Chapter 7

Perspective on Therapeutic Strategies of Leukemia Treatment — Focus on Arsenic Compounds

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Additional information is available at the end of the chapter

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Abstract

Leukemia is a type of cancer of the body's blood-forming tissues, including the bone marrow and the lymphatic system. Treatments for leukemia are complex, depending upon the type of leukemia and other factors. Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) and accounts for approximately 10-15% of all cases of AML in adults. Arsenic and its compounds are widely distributed in the environment and have been used medicinally for over 2,000 years. In fact, investigators from China and the USA have demonstrated that treatment with ATO (As_2O_3, As^{III}) results in complete remission in 90% of relapsed APL patients since mid-1990s. Moreover, As_2S_2 or As_4S_4, also known as realgar, has been gaining increasing attention and is traditionally used to treat certain types of hematological disorders including chronic myeloid leukemia (CML), AML, myelodysplastic syndrome (MDS) and MDS/AML in China. In this chapter, we first highlight the pharmacokinetics of ATO and realgar in leukemia patients and/or healthy volunteer. We will further summarize the detailed mechanisms underlying the cytocidal effects of these arsenic compounds. We also provide detailed insight into potential future clinical application of those promising candidates endowed with potent antitumor activities in view of combination with arsenic compounds.

Keywords: leukemia, arsenic trioxide, realgar, combination therapy, naturally derived substances

1. Introduction

Leukemia is a type of cancer of the body's blood-forming tissues, including the bone marrow (BM) and the lymphatic system. Treatments for leukemia are complex, depending on the type of leukemia and other factors. At present, treatment may include some combination of chemotherapy, radiation therapy, targeted therapy, and hematopoietic stem cell transplantation, in addition to supportive care and palliative care as needed.

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) and accounts for approximately 10–15% of all cases of AML in adults. Morphologically, it is identified as AML–M3 by the French–American–British classification. Cytogenetically, APL is characterized by a balanced reciprocal translocation between chromosomes 15 and 17, generating a promyelocytic leukemia (PML)/retinoic acid receptor α (RARα) fusion gene, which is thought to play a central role in the initiation of leukemogenesis [1–4]. The oncogenic fusion protein PML-RARα has been demonstrated to recruit corepressor (CoR) complexes containing nuclear receptor CoRs, histone deacetylases (HDACs), resulting in myeloid differentiation arrest observed in APL [5, 6]. An introduction of all-*trans* retinoic acid (ATRA) since 1986 has dramatically improved the outcome of treatment of this disease [5]. It has been reported that ATRA induces differentiation of APL cells through not only dissociating CoRs from the PML-RARα oncoprotein but also recruiting coactivators that possess histone acetylase activity [5–7]. For detailed mechanisms underlying the efficacy of ATRA, please refer to some excellent research and review articles [6, 8–11]. In fact, in 90% of *de novo* APL patients, administration of ATRA induces differentiation of leukemic blasts and clinical remission, and 70% of them have been cured by ATRA administration in combination with chemotherapy [4, 6, 12]. Due to its good clinical outcomes, ATRA is used as a first-line administration for *de novo* APL patients. Nevertheless, the remaining 30% of patients relapse and often become resistant to this conventional treatment [4, 6, 12].

Arsenic and its compounds are widely distributed in the environment and exist in organic and inorganic forms. There are three inorganic forms of arsenic: yellow arsenic (As_2S_3, also known as orpiment and Cihuang "female yellow" in China); red arsenic (As_2S_2 or As_4S_4, also known as realgar and Xionghuang"male yellow" in China); and white arsenic or ATO (As_2O_3, As^{III}), which is made by burning realgar or orpiment [6]. Although a well-known poison, arsenic has been used medicinally for over 2 000 years. Since the mid-1990s, investigators from China and the USA have demonstrated that treatment with ATO results in complete remission in 90% of relapsed APL patients [4, 13–15]. From then on, a dramatic clinical efficacy of ATO-based regimens against APL has been reported around the world. An impressive drug efficacy against APL led to its approval in the USA and in Europe under the brand name Trisenox for "the induction of remission and consolidation in patients with APL whose conditions are refractory to, or who have relapsed from retinoid and anthracycline chemotherapy, and whose APL is characterized by the presence of the t(15:17) translocation or PML-RARα gene expression" [13]. Moreover, As_2S_2 as another important arsenic compound has been gaining increasing attention and traditionally used to treat certain types of hematological disorders, including chronic myeloid leukemia (CML), AML, myelodysplastic syndrome (MDS) and MDS/AML in

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China [16–18]. Although realgar has not yet been approved by the U.S. Food and Drug Administration (FDA) and other major countries for clinical use, it has become another research for focus following ATO due to its good therapeutic efficacy and perceived low toxicity [19, 20].

In this chapter, we first highlight the pharmacokinetics of ATO and realgar in leukemia patients and/or a healthy volunteer. We will further summarize the detailed mechanisms underlying the cytocidal effects of these arsenic compounds. We also provide detailed insight into potential future clinical applications of those promising candidates endowed with potent antitumor activities in view of combination with arsenic compounds.

