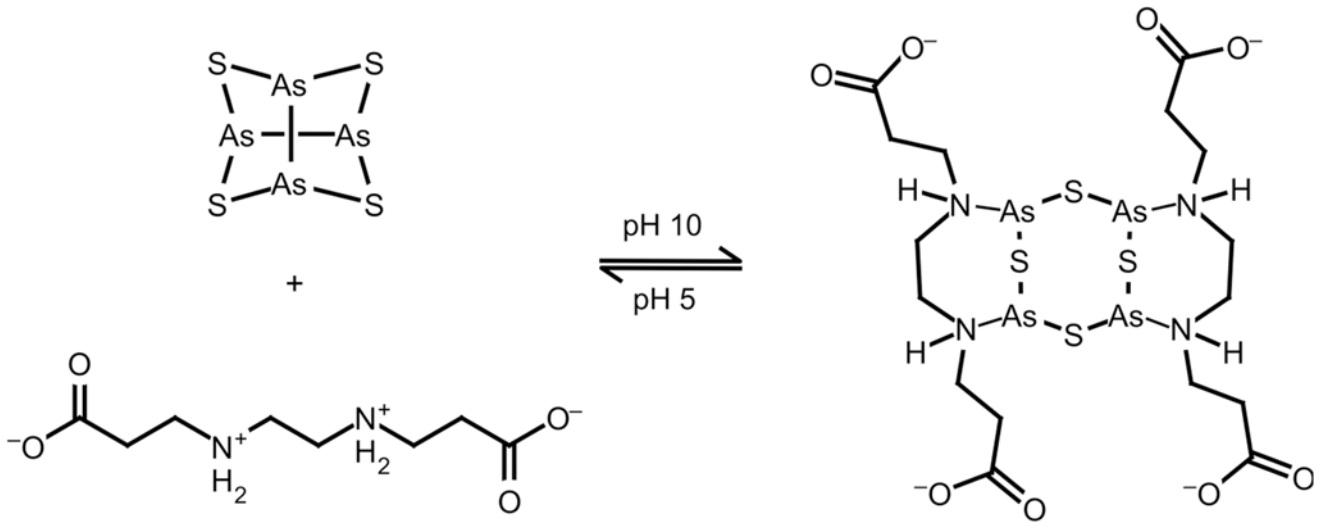


Figure 6.
Arsenic sulfide nanogel assembly is driven by coordination of the arsenic centers by nitrogen ligands and hydrogen bonds between the chelator molecules.[122]

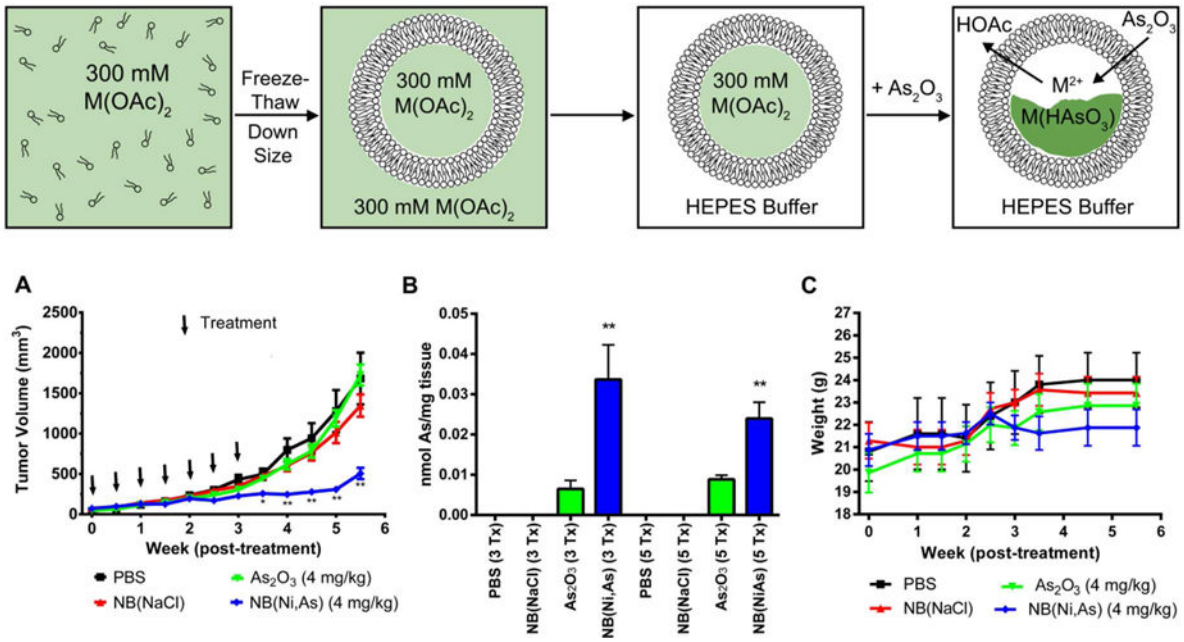


Figure 7.

