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Arsenic compounds: revived ancient remedies in the fight against human malignancies

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Arsenic, the 20th most abundant element in the earth crust, is one of the oldest drugs in the world. It was used in the 18th century in treating hematopoietic malignancies, discarded in 1950s in favor of chemotherapeutic agents (busulphan and others), and was revived in the 1970s due to its dramatic efficacy on acute promyelocytic leukemia (APL) driven by the t(15;17) translocation-generated PML–RARα fusion. Arsenic represents the most potent single agent for APL, and achieves a five-year overall survival of 90% in APL patients when combined with all-*trans* retinoic acid (ATRA) and chemotherapy (daunorubicin and cytarabine), turning this disease from highly fatal to highly curable. Arsenic triggers sumoylation/ubiquitination and proteasomal degradation of PML–RARα via directly binding to the C3HC4 zinc finger motif in the RBCC domain of the PML moiety and induction of its homodimerization/multimerization and interaction with the SUMO E2 conjugase Ubc9. Because of its multiplicity of targets and complex mechanisms of action, arsenic is widely tested in combination with other agents in a variety of malignancies. Other arsenic containing recipes including oral formulations and organic arsenicals are being developed and tested, and progress in these areas will definitely expand the use of arsenicals in other malignant diseases.

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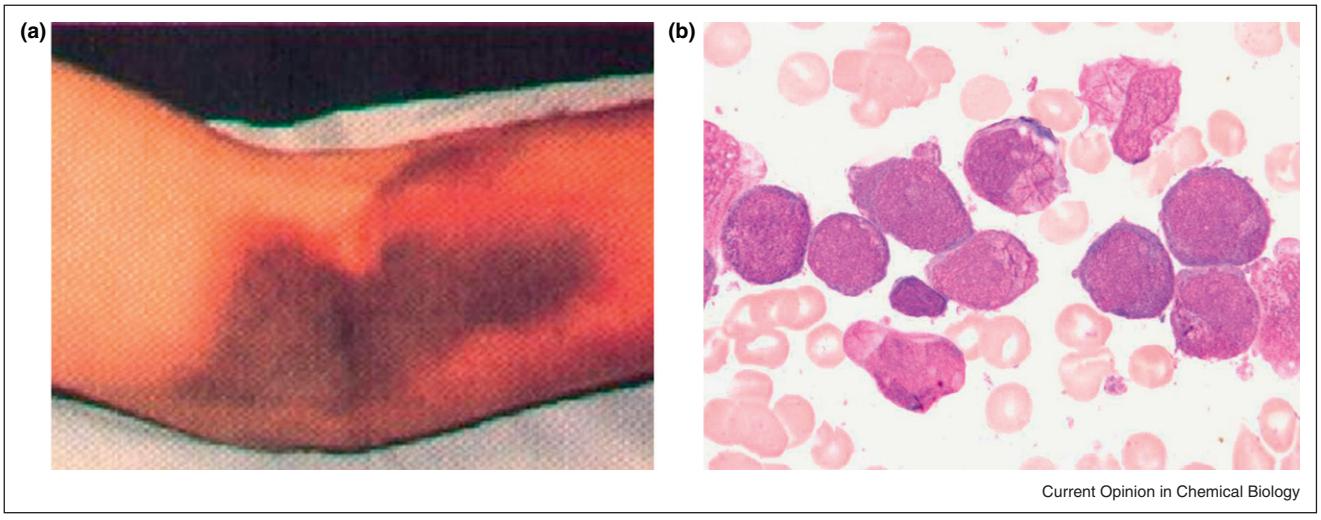
Introduction

