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Realgar and arsenene nanomaterials as arsenic-based anticancer agents

Sophia E. Hollow and Timothy C. Johnstone

Abstract

Arsenic trioxide (ATO) is an approved therapy for the treatment of acute promyelocytic leukemia, but the extension of arsenic-based therapies to other types of malignancies, notably tumor-forming cancers, has been slow. Nanodelivery vehicles offer a means of effectively delivering ATO to tumors. Very recently, there has been a series of developments in the formulation of arsenic-based nanomedicines that are not simply loaded with ATO. Realgar nanoparticles are comprised of molecular As_4S_4 units. Current studies suggest that realgar nanoparticles ultimately act in a manner similar to ATO, but with greatly attenuated toxic side effects. A drastically different approach is taken with arsenene nanosheets, a 2-dimensional form of elemental As. The electronic properties of this material allow it to mediate both photothermal therapy and photodynamic therapy. The exploration of these nanomaterials is still in its infancy but is poised to allow arsenic-based therapy to make yet another significant impact on cancer treatment.

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Keywords

Arsenic, Realgar, Arsenene, Nanoparticle, Anticancer.

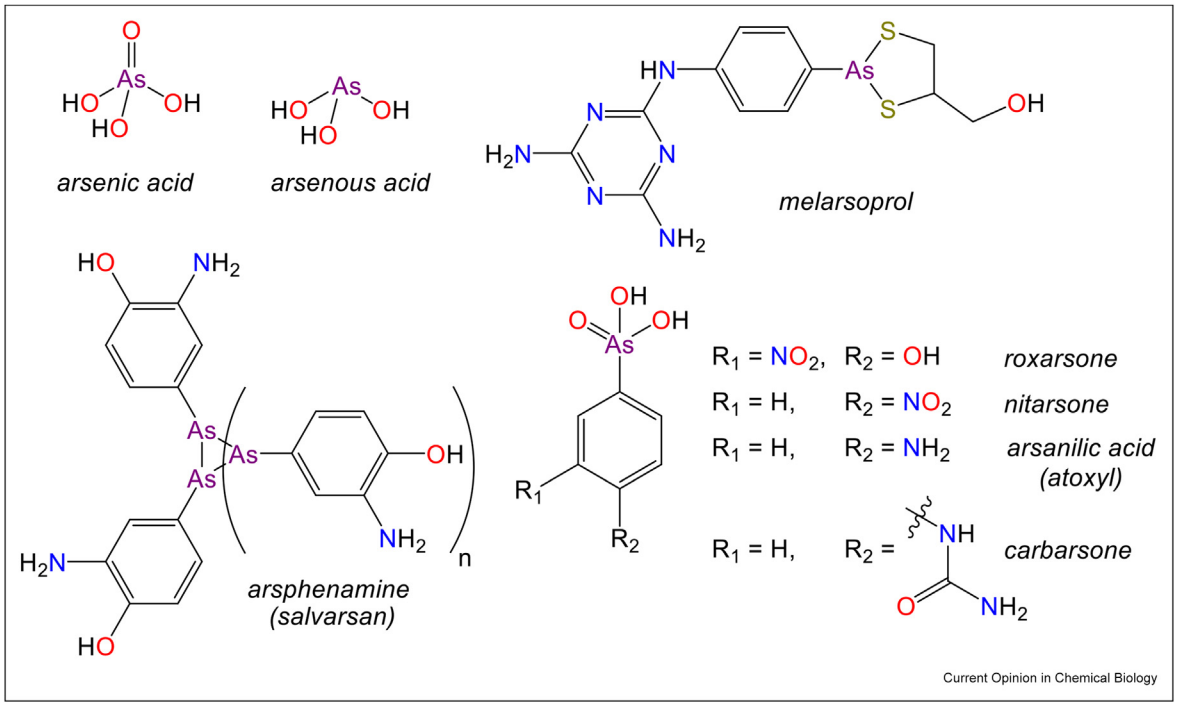
Introduction

Arsenic compounds have a long and complex relationship with human health, which has been recently reviewed [1,2]. Extensive toxicological and medicinal studies have been performed on compounds containing As(III) and As(V), including arsenic acid (H_3AsO_4) and arsenous acid (H_3AsO_3), their oxyanion conjugate bases, and their organic derivatives. Examples include the As(III)-containing antitrypanosomal agent melarsoprol [3]; the antiparasitic As(V)-containing arsonic acids roxarsone, nitarsone, carbarsone, and arsanilic acid [4]; and the As(I)-containing antisyphilitic species arsphenamine (Figure 1) [5]. Although organoarsenic compounds have provided a rich source of biological activity, the most successful example of the modern use of arsenic in medicine is the discovery that arsenic trioxide (ATO) can be used to treat acute promyelocytic leukemia (APL). The advent of combination therapies employing ATO and