2. Pharmacokinetics of arsenic compounds in leukemia patients

2.1. Pharmacokinetic studies of ATO in Peripheral Blood (PB)

It has been established that biomethylation, which primarily occurred in the liver [21, 22], is a major metabolic pathway for inorganic arsenic in human and many animal species [23], by which arsenic undergoes metabolic conversion by the reduction of As^{V} to As^{III} with subsequent methylation, yielding monomethylated and dimethylated metabolites [24, 25]. A postulated metabolic pathway is as follows: As^{V} → As^{III} → methylarsonic acid (MAs^{V}) → methylarsonous acid (MAs^{III}) → dimethylarsinic acid ($DMAs^{V}$) → dimethylarsinous acid ($DMAs^{III}$) (Figure 1). It has been demonstrated that, following arsenic exposure, 40–60% of arsenic intake is eliminated through urine. The standard profile of urinary arsenic in human is comprised of 10–30% inorganic arsenic, 10–20% monomethylated arsenic (MAs: MA^{III} + MA^{V}), and 60–80% dimethylated arsenic (DMAs: DMA^{III} + DMA^{V}) [26, 27].

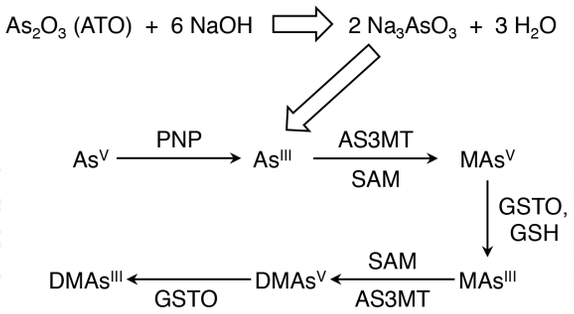

Figure 1. Postulated pathways of the biotransformation of arsenic in mammalian systems. Biomethylation, which primarily occurs in the liver, is a major metabolic pathway for inorganic arsenic in human and many animal species. In human, dimethylarsinous acid ($DMAs^{III}$) and dimethylarsinic acid ($DMAs^{V}$) appear to be the end products of this pathway [133]. In the pathway, arsenate reductases, such as the omega isoform of glutathione S-transferase (GSTO) and purine nucleoside phosphorylase (PNP), catalyse the reduction of arsenate species [4, 22]. Human arsenic (+ 3 oxidation state) methyltransferase (AS3MT) catalyse the methylation of arsenite [4, 22]. S-adenosylmethionine (SAM) is the methyl donor [4, 22].

In order to provide an effective treatment protocol for individual APL patients, detailed studies have been conducted on the pharmacokinetics of As^{III} in APL patients using biological samples

such as peripheral blood (PB), cerebrospinal fluid (CSF), BM, and urine [14, 28–31]. Compared with a limited understanding of metabolic profiles of realgar and its pharmacokinetics, many detailed systematic analyses of the metabolites of ATO in blood cells and plasma of APL patients have been performed. In this regard, by using high-performance liquid chromatography (HPLC)/inductively coupled plasma mass spectrometry (ICP-MS), we have conducted studies on the total arsenic and speciation of ATO metabolites in an APL patient when 0.15 mg/kg/day was infused. Blood samples were obtained from the patient at various time points after remission induction therapy and during consolidation therapy [31]. Of note, biological samples, such as blood and urine, contain much higher concentrations of chloride ion, which interferes with arsenic detection at *m*/*z* 75 due to the formation of argon chloride ($^{40}Ar^{35}Cl^{+}$: *m*/*z* 75) in argon plasma [28, 32]. Therefore, arsenic was detected as the adduct ion of $^{75}As^{16}O^{+}$ (*m*/*z* 91), which is produced by the oxidization of arsenic in the oxygen atmosphere with the dynamic reaction cell techniques [31].

We first demonstrated that, in all blood samples collected either after the remission induction therapy or during the consolidation therapy, approximately 80–90% of the total arsenic in the blood samples was observed in the blood cells, suggesting that most of the blood arsenic is bound to hemoglobin [31]. These results are in good agreement with a previous report showing that 90% of blood arsenic is bound to hemoglobin [33]. These findings suggest that careful attention should be paid to profiles of total arsenic in blood cells and consequently provide valuable insight into clinical applications of As^{III}. We further clarified that, during the drug withdraw period, the amount of As^{V} in plasma was more readily eliminated among all arsenic metabolites [31], as reported by other groups [32, 34]. We also demonstrated that As^{III} concentrations in plasma initially declined more quickly than those of MAs^{V} and $DMAs^{V}$ [31]. These results suggest that methylated metabolites (MAs^{V} and $DMAs^{V}$) are major metabolites in plasma and are similar to those reported by other groups [23–25]. Furthermore, we demonstrated that the concentrations of methylated metabolites (MAs^{V} and $DMAs^{V}$) as well as inorganic arsenic (As^{III} and As^{V}) in plasma increased with multiple administration during the consolidation therapy period [31]. In comparison, Fujisawa et al. demonstrated no increase in the maximum concentrations of inorganic arsenic (As^{III} and As^{V}) despite multiple administrations, suggesting that inorganic arsenic plasma may reach a steady state after multiple administration [28]. It is noteworthy that only one of the 14 patients enrolled in the study of Fujisawa et al. was in the first relapse, and the remaining patients were in the second to fifth relapse [28]. In contrast, the patient in our study was in the first relapse [31]. Although the exact reason for the above-mentioned apparent differences in the profiles of the concentrations of inorganic arsenic (As^{III} and As^{V}) in the plasma of APL patients is not clear, the differences in patient characteristics may explain the discrepancy in the concentrations of inorganic arsenic. These results also suggest that understanding the differences in metabolism among patients is very useful for providing an effective treatment protocol for individual patients with leukemia.