Arsenic is a metalloid element widely distributed on earth, mostly found in minerals in conjunction with sulfur and metals. Arsenic exists in either inorganic or organic (arsenic compounds containing carbon) forms. There are 3 common inorganic arsenic compound forms in nature, realgar (As_4S_4, also known as red arsenic), orpiment (As_2S_3, also known as yellow arsenic), and arsenic trioxide (ATO, As_2O_3, or white arsenic). Several species of bacteria use arsenic compounds as respiratory metabolites, but arsenic is toxic and carcinogenic to humans and animals [1]. Even today, millions of people worldwide are exposed to environmental arsenic pollution mainly due to contaminated drinking water [1,2]. However, arsenicals have been used as a medicine for over two thousand years both in the Western society and in Chinese traditional medicine [3,4]. In the 18th century, Thomas Fowler empirically used an arsenic trioxide solution to treat a variety of disorders including hematological malignancies. In 1878, it was found that Fowler's solution was able to reduce the white blood cell (WBC) count in chronic myelogenous leukemia (CML) [5]. Subsequently, arsenic became the mainstay antileukemia therapy, but gradually was phased out when radiotherapy and cytotoxic chemotherapy became available in the early to middle 20th century [3–5]. However, since the 1970s, Chinese scientists have been using ATO to treat acute promyelocytic leukemia (APL, Figure 1) with remarkable therapeutic efficacy [3,4,6,7••,8,9•], which has been confirmed by investigators in many countries, and in 2000, ATO (trisenox) was approved by the US FDA for the treatment of relapsed APL [10,11]. More recent data indicate that ATO (Figure 2), either as a single agent or in combination with all-*trans* retinoic acid (ATRA, Figure 2), gave a superior outcome to all-*trans* retinoic acid (ATRA) alone as frontline therapy in treating newly diagnosed and relapsed APL cases, and when added to consolidation regimens [9,12,13•,14–19]. Because of its multiple mechanisms of action, arsenicals are being tested in other hematological malignancies and solid tumors [20,21]. This review will focus on recent advances in the application of arsenical compounds for APL, their mechanisms of action, and potential applications in other malignancies.

A cure for APL: revival of an old recipe

APL (Figure 1) is one of the most fatal subtypes of acute myeloid leukemia (AML). Traditionally, APL was treated with chemotherapy consisting of an anthracycline and cytarabine. Complete response rate was between 65% and 80% for newly diagnosed cases, but over half of the responders relapsed, and the two-year survival rate was below 50%. Death most often resulted from hemorrhagic complications during induction therapy [22]. In the 1980s,

Figure 1



APL is characterized by severe bleeding tendency **(a)**, the accumulation of abnormal promyelocytes in blood and bone marrow **(b)**, and the specific chromosomal translocation t(15;17)(q22;q21).

Figure 2

The magic bullets for fighting APL. **(a)** The chemical structure of ATRA and ATO. **(b)** Retinoic acid capsules (left) and arsenious acid-sodium chloride injection (right) firstly used in China.

all-*trans* retinoic acid (ATRA) was successfully used at Shanghai Institute of Hematology for the induction of terminal differentiation and complete remission (CR) in APL [23,24••]. This prompted extensive studies to investigate ATRA either as a single agent or in combination with chemotherapy [25–27]. When used alone, ATRA achieved a CR rate as high as 90%, but most cases relapsed if no subsequent consolidation therapy was given [22,28]. Several randomized trials combining ATRA and chemotherapy showed better outcomes with reduced relapse rates [28,29]. Thus, the combinatory use of ATRA and chemotherapy was recommended as the standard induction therapy for most APL patients [28–30]. The use of arsenicals (including Realgar and orpiment) has a long history both as a component in Chinese medicine and as a poison for murder. The paradox of arsenic was best

reflected in a Chinese proverb, combat the evil with evil. This ancient philosophical idea was fully translated by Chinese physicians into a modern weapon against cancer. In the 1970s, physicians at Harbin Medical College, China, formulated an intravenous solution containing 1% ATO and trace amount of mercury chloride based on a Chinese traditional recipe. This so-called 'Ai-Ling #1 (Magic bullet for cancer #1)' was tested on a variety of malignancies with APL showing the best response [3,4,6,7,22]. Systematic studies on its metabolism and safety, and subsequent clinical trials were performed at Shanghai Institute of Hematology [8,9•,22]. CR rates reached as high as 90% in relapsed cases when ATO was used as a single agent, some with molecular remissions which were not observed with ATRA based induction therapies [8]. ATO was also tested in *de novo* APL cases, but the CR rate was not as high when used alone compared to ATRA plus chemotherapy [9•]. On the basis of possible synergistic mechanisms of action (see 'Mechanism of action' below), clinical trials using combination regimens of ATRA and ATO have been carried out at multiple sites. Our data showed that ATO combined with ATRA achieved similarly high CR rates, but the time to achieve CR was shorter, recovery of platelet count was faster, the copy number of PML–RARα transcripts was reduced, and relapse was lower compared with either ATRA or ATO alone for newly diagnosed cases [13]. The long-term efficacy and safety of the combination regimen was also demonstrated [31••]. In more recent studies, ATO also improved outcome when incorporated in consolidation and maintenance therapies [32]. This raises the possibility of minimizing or even eliminating conventional chemotherapy in APL, especially in low-risk to intermediate-risk diseases (presenting WBC $< 10 \times 10^9$/L), thus avoiding the side effects of chemotherapy like secondary tumors [15,31,33–35]. The combination regimen of ATRA plus ATO is also an alternative option for APL patients considered inappropriate for chemotherapy, and those with severe comorbidities (e.g. severe organ failure) [33–36]. However, it is recommended that if presenting leukocyte count is high (WBC $> 10 \times 10^9$/L), chemotherapy should still be incorporated to prevent leukocytosis and differentiation syndrome [28–30]. ATO is well tolerated by most patients, and only mild toxic effects have been observed when used at the recommended dosage of 0.15 mg/(kg d). Major complications include differentiation syndrome and prolongation of the QT interval, which are readily manageable according to recommended procedures [2,4]. Thus, the introduction of arsenicals changed the profile of APL treatment, and combination regimens likely will make cytotoxic chemotherapy unnecessary in most APL patients in the near future.