all-*trans* retinoic acid (ATRA) has allowed complete remission rates for APL to exceed 90% and 5-year overall survival rates to reach 85–99% [6–8]. We note that although ATO is a solid with the composition As_2O_3, it dissolves in water to form arsenous acid (Figure 1). The history of the modern application of ATO to treat APL and the origins of this therapy in traditional Chinese medicine have been well documented [9,10].

Like many other medicinal inorganic compounds, arsenic drugs have benefited from the advancements in nanoparticle drug delivery that began near the turn of the 21st century. Arsenic compounds have been delivered using, among others, liposomes, polymer nanoparticles, and inorganic nanoparticles [11,12]. Conjugation of targeting units to the surfaces of these nano-sized objects allows them to be targeted to cancer cells both *in vitro* and *in vivo* [12]. These constructs are typically loaded with the arsenic agent in the form that it would otherwise be administered, which is then released in a controlled fashion.

Here we will discuss recent developments in the anticancer potential of nanoparticles formed from realgar (As_4S_4) and arsenene (a 2-dimensional allotrope of elemental As) (Figure 2). In these works, the arsenic-containing species is not a molecule that is loaded into a nanoparticle delivery device, but is rather the solid that comprises the nanoparticle itself. Compared to a vehicle/cargo paradigm, the reduced number of components can potentially increase robustness, but can also reduce the capacity of the construct to be tuned. In the case of realgar nanoparticles, biological activity is proposed to stem from conversion of the formally As(II) centers in the solid to As(III) and release of biologically-active arsenous acid. In contrast, arsenene nanoparticles

Figure 1



A selection of prominent arsenic compounds whose biological activity has been exploited.

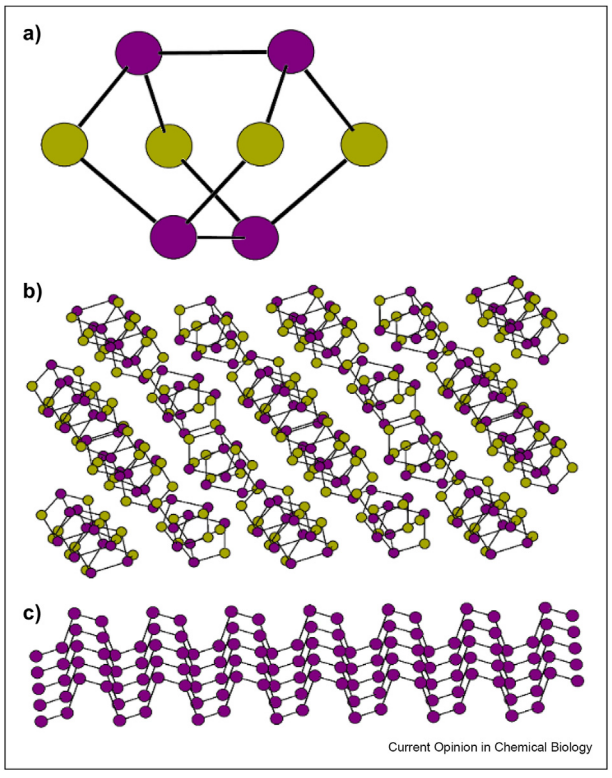
do not release bioactive As centers but instead function as photodynamic therapy agents because of their unique electronic properties. Herein, we will discuss reports of the anticancer applications of these materials that have appeared within the past two years. We will also provide our assessment of the potential for further development in this area.