2.2. Pharmacokinetic studies of ATO in the CSF

Although the central nervous system (CNS) relapse of APL occurs in 1–5% of patients, the optimal therapy for this case remains unclear [35, 36]. Fortunately, several clinical data have

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demonstrated that ATO seems to be capable of crossing the blood–brain barrier in humans and can be considered as an effective treatment strategy for the CNS relapse of APL [37–40]. We have recently determined the total arsenic and speciation of ATO metabolites in the CSF and PB plasma samples from three patients with APL who were treated with intravenous ATO as salvage therapy [0.15 mg/kg/day ATO + intrathecal chemotherapy (methotrexate + cytosine arabinoside + prednisolone)] [29]. In this study, PB was collected before and after the infusion, and the CSF was collected after the infusion, respectively. Furthermore, in order to prepare samples for arsenic speciation, the PB plasma was ultrafiltered with a 10-kDa molecular mass cutoff. The filtrates were thus defined as low-molecular-weight fraction (LMW-F) and subjected to arsenic speciation analysis. The remains trapped in filters were defined as high-molecular-weight fraction (HMW-F) and subjected to total arsenic determination [29]. We first demonstrated that not only inorganic arsenic (As^{III} and As^{V}) but also methylated arsenic metabolites (MAs^{V} and $DMAs^{V}$) existed in the CSF and that the total CSF arsenic concentrations ranged from 148 nM to 250 nM (mean: 199 nM) [29]. It has been demonstrated that ATO exerts a dual effect on APL cells; that is, ATO induces apoptosis at relatively high concentrations, ranging from 1 μM to 2 μM, whereas trends to promote differentiation of APL cells at low concentrations range from 0.1 μM to 0.5 μM [4, 6, 41]. Therefore, the beneficial effects of arsenic on the CNS relapse of APL patients might be attributed to its differentiation induction, rather than apoptosis induction. Furthermore, we found that the total PB plasma arsenic concentration is about twice of the sum of the amount of each arsenic metabolite present in LMW-F [29]. We also demonstrated that the total arsenic concentration in HMW-F accounted for approximately 50% of the total PB plasma arsenic concentration, suggesting that considerable amounts of arsenic species exist in clinical samples as a protein-bound complex.

Similar to previous reports showing that arsenic concentrations in the CSF were about 10% of those in whole blood or plasma [38, 40], we also demonstrated that the arsenic concentrations of the CSF were 8–17% of the plasma levels [29]. Taken together, it is possible to use a combination of arsenic with other chemotherapeutics to achieve a favorable clinical outcome in APL patients with CNS relapse, although a further larger scale randomized study must be conducted to reach a firm conclusion. It is noteworthy that Meng et al. have developed a non-invasive method via a concomitant with 20% mannitol intravenous bolus injection to help ATO enter into the CNS [42]. Their regimens include 125 ml of 20% mannitol bolus through the medial cubital vein at the rate of 12 ± 30 ml/min, followed with 250 ml of mixed solution (including 20% mannitol and ATO 0.08 mg/kg/day) through intravenous infusion at the rate of 6 ml/min, followed by ATO 0.08 mg/kg/day + 5% glucose 250-ml infusion at the rate of 0.5 ml/min in the total dosage of ATO (0.16 mg/kg/day) [42]. Compared with the general ATO intravenous infusion, the mannitol-assisted ATO penetration followed by the slow-speed continuous ATO intravenous infusion can not only increase the elemental arsenic concentration in the CNS but also keep the plasma arsenic at prolonged effective therapeutic levels without remarkable plasma arsenic peak. Therefore, the non-invasive method was supposed to be more beneficial to the CNS relapse of APL and increase the prevention and treatment efficiency of APL marrow relapse, as well as less side effects to normal tissues [42]. The same group has recently extended the application of the non-invasive method to additional patients [43]. They demonstrated that, in 16 of the 17 patients examined, abnormal blasts/promyelo-

cytes from the CSF were eliminated in 18 to 32 days (median: 24 days) after the start of induction treatment and that all the patients tolerated the induction well. Importantly, there were no complaints of side effects associated with the use of mannitol [43]. Of note, over the course of the entire induction treatment process, the concentrations of arsenic in the blood and CSF were fairly stable in each patient. For each individual, the arsenic level in the CSF was ~99.7% of those in the paired blood samples, although the arsenic levels in different individuals were highly variable in the blood and CSF [43].