Preparation and use of arsenic nanobins. Top: Synthesis scheme of arsenic nanobins. M = Ni^{2+}, $[Pt(NH_3)_2(OH_2)_2]^{2+}$ A)Tumor volume plot of human triple-negative orthotopic xenograft tumors implanted in the 4th mammary fat pad of female nude mice showing decreased tumor volume in nanobin treated mice vs. mice treated with arsenic trioxide. B) Nanobin treatment increases total amount of arsenic in the tumor compared to arsenic trioxide treatment. C) Stable animal weight during treatment indicates all treatments were well tolerated.[126]

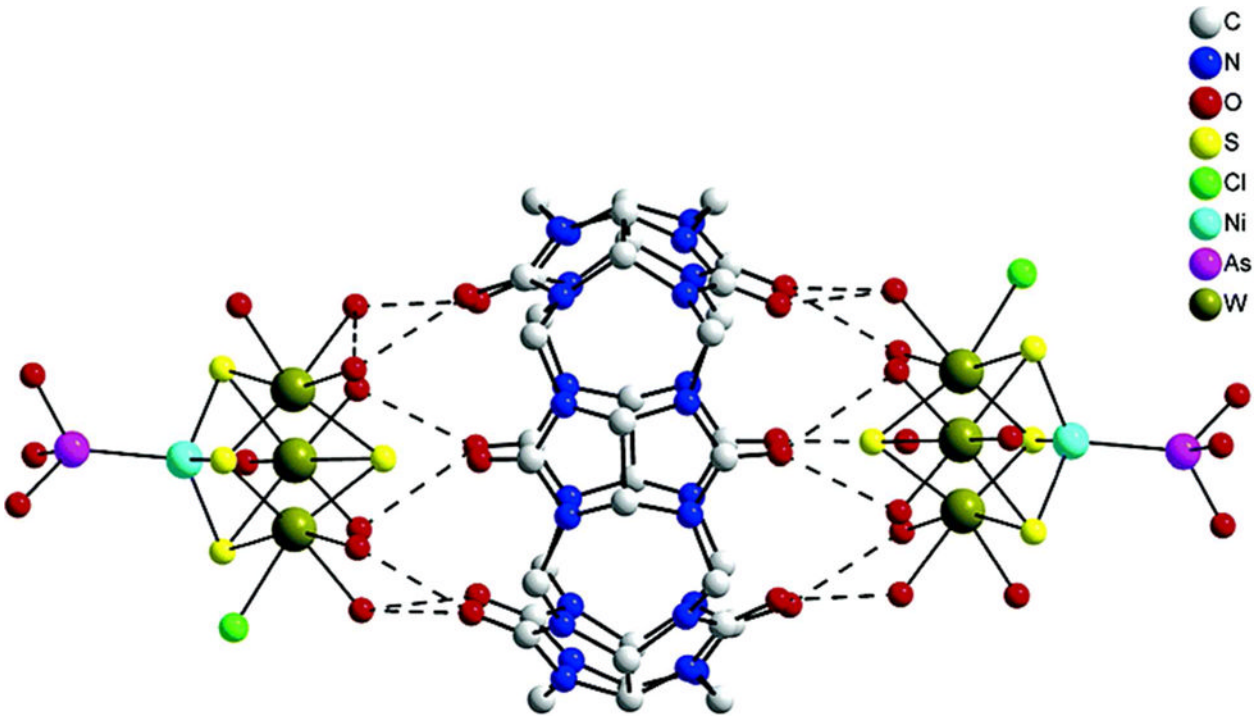


Figure 8.
Structure of supramolecular complex including arsenic-nickel coordination.[132]