Realgar

Realgar is a mineral consisting of molecular As_4S_4 units. Like ATO, the anticancer application of realgar has its roots in traditional Chinese medicine. It is believed to act as a controlled-release formulation of the same reactive As(III) centers that are generated by ATO (Figure 3) [13]; knockdown of proteins that mediate uptake of soluble As(III) centers decreases the efficacy of realgar [14]. Although many of the studies on realgar focus on its use to treat APL, we note that recent studies have also examined the clinical efficacy of realgar in the treatment of myelodysplastic syndrome [15–17]. Based on its historical formulation in traditional Chinese medicine, realgar is typically administered as realgar/indigo naturalis formulation (RIF): a mixture of realgar, indigo naturalis (a mixture containing, *inter alia*, indigo and indirubin), and extracts from red sage (*Salvia miltiorrhiza*) and false starwort (*Pseudostellaria heterophylla*). The clinical efficacy of orally administered RIF has been studied extensively [18]. The most recent guidelines for APL treatment endorsed by the Chinese Society of Hematology and the Chinese Medical Association, include not only ATO but also "compound huangdai tablets," an orally administered form of RIF that is commercially available in China [19]. A very recent meta-analysis of data from 12 studies involving a total of 775 patients with APL revealed that oral RIF was as effective as intravenous ATO with regards to complete remission rate, relapse rate, mortality, complete remission time, and 2-year disease-free survival [20]. RIF may also be particularly advantageous for pediatric APL patients [21]. It is notable that the dosages of RIF can approach up to 10 g d^{-1}, as compared to a typical ATO dosage of approximately 10 mg d^{-1} [19]. We note that the larger doses used in RIF treatment stem from the lower bioavailability of the As when realgar is administered orally.

To combat this low bioavailability, realgar nanoformulation strategies have been developed, including wet processes [22], gel methods [23], and direct grinding. Within the two-year scope of this review, synthetic developments have centered around the latter method as described below. Realgar nanoparticles with diameters ranging from 70 to 150 nm were able to effectively inhibit the proliferation of lung cancer stem cells *in vitro* and lung tumor xenograft growth in a mouse model [24]. The suspension of nanoparticles was

Figure 2



Ball-and-stick structures of the nanomaterials that will be discussed in this Current Opinion: (**a**) the molecular As_4S_4 unit of realgar, (**b**) the extended solid-state structure of realgar, and (**c**) the corrugated-sheet solid-state structure of arsenene. Color code: As purple, S yellow.

administered by oral gavage but it is noteworthy that no comparison was made to a typical realgar administration, so it is unclear how much of the effect derives from the nanoformulation. Another study demonstrated that realgar nanoparticles are able to inhibit the migration and invasion of breast cancer cells in a mouse model [25]. A recent study drew attention to the fact that if nanoparticulate realgar is prepared via ball-milling in an aerial atmosphere, then a significant amount of ATO is generated, which impacts the biological activity of the material. If that nanoparticulate realgar is, however, subsequently ground and rinsed with acidic water, the level of ATO contamination is drastically reduced and the effects of this contamination on the growth of cultured cancer cells is reduced [26]. The influence of nanoparticle size on cellular response has yet to be systematically studied, but cultured cells treated with 6 nm diameter realgar nanoparticles increased their expression of autophagy-related proteins and elicited a microscopically observable autophagic phenotype [27].