Mechanisms of action

Our initial study indicated that ATO induced partial differentiation at lower concentrations (<0.5 μM) and apoptosis at higher concentrations (0.5–2 μM) in APL cells [37••,38••]. Subsequent studies indicated that the ultimate outcome may depend upon the specific cell type as well as the dose and duration of ATO exposure [4,39,40].

In APL with the typical t(15;17) translocation, the fusion protein PML–RARα recruits corepressors SMART/N-CoR and histone deacetylase (HDAC) that interfere with the proper expression of target genes crucial in myeloid cell differentiation. ATO induces phosphorylation of SMART/NCoR via the MAPK pathway, leading to the dissociation of the repressor complex from PML–RARα, and the release of the differentiation block [22,40]. PML is normally localized in a specialized nuclear structure called nuclear bodies (NBs) in normal myeloid cells. It functions as a tumor suppressor, regulating cell proliferation, apoptosis and senescence. The fusion protein PML–RARα disrupts the structure, distribution and function of normal NBs, contributing to cellular transformation in APL [40]. Studies show that ATO induces degradation of PML–RARα [38••,40,41]. The trivalent arsenic in ATO has a high affinity for thiol groups in proteins containing cysteines. Therefore, proteins depending on multiple cysteines for functional conformation are readily attacked by ATO. Our study demonstrated that arsenic binds directly to the cysteines of the RING domain in the PML moiety of PML–RARα, replacing the zinc ion [20,42••]. The substitution of zinc by arsenic induces conformational changes of PML–RARα, facilitating oligomerization of PML–RARα and its interaction with UBC9, a small ubiquitin-like modifier (SUMO) E2 conjugase, which promotes addition of SUMO to the fusion protein that leads to recruitment of 11S proteasome and subsequent degradation of the fusion protein [20,42••,43]. Furthermore, our group also provided solid evidence that arsenic targets the cysteines of the RING finger domain in c-CBL, an E3 ligase mediating ubiquitination and degradation of breakpoint cluster region (BCR)–Abelson (ABL), the leukemogenic oncoprotein in CML [44]. Contrary to PML–RARα, binding of arsenic ion to c-CBL results in suppression of its self-ubiquitination and degradation, but leading to increased ubiquitination and degradation of the oncoprotein BCR–ABL [20,44]. Thus, a plausible explanation for the mechanism of action for the legendary Fowler's solution, which had been used empirically to treat CML, may have been found after more than 130 years. This also provides a theoretical basis for combined use of arsenic and the tyrosine kinase inhibitor (TKI) imatinib mesylate in the treatment of CML. In fact, synergistic effect was indeed observed between arsenic and imatinib in *in vitro* studies and animal models [45].