2.3. Pharmacokinetic studies of ATO in the BM

Although an initial report demonstrated that the total arsenic concentrations in the plasma of a BM sample from five relapsed APL patients were close to levels for differentiation induction, the analysis was conducted for the BM sample collected at only one collection time point [44]. Moreover, despite the fact that BM is a vital site for regulating the production of blood cells, no speciation analysis of arsenicals in the BM from APL patients undergoing long-term administration of ATO has been done before. In this regard, in order to gain more detailed information on the distribution of arsenic, we have recently investigated, for the first time, the arsenic speciation in the plasma of the BM and compared the arsenic speciation profiles between the PB and BM collected before and after the start of administration of 0.15-mg/kg/day ATO [30]. In this study, we first demonstrated a time-dependent increase of the total arsenic concentrations in the BM plasma, which was similar to that in the PB plasma. Furthermore, the total arsenic concentration levels tended to be higher in the BM plasma than those in the PB plasma, raising clinical concerns and inspiring us to unravel the detailed information on the distribution of arsenic as well as its speciation in these biological samples [30]. In this study, the concentrations of MAs^{V} and $DMAs^{V}$ substantially increased after the start of administration, whereas those of As^{III} were still kept at a low level until day 10, followed by a substantial increase from day 14 after the start of administration. As mentioned in Section 2.1, we have previously demonstrated that the PB plasma concentrations of both methylated arsenic metabolites (MAs^{V} and $DMAs^{V}$) and inorganic arsenic (As^{III} and As^{V}) remarkably increased after the start of administration in a Japanese APL patient undergoing consolidation therapy [31]. Compared with the APL patient in our previous study [31], the patient enrolled in this study appeared to have relatively higher metabolic efficiency, probably due to her relatively young age or without clinical complications. Collectively, our findings suggest that the efficiency of drug metabolism is obviously different in individual patients with different backgrounds, such as age range, with or without organ failure or disseminated intravascular coagulation (DIC), which, in turn, affect clinical outcomes and appearance of side effects. Of note, a close similarity of the arsenic speciation profiles between the PB and BM plasma was observed throughout the remission induction therapy, suggesting for the first time that the arsenic speciation analysis of the PB plasma could be predicative for BM speciation without applying BM aspiration [30]. Investigation of the total amount of arsenic in HMW-F trapped in a 10-kDa molecular mass cutoff filters further demonstrated that arsenic concentrations were much higher in the BM plasma than those in the PB plasma. One important biological effect of arsenic has been suggested to be mediated by reaction with closely spaced cysteine residues on a critical cell protein [45]. Several proteins such as tubulin, thioredoxin reductase, human

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arsenic methyltransferase (AS3MT, responsible for arsenic methylation) with a high cysteine content, and accessible thiol group are candidates for interactions with arsenic [4, 46]. In fact, arsenic bound to high molecular weight proteins (MW > 10-kDa) has been detected in livers and kidneys in rats after an intravenous injection of arsenite [47]. In view of the vital role of the BM microenvironment in maintaining the homeostasis of hematopoietic system, we assumed that a higher amount of proteins (MW > 10-KDa)-bound arsenic complex contributes to the protection effect from the attack of free arsenic species. Likewise, patients with low-proteinemia besides liver and/or renal dysfunctions might frequently develop arsenic-mediated side effects. Understandably, further investigation of the detailed information about these proteins is needed.

2.4. Pharmacokinetic studies of ATO in urine

The pharmacokinetic studies in APL patients have been well discussed by using urine samples [14, 28, 32, 48]. Previous studies on urinary arsenic excretion profiles demonstrated that there are large variations among individual patients with regard to the arsenic metabolic profiles and excretion patterns. For instance, an initial study demonstrated that urinary arsenic contents slightly increased during drug administration and the total amounts of arsenic excreted daily into the urine accounted for approximately 1–8% of the daily dose [14]. However, a previous report revealed that the mean total arsenic excretion rate including inorganic arsenic and methylated arsenic was about 20% of the daily dose on day 1 and remained at about 60% of the daily dose during subsequent weeks [28]. A clinical pharmacokinetic study performed in an APL patient showed that, in the urine sample collected for 24 h after administration of ATO, the total amount of inorganic arsenic and the methylated metabolites was almost 30% more of the daily dose [32]. Furthermore, Wang et al. [48] have demonstrated that there is an interindividual difference in excretion profiles and the relative concentrations of major arsenic species in the urine among four Chinese APL patients undergoing ATO treatment. In addition, other pathways of excretion, such as through the bile, have also been suggested to play a partial role in the elimination of arsenic [14, 48]. Therefore, systematic monitoring of the speciation of arsenic compounds in not only urine samples but also other biological samples such as the PB plasma has important implications for achieving favorable outcomes and minimizing side effects in leukemia patients treated with arsenic-based regimens.

2.5. Pharmacokinetic studies of realgar in leukemia patients as well as a healthy volunteer

Realgar has been widely used clinically in China [17, 18]. Moreover, an As_2S_2-containing formula, Qinghuang powder (QHP), was used as a folk medicine recorded in a famous traditional Chinese medicine (TCM) book, Shi Yi De Xiao Fang, published in 1345. Despite this, the systematic study on metabolic profiles of realgar and its pharmacokinetics have not yet been fully investigated. Compared with intravenous administration of ATO, oral administration of realgar is advantageous and would be more suitable for consolidation and maintenance therapy and consequently make better patient compliance and quality of life [16]. In this regard, Lu and colleagues designed a pilot study in which 129 APL patients with