Very recently, a theranostic platform was developed in which ethanolamine-modified realgar nanoparticles were encapsulated by a PEG-linked phospholipid and further functionalized with hyaluronic acid [28]. The authors exploited the intrinsic fluorescence of the realgar nanoparticles to follow their accumulation in tumor tissue and their inhibition of tumor growth in a mouse xenograft model.

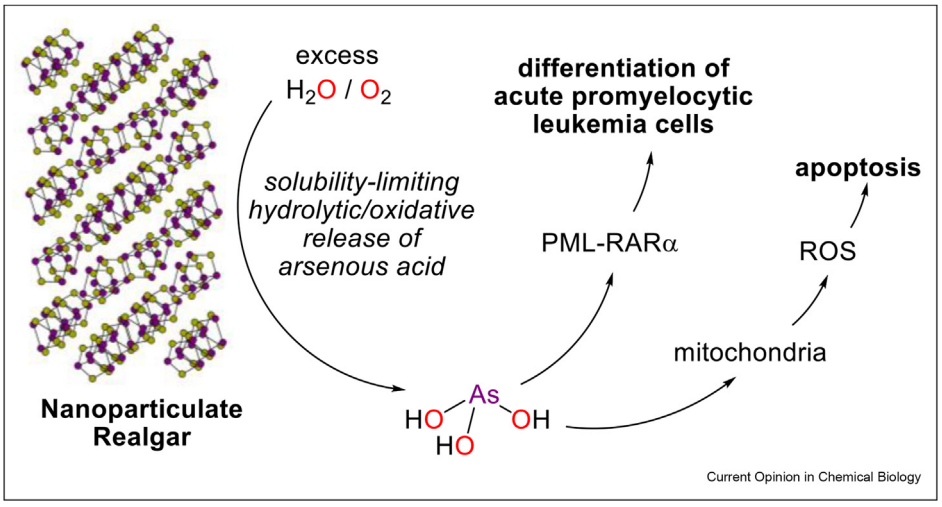
A final study investigated the combined effect of treatment with realgar nanoparticles and solid lipid nanoparticles that were loaded with toad venom [29]. The toad venom comprises secretions from *Bufo bufo gargarizans* Cantor and *Bufo melanostictus* Schneider, the primary active components of which are cinobufagin and resibufogenin. Prior *in vitro* and *in vivo* studies had demonstrated that these agents can be effective in inhibiting the growth of gynecological tumors. The solid lipid nanoparticles and realgar nanoparticles were co-embedded within a thermosensitive hydrogel made from poloxamers 188 and 407. Release of the drugs obeyed first-order kinetics and the drug-loaded hydrogel exhibited robust bioadhesion to vaginal tissue in mice [29]. Further work needs to be done to assess whether this formulation exhibits any synergism between the realgar and the toad venom.

Arsenene

Arsenene has many interesting optical and electronic properties, and we direct the reader to an excellent recent review of the synthesis and properties of this substance [30]. Arsenene has been heavily investigated for its materials applications, but in 2020 liquid-exfoliated arsenene nanosheets were investigated as anticancer agents for the first time [31]. Incubation of NB4 APL cells with these arsenene nanosheets resulted in the generation of reactive oxygen species (ROS) and cell death via apoptosis. In contrast to following work, this ROS generation occurred in the absence of photostimulation. We also note that Raman spectroscopy demonstrated that, during the liquid exfoliation process, the arsenene was partially converted to an oxidized form. A separate study of arsenene nanoparticles that were half-coated with elemental Pt found them to be non-toxic to cultured cancer cells up to 1.25 mg mL^{-1} [32]. The half-coating with Pt allowed these Janus particles to act as molecular motors that consume H_2O_2 as fuel, which was purported to help the particles deliver doxorubicin into A549 cells, but too little detail was provided to assess the validity of these claims.