ATO induces apoptosis of APL cells and a variety of other malignant and non-malignant cell lines [21,22,38••,39,40]. Evidence indicates that ATO directly binds the thiol

groups of the permeability transition pore complex (PTPC) in the mitochondrial membrane, and thus opens PTPC, releasing cytochrome c and other pro-apoptotic proteins, leading to activation of the caspase apoptosis cascade [4,39,46]. ATO also induces overproduction or accumulation of reactive oxygen species (ROS), which causes damage to proteins and DNA, eventually resulting in apoptosis [2,4,39,47]. The overproduction of ROS may also account for some of the pathological changes in chronic exposure to arsenicals. The exact mechanisms for ROS generation stimulated by arsenicals are yet to be fully explored, but multiple targets may be involved, including activation of NADPH oxidase and NO synthase, perturbation of the mitochondrial electron transport chain, and inhibition of antioxidant enzymes such as thioredoxin reductase and glutathione peroxidase [39,48–50]. APL cells have a lower level of cellular glutathione content, and thus a higher sensitivity to arsenic-induced ROS. Multiple signaling pathways and some transcription factors are sensitive to or regulated by cellular ROS levels, including mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinase 1/2(ERK1/2), Jun N-terminal kinase (JNK), the Akt–mTOR pathway, NFκB and AP-1 [4,39,40,50]. In APL cells, in addition to direct binding to PML–RARα and inducing its degradation, ATO-induced ROS also facilitates disulfide bond formation between PML–RARα and/or PML, forming homo-multimers or hetero-multimers that are subsequently degraded in the proteasome [41,42••]. Alternatively, ATO may also induce apoptosis through inhibition of Bcl-2 expression and activation of pro-apoptosis proteins including Bax and BH3 only proteins [37••,39,50].

Autophagy is a catabolic process that degrades cellular structures/components via the lysosome machinery. It plays complex roles in normal and disease conditions, and may result in distinct outcomes in different contexts [51]. Recent studies suggest that the antileukemia effect of ATO may also be attributed to induction of autophagy [20,52,53]. Some studies observed direct autophagic cell death induced by ATO both in leukemia and solid tumors. Different mechanisms have been proposed for the activation of the autophagy pathway, including activation of the MEK/ERK pathway in APL, a Beclin 1-independent activation of the autophagic pathway by the modulation of SnoN/SkiL expression in ovarian cancer, and others [39,54]. Intracellular ROS may also play a regulatory role in autophagy [54]. Degradation of the PML–RARα fusion protein in APL may also involve autophagy activities [55,56]. These studies provide new targets for developing selective cancer therapeutics [54].

Leukemia cells are believed to be derived from progenitors with stem cell properties, the so-called leukemia initiating cells (LICs) or leukemia stem cells, which are generally resistant to radiotherapy and chemotherapy [57]. Although there have been numerous efforts to study LICs, this population of malignant cells has not been well defined yet. Relapse after remission is attributed to failure to eliminate the LICs population. The difficulty lies in that these cells are rare and mostly in a quiescent state, allowing them to escape conventional chemotherapy [57,58]. In APL, ATRA induces terminal differentiation of leukemia cells (promyelocytes) but is unable to target and eliminate the LICs; thus, relapse after induction of remission with ATRA frequently occurs if no further treatment (consolidation) is initiated [59]. Recent studies demonstrated that PML–RARα contributes to self-renewal and survival of LICs, and ATO targets PML–RARα and thus diminishes the leukemogenic potential of leukemic cells [60–62].

ATO has also been shown to induce cell cycle arrest at G1 or G2-M, likely by targeting cell cycle inhibitory proteins including P21 and P27 [4,39,62]. It also inhibits angiogenesis in leukemia and solid tumors through inhibition of VEGF and induction of apoptosis of vascular endothelia cells [63,64]. Thus, it seems that arsenic exhibits a difference in the spectrum of targets in different cell types. The complex mechanisms of action and multiple targets provide clues for the use of arsenic compounds in a variety of malignancies, and various cell type specific combinations are being tested based on potential synergistic effects [4,21,65]. These mainly include ROS modulators (e.g. ascorbic acid), inhibitors of signaling molecules (e.g. imatinib), chemotherapeutic agents (e.g. melphalan), radiotherapy, and others (e.g. the proteasome inhibitor bortezomib) [65].