different disease stages were enrolled and received oral administration of highly purified crystalline realgar (As_4S_4) [19]. In this pilot study, encouraging responses such as high complete remission rate, long disease-free survival period, and tolerable side effects after oral administration of realgar alone were observed [19]. Furthermore, clinical pharmacokinetic studies on seven volunteers with APL and hematologic complete remission (HCR) by using 60 mg/kg oral As_4S_4 in a single dose demonstrated that arsenic could be detected in the blood 30 min after oral administration of As_4S_4. The peak time (T_{peak}) was 3.4 ± 1.4 h, and the maximum concentration (C_{max}) was 24.9 ± 8.0 μg/l. As expected, there was a wide interpatient variation in area under the concentration–time curve ($AUC_{0\text{-infinity}}$) (899.01 ± 705.64 μg/h per liter) and elimination half-life ($t_{1/2}$) (30.1 ± 11.1 h). These pharmacokinetic data revealed that rapid absorption of arsenic occurred after oral administration of As_4S_4 [19]. In addition, most urinary arsenic excretion occurred within the first 24 h. Measurement of blood arsenic levels in eight patients who were given oral As_4S_4 at a dosage of 50 mg/kg/day for 2 weeks followed by a break of 2 weeks during the first year of treatment demonstrated that blood arsenic levels declined during the drug withdraw period [19]. Arsenic concentrations in plasma and red cells were also measured in five patients with newly diagnosed APL and showed that the red-cell arsenic level was approximately two times higher than the plasma level, similar to the findings observed in an APL patient treated with ATO. Similar to the behavior of blood arsenic levels, urinary arsenic levels also quickly declined after discontinuation of As_4S_4. Of note, the arsenic level in the CSF in nine patients on the 10th day of treatment was 5.6–14.6 μg/l, a level similar to that in plasma, indicated that oral As_4S_4 is capable of penetrating into the CSF, suggesting the usefulness of As_4S_4 for the CNS relapse of APL [19]. These previous findings suggested that As_4S_4 treatment alone is highly effective and safe in both remission induction and maintenance therapy in patients with APL, regardless of disease stage.

Recently, the therapeutic effects of QHP have been evaluated in MDS patients with different karyotypes, including normal karyotype, trisomy 8 karyotype, and other cytogenetic abnormalities after receiving one to two 3-month courses of oral administration of QHP (containing realgar 0.16 g/capsule/day). Furthermore, the PB samples were collected 10–12 h after ingestion, and the total arsenic concentrations in the PB or PB plasma were determined using HPLC/ICP-MS. A positive correlation was found between the efficacy of QHP and total arsenic concentrations in the PB, but not in the PB plasma. Compared with patients with other cytogenetic abnormalities, much better clinical efficacy was observed in patients with normal or trisomy 8 karyotype, in agreement with our previous findings [49]. Surprisingly, no significant difference in the total arsenic concentrations in the PB was observed between the high-efficacy groups (patients with normal or trisomy 8 karyotype) and low-efficacy groups (patients with other cytogenetic abnormalities). Furthermore, no correlation between total arsenic concentrations in the PB or PB plasma and administration period was found in the study, suggesting that realgar might have a relatively low absorption/accumulation rate. Interestingly, compared with patients who did not receive oral administration of QHP, no apparent alteration in the mitochondria membrane potential ($\Delta\Psi m$) was found in primary blasts from the BM in patients treated with QHP. Moreover, among 28 patients treated with QHP, no correlation between $\Delta\Psi m$ and total arsenic concentrations in the PB was observed, suggesting that the mitochondria might not be the main target for QHP, although further detailed studies are obviously needed.

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Currently, as much as 49 items among approximately 500 TCMs contain realgar [16]. Furthermore, there are 22 registered oral formulae containing realgar in the *Chinese Pharmacopoeia 2010*, which account for nearly 50% of the 49 realgar-containing formulae listed in the Pharmacopoeia [16]. Since TCM has been evolved and developed through long-term historical clinical practice, many TCMs are available over the counter. However, the lack of scientific evidence, especially in pharmacokinetics, results in a difficulty in its clinical application. In this regard, Koch and colleagues investigated the bioaccessibility (a surrogate measurement for bioavailability) and excretion of *Niu Huang Jie Du Pain* (translated as cow bezoar detoxifying pills), one of the most commonly used over-the-counter TCMs containing about 7% of arsenic in the form of realgar [50]. Analysis of the amount of arsenic available for absorption using three different bioaccessibility methods demonstrated that, although the total arsenic exist as high as 28 mg in a single pill, only 4% of it was available for absorption into the bloodstream (1 mg of arsenic per pill), suggesting that the bioaccessibility of realgar is very poor [50]. In fact, the poor bioaccessibility and/or bioavailability of realgar has also been reported by other researchers [51, 52]. Therefore, these observations raise the possibility that mild toxicity of the clinical application of realgar reported might be attributed to poor bioaccessibility and/or bioavailability, rather than its low toxic nature. Obviously, in order to draw a firm conclusion, more detailed studies are needed.

Koch and colleagues also evaluated arsenic concentrations and their speciation in urinary samples collected from a volunteer aged 70 years before and after ingestion of one pill of *Niu Huang Jie Du Pain*. [50]. The maximum value of the total arsenic species excreted after the ingestion of the one pill appeared approximately 14 h after ingestion, and methylated arsenic metabolites (MAs^{V} and $DMAs^{V}$) were the predominant species. The proportion of methylated arsenic metabolites in all arsenical species are in total agreement with previous arsenic metabolic profiles observed in other clinical samples, including the PB plasma, the CSF, and BM obtained from APL patients undergoing ATO treatment [4, 29–31], and healthy individuals who have drunk water containing inorganic arsenic [26, 27], reconfirming that biomethylation is a major detoxification pathway for inorganic arsenicals, regardless of different forms of arsenic. Interestingly, compared with previous findings showing that MAs^{III} and $DMAs^{III}$ have been detected in the urine of APL patients undergoing ATO treatment [48] and healthy individuals who have drunk water containing inorganic arsenic [25, 53], no traces of MAs^{III} and $DMAs^{III}$ were detected in the urine collected at any time after ingestion of one pill of *Niu Huang Jie Du Pain*, although two different HPLC/ICP-MS conditions, which were certified as the optimal condition for distinguishing MAs^{III} and $DMAs^{III}$ from other arsenic species, were used [50]. It is important to note that there should be some differences in metabolic profiles between taking a single dose or multiple doses. Since the aforementioned sampling of the urine was from a single individual who just took only one dose of *Niu Huang Jie Du Pain*, further research is desirable to study the more realistic exposure of multiple chronic doses, as well as to include more individuals to gain more statistical strength for populations consuming these medicines [50].