In 2021, arsenene nanosheets were functionalized with PEG to enhance dispersibility, conjugated to PSMA-targeting antibodies to confer prostate cancer specificity, and loaded with doxorubicin for proof-of-concept combination treatment [33]. The construct was able to induce cell death in cultured prostate cancer cells via ferroptosis, a cell-death pathway characterized by excessive lipid peroxidation via ROS. Again, this activity was achieved in the absence of direct light stimulation.

Figure 3



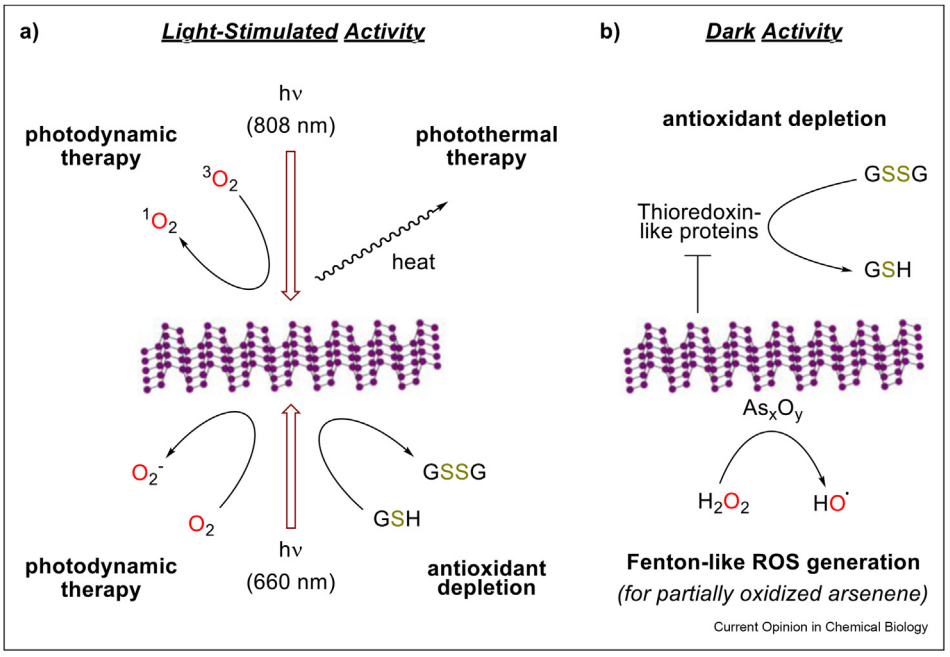
Proposed mechanism of action of realgar as a controlled-release delivery vehicle for arsenous acid, the active species generated by ATO.

In a PC3 xenograft model, the construct was able to elicit greater tumor growth inhibition than any of the components alone and immunohistochemical analysis of the tumor tissue confirmed that ferroptosis was the active pathway. A very recent study combining proteomic and single-cell RNA sequencing techniques revealed that arsenene disrupted cellular redox balance by interacting with thioredoxin-like proteins and ultimately led to a remodeling of the tumor microenvironment by recruiting immune cells to the tumor area [34]. This work highlighted the important role that immunogenic cell death can play in the action of arsenic-based nanomedicines, an area that deserves increased research attention.

To address whether the formation of oxidized As centers at the surface of arsenene nanosheets plays a role in anticancer activity, the authors of a subsequent study included ascorbic acid as a reductant in the exfoliation step [35]. The resulting 3 nm diameter arsenene particles were treated with DSPE-PEG to facilitate water dispersion and exhibited a dose-dependent killing of cultured cancer cells. Cells treated with the nanoparticles exhibited increased ROS production and the construct was able to induce cell-cycle arrest, metabolic alteration, growth inhibition, and, ultimately, cell death. Importantly, this work demonstrated for the first time that, in addition to their apparent light-independent activity, the unique electronic properties of arsenene allow it to act as a photothermal therapy agent (Figure 4). Irradiation of an aqueous suspension of the nanoparticles with 808 nm light (1 W cm^{-2} for 5 min) could raise the temperature of the water by nearly 40 °C. Very high doses (10 mg kg^{-1}) were found to be non-toxic in mice, even upon repeated daily injection. When injected into the tumors of mice bearing 4TI tumors (40 μL of a 1 mg mL^{-1} suspension) and irradiated with 808 nm light at 1 W cm^{-2} for 30 s, the temperature of the tumor rose to 60 °C. This treatment successfully eliminated the tumors and there was no recurrence after 2 weeks.