Application in other hematological malignancies and solid tumors

Because of the complex mechanisms and potential multiplicity of targets, arsenic has been tested either as a monotherapy or in combination with other agents in a variety of hematologic malignancies other than APL, including CML, multiple myeloma, lymphoid leukemias and lymphomas, myelodysplastic syndrome (MDS), and a number of solid tumors, including ovarian cancer, gastric cancer, hepatocellular carcinoma, esophageal cancer, prostate cancer, lung cancer, breast cancer, and melanoma [21,65]. Antitumor activity has been demonstrated in most of the cell lines derived from these neoplasms, but clinical trials turned out to be disappointing in most cases [21]. A combinatory approach is now being adopted in many clinical trials to target the many aspects of the complex mechanisms of carcinogenesis. For example, several clinical trials on MDS using arsenic as monotherapy showed moderate response with manageable adverse effects [21,65,66]. Various combination regimens incorporating ATO (thalidomide, granulocyte-macrophage colony-stimulating factor (GM-CSF), etanercept, and chemotherapeutic agents), are being tested for MDS, some of which showed significant benefits [21,65]. In

CML, ATO acts synergistically with tyrosine kinase inhibitor imatinib mesylate to induce apoptosis of BCR-ABL positive cells. The strategy of combining arsenic and imatinib is warranted in further clinical trials [21]. Since AML other than APL is less sensitive to ATO, a combination approach is adopted, including arsenic plus ascorbic acid or chemotherapy. ATO also induces apoptosis of multiple myeloma cells, inhibits angiogenesis and results in tumor necrosis in animal models. Clinical trials showed a 30–40% response rate. Various combination regimens are being tested in refractory/relapsed myeloma cases [21,65,67]. Generally, ATO as monotherapy produced unsatisfactory results in most clinical trials on solid tumors [4,21,68]. Combination of arsenic with radiotherapy or chemotherapy is also being tested.

Other forms of arsenicals in fighting malignant neoplasms

Other forms of arsenic containing compounds, including organic forms, are also being explored. These arsenic compounds may exhibit differences in the mechanism of action, metabolism, efficacy and/or specificity against certain tumor cell types. Similar to ATO, realgar is also an important component in Chinese traditional medicine [69]. A composite Realgar-Indigo Naturalis formula was tested by our group in which realgar acts as the primary component, indigo naturalis, salvia miltiorrhiza, and radix pseudostellariae are accessory components acting synergistically with realgar to induce degradation of PML–RARα and apoptosis of APL cells [70•]. One clinical trial testing this formula in 204 APL cases produced a CR rate of 96%, and a five-year overall survival (OS) rate of 87% [71]. Less toxicity and the convenience of oral administration make it a good choice to substitute for ATO.

Organic arsenicals are generally more stable and less toxic compared to inorganic counterparts, and thus have been tested as alternatives for ATO in cancer treatment. Examples include melarsoprol, GSAO, dimethylarsinic acid, and ZIO-101. Melarsoprol is mainly used to treat African trypanosomiasis. It induces apoptosis of both myeloid and lymphoid leukemia cells in vitro more effectively than ATO, but results from clinical investigations were not satisfactory due to serious side effects [21,72]. GSAO is a tripeptide trivalent arsenical which induces apoptosis and growth arrest of proliferating vascular endothelial cells, thus inhibiting angiogenesis of tumor tissues [21,73]. Clinical trials with GSAO are recruiting advanced solid tumor patients (http://clinicaltrials.gov/ct2/show/NCT01147029). Dimethylarsinic acid is not only less toxic but also less effective than ATO in inducing apoptosis and inhibiting cell growth in leukemia and multiple myeloma cell lines [74]. ZIO-101 (S-dimethylarsino-glutathione, SGLU-1), also called Darinaparsin, is a conjugate of glutathione and dimethylarsinous acid that blocks the cell cycle at G2-M and induces apoptosis via perturbation of the mitochondrial membrane potential and activation of caspases [75]. Of interest is that it is even effective against cell lines resistant to ATO, reflecting a difference in mechanism of action [75,76]. Darinaparsin exhibited significant activity in a broad spectrum of hematologic and solid tumors in preclinical models. Initial clinical trials in both hematological malignancies and solid tumors showed some response and fewer side effects compared to ATO [75,77,78].

Conclusions and perspectives

The application of ATO together with ATRA has turned the most fatal subtype of AML into a curable disease. Because of its multiplicity of targets and complex mechanisms of action, ATO is widely tested in combination with other agents in a variety of malignancies, some of which have given promising results. Other arsenic containing compounds or composite recipes are being developed and tested, including oral formulae and organic arsenicals. Progress in these areas will definitely expand the use of arsenicals in other malignant diseases. Further understanding of the complex mechanism and targets of arsenic compounds in specific malignancies will surely shed light on rational design of cell type specific combination regimens with other antitumor agents.

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