Due to the poor bioaccessibility and/or bioavailability of realgar mentioned above, realgar nanoparticles (NPs) were designed to improve its pharmacological and toxicological profiles [16]. It has been demonstrated that, compared with commercially used coarse realgar powder, realgar NPs with a size less than 200 nm prepared even by different methods show much higher

efficiency in cytotoxicity associated with apoptosis induction and differentiation induction in leukemic cells such as HL-60 and U937 [54–57]. Although the detailed pharmacokinetic studies on realgar NPs have not yet been investigated in human beings, an *in vivo* bioavailability of realgar NPs prepared by cryo-grinding with polyvinylpyrrolidone (PVP) and/or SDS was evaluated in rats [58]. In this study, a remarkable increase in the urinary recovery of arsenic was observed in rats after a single oral administration of the cryo-ground realgar particle suspension. A range of 58.5–69.6% of the administered dose of arsenic was recovered in urine in the first 48 h from the PVP and/or SDS co-ground preparations; whereas the original realgar powder gave a urinary recovery of only 24.9%, suggesting that size reduction of realgar particles to nano levels could substantially enhance their bioavailability [58]. Furthermore, the cytotoxicity of the realgar NPs to human gynecological cell lines such as GI80-13S, Hela cells was comparable with the ATO observed previously [58]. Interestingly, realgar NPs were also found to be successfully delivered by transdermal administration, which gave more therapeutic efficiency than intraperitoneal administration in a model of treating melanoma skin tumor-bearing C57BL/6 mice, raising the possibility of its new clinical use [59].

It is well known that, besides administration of arsenic to patients in clinical practice, arsenic can also enter the body through food chains. The most common exposure to high levels of arsenic in food is through marine products in the form of arsenobetaine or plant products in the form of various arsenosugars [4]. Arsenobetaine, a trimethylarsenic compound, is one of the major organic arsenic in seafood and is not produced by the metabolism of As^{III} in human [34, 60]. Once arsenobetaine is ingested during periods of arsenic-based therapy, it will be excreted from the body in the same form and consequently interrupt the accuracy of evaluation of the pharmacokinetics of arsenic species. Therefore, controlling the daily diet, in particular seafood, during the periods of remission induction therapy and/or consolidation therapy is very important to accurately monitor the metabolic profiles of arsenic.

3. Mechanisms underlying the cytocidal effects of arsenic compounds

3.1. Involvement of oxidative stress in arsenic-mediated apoptosis induction

The remarkable clinical results achieved with ATO and realgar in relapsed as well as newly diagnosed APL patients have promoted investigations to determine the mechanisms underlying their activity. Accumulating evidence has shown that ATO exerts dose-dependent dual effects in APL cells such as NB4, with preferential apoptosis at relatively high concentrations ranging from 0.5 μM to 2.0 μM and partial differentiation at relatively low concentrations ranging from 0.1 to 0.5 μM [4, 61, 62]. The apoptosis-inducing effect is primarily associated with the mitochondrial-mediated intrinsic apoptotic pathway, whereas the extrinsic pathway through death receptors, such as the tumor necrosis factor receptor (TNFR) and Fas, have also been reported to be implicated in ATO-induced apoptosis in human leukemia, lymphoma, as well as glioma cell lines [63–65]. Moreover, a third pathway involving endoplasmic reticulum and caspase-12 has been reported to associate with ATO-mediated apoptosis in the chronic myeloid leukemia cell line K562 [66].

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It has become clear that oxidative damage is one of the main mechanisms by which arsenic induces apoptosis [4, 6, 55, 61, 62, 67]. This idea is consistent with the binding capacity of arsenite to adjacent sulfhydryl (SH) groups present in many vital biomolecules [4, 6, 61, 62, 67] and also strongly supported by clinical data that levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dGuo), one of the most abundant oxidative products of DNA, are increased in plasma from APL patients after remission induction and consolidation therapy with ATO [68]. Similar to ATO, realgar NPs also caused elevated urinary 8-OH-dGuo excretion in rats from day 1 after oral administration [69]. ATO has been demonstrated to disrupt mitochondrial respiration through blockading of electron flow at complex III and IV to elevate the generation of oxygen free radicals [70]. It has also been reported that reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived reactive oxygen species (ROS) are responsible for the susceptibility to arsenic cytotoxicity in leukemia cells [71, 72]. Furthermore, downregulation of the ROS elimination system, comprising glutathione (GSH), thioredoxin, and anti-oxidative enzymes including superoxide dismutase (SOD), catalase, as well as glutathione peroxidase (GPx), has been reported to be involved in cytocidal effects of arsenic compounds [73–78]. In line with these findings, GPx inhibitors such as mercaptosuccinic acid [79], catalase inhibitors such as 3-amino-1,2,4-trizaole [79], and SOD inhibitors such as 2-methoxyestradiol have been reported to potentiate the apoptosis-inducing activity of arsenic in NB4 cells as well as primary leukemia cells from patients with chronic lymphocytic leukemia [70, 73, 80]. Collectively, through not only enhancing the ROS production system but also impairing the ROS elimination system, arsenic compounds are able to induce intracellular ROS accumulation, which, in turn, activates apoptotic pathways in hematological cell lines including the APL cell line [41, 73, 74, 81–84].