In a subsequent study, another group of investigators chose to exploit the partial surface oxidation of arsenene nanosheets that were approximately 90 nm wide and 3 nm thick [36]. The partial oxidation was achieved simply by performing the exfoliation under aerobic conditions. Raman spectroscopy, XPS, and XRD confirmed that regions of both As_2O_3 and As_2O_5 formed. Electrons that are excited from the conduction band of the arsenene with 660 nm light can transfer into the conduction band of the As_xO_y, which can then reduce O_2 to O_2^-, increasing levels of oxidative stress (Figure 4). The holes remaining in the valence band of the arsenene can react with cellular antioxidants such as glutathione, reducing the ability of these species to deactivate the newly generated superoxide (Figure 4). Energy conversion pathways are also operative and can generate $^1O_2$ at As(0) centers of the nanosheets. These partially oxidized nanosheets still maintain the photothermal capabilities described above when excited with 808 nm light. In the absence of light, they disrupt the redox balance of the cells by interfering with thioredoxin-like proteins, as described above, and also engage in Fenton-like chemistry at the sites of partial oxidation (As_xO_y). Intravenous administration (2 mg kg^{-1}) followed by irradiation with 660 nm and 808 nm light resulted in complete eradication of the tumor tissue in an MCF-7 xenograft model with no recurrence over 2 weeks and 100% survival.

Figure 4



Proposed photo-stimulated and dark modes of anticancer action of arsenene nanosheets and partially oxidized arsenene nanosheets. **a**) Arsenene nanosheets are active upon irradiation with 808 nm light, whereas partially oxidized arsenene nanosheets are active upon irradiation with either 808 or 660 nm light. **b**) Arsenene activity in the absence of light stimulation.

Outlook

The approval of ATO for the treatment of APL in the United States at the turn of the 21st century and the approval of darinaparsin for the treatment of relapsed or refractory peripheral T-cell lymphoma in Japan this past year highlight that arsenic does not exist on the fringes of medicine. Although the chemistry of this element can confer toxicity, it can also be harnessed for therapeutic benefit. In this Current Opinion, we have described recent promising developments in arsenic-based nanotherapeutics. Realgar has been shown to act as an orally-available alternative to intravenous ATO. Although it stands to reason that the higher surface area of nanoparticulate realgar would serve to increase As bioavailability, our understanding of the specific advantages conferred by nanoformulation would be improved if studies describing novel nanoformulations consistently included conventional realgar as a comparator. The use of arsenene represents a dramatic departure from the conventional paradigm in which As anticancer agents exploit the soft Lewis acidic character of low-valent As centers. Instead, arsenene nanosheets exploit the unique electronic structure of this novel 2-dimensional material to generate cell-killing heat and ROS. We anticipate that future work will focus on the further functionalization of these nanosheets with targeting moieties that will confer yet further selectivity. Nanoparticulate realgar and arsenene nanosheets differ from most prior arsenic nanodelivery constructs in that they are not an inert delivery vehicle loaded with molecular As-based warheads, but rather single-component systems that serve as both the nanosized object and the active agent. Such a reduction in system complexity has the potential to make these systems more robust, but we note that it can also limit their engineerability. Nevertheless, the studies described above highlight that there are many avenues that remain to be explored in the medicinal chemistry of arsenic and its compounds.

Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Timothy C. Johnstone reports financial support was provided by University of California Cancer Research Coordinating Committee.

Data availability

No data was used for the research described in the article.

Acknowledgments

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