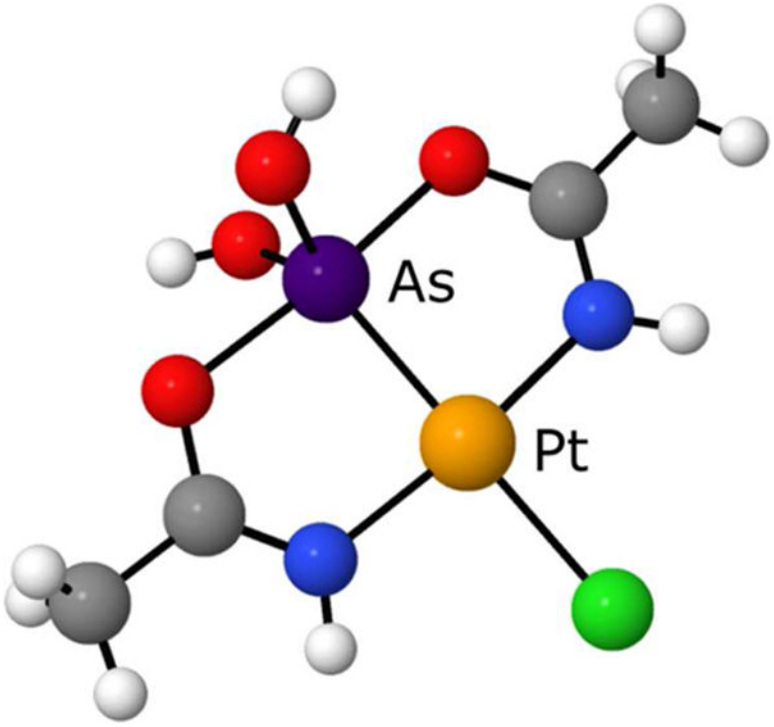
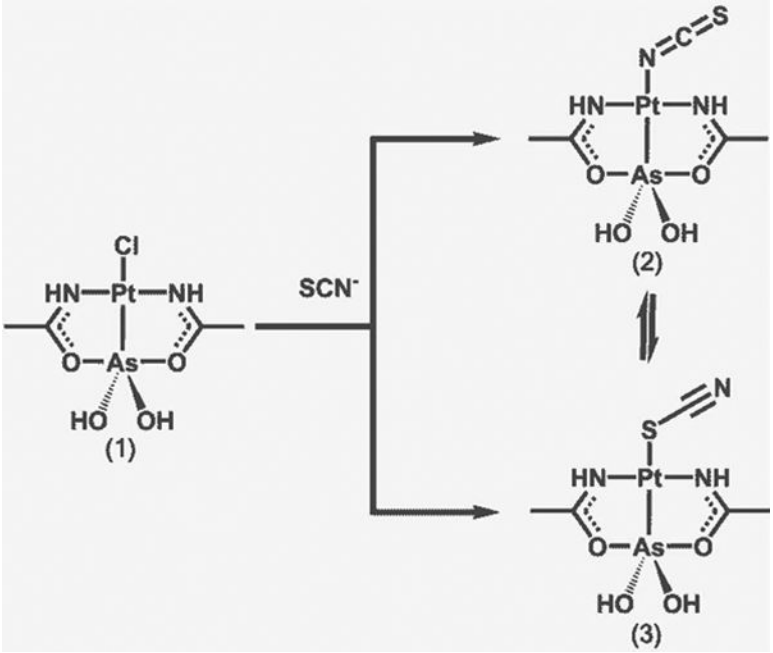


Figure 9.
Structure of Arsenoplatin 1 based on single-crystal X-ray crystallography. Unlabeled atoms are colored according to the key in Figure 8.[135]



Scheme 1.

Stability of the As-Pt bond in Arsenoplatin 1. The strong *trans* effect of As enables rapid displacement of the chloride ligand with thiocyanate. Both the thiocyanate and isothiocyanate species are present in DMSO.[135]

Table 1

Clinical trials for arsenical drugs in cancer registered with the Food and Drug Administration. For more details see http://www.clinicaltrials.gov

Malignancy	Number of Trials	Treatment	Comments	Ref
Hematological	**86**			
Acute Promyelocytic Leukemia (APL)	18	ATO most commonly used for induction of refractory APL or as part of combination therapy (induction and/or consolidation)for newly-diagnosed APL	Most effective single-agent therapy; very effective with no major side effects	15,16,92-94
Multiple Myeloma	16	ATO monotherapy or in combination with ascorbic acid and/or thalidomide; ZIO-101	Moderate activity and reasonably well tolerated; NR	95-98
Myelodysplastic Syndromes (MDS)	13	ATO monotherapy or in combination with chemotherapy	Moderate activity and reasonably well tolerated	99-102
Lymphoma	11	ATO monotherapy or in combination with other agents	Partial and/or transient response with variable toxicity	103,104
Acute Myeloid Leukemia (AML)	8	ATO monotherapy	Variable response	105-109
Chronic Myeloid Leukemia (CML)	6	ATO monotherapy or in combination with imatinib	Partial response and well-tolerated	110
Solid Tumors	**25**			
Glioma	5	ATO and radiotherapy	Effect on tumor vasculature; well-tolerated	111,112
Various Advanced Solid	3	ATO; ZIO-101; GSAO	NR; Early evidence of clinical activity; NR	91
Liver	2	ATO monotherapy	Not active	113
Lung	2	ATO monotherapy	NR	
Skin	2	ATO monotherapy	Well-tolerated; not active	114
Breast	1	ATO monotherapy	NR	
Colorectal	1	ATO and 5-Fluorouracil	Moderate activity in refractory cases	115
Pancreatic	1	ATO monotherapy	Not active	116
Cervical	1	ATO monotherapy	NR	
Prostate	1	ATO monotherapy	Decreased PSA markers	117
Kidney	1	ATO monotherapy	No response	118