3.2. Differentiation induction

In vitro and *in vivo* studies have demonstrated that a low-dose of ATO (0.1 ~ 0.5 μM) induces differentiation of NB4, a human APL cell line with t(15;17), as well as fresh APL cells [15, 81, 85]. Morphological changes such as condensation of chromatin, lobulation of nuclei, and the expression profiling of surface markers have been used as markers of the differentiation of APL cells [15, 81, 85]. Consistent with previous findings, we have recently demonstrated that 0.125 μM ATO also induced differentiation in HT93A cells, another t(15;17)-positive APL cell line established from the peripheral blood of a patient with APL [86], as evidenced by the appearance of jelly-bean-shaped nuclei in almost all cells, accompanied by a significant increase in CD11c and CD15 expression [87]. Furthermore, we demonstrated that As_2S_2 induced granulocyte differentiation in HL-60 cells, as evidenced by the increment in CD11b expression, in which oxidative stress associated with GSH depletion and p38 mitogen-activated protein kinase (MAPK) activation was involved [77, 84]. More interestingly, we have recently found that treatment with As_2S_2 induced erythroid differentiation in F36p, derived from MDS/AML, and HL-60 cells, as evidenced by a dose-dependent increase in the expression level of CD235a, a marker for the detection of the erythroid cell lineage [88, 89]. We further demonstrated that, although the alteration in the expression level of phosphorylated and total p38 MAPK was observed to some extent in parallel with As_2S_2-induced erythroid differentiation, no alteration was observed in the expression level of CD235a, regardless of the presence

or absence of SB203580, a specific inhibitor for p38 MAPK, suggesting that p38 MAPK plays a small role in As_2S_2-induced erythroid differentiation in HL-60 cells [89]. Therefore, our findings suggest that p38 MAPK plays a critical role in As_2S_2-induced differentiation into granulocytes, rather than erythroid differentiation.

It has been demonstrated that higher DNA methylation levels at a few CpG sites in some erythroid specific genes correlated with a decreased erythroid differentiation capacity of K562 cells, which has been proposed as a very useful *in vitro* model system for studying erythroid differentiation [90, 91]. We have recently evaluate the effects of the As_2S_2-containing Chinese herbal formula QHP on the genomic methylation level in primarily diagnosed MDS patients [49], since MDS patients, especially those with high-risk MDS, have been reported to possess abnormal hypermethylation in tumor suppressor genes [92]. In this study, global DNA methylation detection (ChIP-on-chip assays) demonstrated that the number of hypermethylated genes decreased from 1,063 to 75 and that the methylated genes involved in multicellular organismal development, signal transduction, and apoptosis were demethylated after treatment with QHP [49]. We thus suggest that demethylation status, which might be resulted from As_2S_2 treatment, attributed to the above-mentioned erythroid differentiation of F36 and HL-60 cells, although further investigation of a correlation between erythroid differentiation and As_2S_2-triggered demethylation in these cells is needed to draw a more conclusive idea. Collectively, our findings provide a conceptual basis for the establishment of clinical protocols of As_2S_2 for the treatment of hematological disorders, in particular MDS and MDS/AML.

3.3. Degradation of PML-RARα

It is no doubt that PML-RARα plays a central role in the initiation of leukemogenesis, although there is evidence to suggest that the fusion gene expression is not the sole genetic event required for the development of APL [4–6]. It has been clarified that arsenic-mediated modulation/degradation of the PML-RARα oncoprotein is one of the major mechanisms responsible for the efficacy of arsenic compounds in APL [4–6, 93]. Furthermore, the PML moiety, but not the RARα moiety, of the PML-RARα chimera represents the target for arsenic treatment [6, 61]. It has been clarified that both PML and PML-RARα form high-molecular-weight conjugates with a small ubiquitin-related modifier (SUMO)-1 and are recruited from the nucleoplasm to the nuclear body (NB), followed by ubiquitin-mediated proteolysis [94–96]. Degradation of PML-RARα is closely associated with differentiation, growth inhibition associated with the induction of apoptosis, as well as cell cycle arrest in APL cells treated with arsenic compounds [4–6, 93]. In addition, degradation of the PML-RARα protein associated with its redistribution was also reported in fresh APL cells obtained from the PB and BM of APL patients after treatment with As_4S_4 [97]. More intriguingly, Tian and colleagues have recently investigated the effects of As_4S_4 on RA-resistant human APL NB4-R1 cells and found that treatment with As_4S_4 induced apoptosis in cells through the downregulation of expression of the SET gene, which is a natural inhibitor for protein phosphatase 2 (PP2A), a pro-apoptotic protein [93]. They further demonstrated that the addition of As_4S_4 strengthened the SET RNAi-induced upregulation of PP2A and the downregulation of PML-RARα, suggesting that As_4S_4 induces apoptosis through the downregulation of the SET protein expression, which, in turn, increases PP2A expression and reduces PML-RARα expression, consequently leading to the apoptosis of NB4-R1 cells [93].

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4. Potential combination therapies with arsenic compounds

An extensive body of literature has clearly demonstrated superiority in treating APL simultaneously with ATO and ATRA [4–6, 61, 98]. It has been demonstrated that ATRA synergizes ATO activity to provide superior efficacy of combination therapy in patients by promoting the effects of ATO on several signaling pathways such as apoptosis induction, differentiation, as well as the degradation of PML-RARα [4–6]. In this regard, we have recently investigated the effects of ATO, ATRA, and the granulocyte colony-stimulating factor (G-CSF), alone or in combination, on the APL cell line HT93A by focusing on differentiation, growth inhibition, as well as arsenic uptake [87]. Our experimental data demonstrated that ATRA induced greater differentiation in cells than ATO and that G-CSF promoted differentiation-inducing activities of both ATO and ATRA [87]. Similar to a previous report showing that ATRA induced aquaporin-9 (AQP-9), which is a member of the aquaporin superfamily and proposed to be responsible for arsenic uptake [99–101], in HL-60 cells, we also demonstrated that ATRA induced AQP9 expression in a time- and dose-dependent manner in HT93A cells [87]. However, probably due to its cytotoxicity, treatment with 1 μM ATRA decreased arsenic uptake compared with the control subject. Interestingly, the addition of G-CSF recovered the reduced arsenic uptake to the same level as that in controls by increasing the number of viable cells, although G-CSF itself did not affect the expression levels of AQP9 [87]. Collectively, our results indicate that G-CSF not only promotes differentiation-inducing activities of both ATRA and ATO but also makes APL cells vulnerable to increased arsenic uptake, providing new insight into combination therapy using these three agents for the treatment of APL.

Since HDACs play a key role in the transcriptional regulation and pathogenesis of cancer [102, 103], its inhibitors (HDACi) are currently being developed for the therapy of several types of cancer, including leukemia [104]. Furthermore, aberrant recruitment of HDACs through the expression of PML-RARα has been implicated as an initiating tumorigenic event in APL [5–7]. Based on these previous findings, we hypothesized that treatment with ATRA in combination with HDACi could provide therapeutic benefit for patients with APL. In this regard, we investigated the effects of ATRA and valproic acid (VPA), alone and in combination, on the NB4 cells in view of differentiation induction and growth inhibition [105], since VPA is a member of class I HDACi and has shown potential anti-leukemic activities either alone or in combination with other anti-leukemic agents [102, 103, 106, 107]. In this study, we demonstrated that not only ATRA but also VPA induced differentiation in NB4 cells, and their combination further augmented the differentiation activity [105]. We further demonstrated that the upregulation of transcription factors, including CCAAT/enhancer-binding proteins (CEBPα, β, ε) and PU.1, which are known to be critical factors for normal myelopoiesis, granulocytic maturation, and being repressed in APL, concurred with the differentiation induction [105]. Given the importance of CEBPs and PU.1 in myeloid development, our results thus suggest that restoration of the normal function of the myeloid cell transcriptional machinery is a major molecular mechanism underlying the differentiation induction in NB4 cells [105]. It has been suggested that ATO/ATRA degrades the PML-RARα oncoprotein, resulting in the eradication of leukemia-initiating cells [108, 109]. Therefore, as a new therapeutic approach, a multi-target therapy based on a combination of ATRA, ATO, and VPA would be useful and worth evaluating further for its beneficial clinical effects.

Although advances in science and technology have replaced raw herbs and/or herbal compounds with powerful synthetic drugs, including molecular target-based drugs, in cancer therapy, the issue of concern is still resistance, disease relapse, and side effects of drugs in a clinical setting. In the case of arsenic compounds, side effects such as white blood cell count, QT prolongation, as well as liver dysfunction are still a serious concern and limit further clinical application, although the remarkable clinical efficacy of arsenic compound-based regimens against APL has been reported [4, 16]. Therefore, application of new arsenic-based therapies may require the generation of sensitizing strategies for improving the efficacy of arsenic compounds as well as minimizing their side effects. In order to optimize and/or maximize future clinical applications of arsenic compounds in patients with leukemia or even other malignancies, including solid tumors, combination therapy has attracted considerable interest as new therapeutic strategies. In this regard, we have been interested in the effects of naturally derived substances such as flavonoids on different kinds of cancerous cells, including leukemic cells [110–115]. Of these, Vitex, an extract from the ripe fruit of Vitex agnus-castus, has attracted great attention [112, 113, 115, 116]. We have investigated the effects of Vitex and its major component, casticin (Figure 2), on leukemia cell lines, HL-60 and U-937, and found that HL-60 cells were more sensitive to the cytotoxicity of Vitex/casticin compared with U-937 cells [112]. Furthermore, compared with unstimulated HL-60 cells, phorbol 12-myristate 13-acetate (PMA)- and 1,25-dihydroxyvitamin D3 (VD3)-differentiated HL-60 cells acquired resistance to Vitex/casticin. Based on the observation that the HL-60 cell line is more immature than the U-937 cell line, our results suggest that the levels of cytotoxicity of Vitex/casticin were largely attributed to the degree of differentiation of leukemia cells; that is, cell lines with less differentiated phenotype were more susceptible than the differentiated ones [112]. More importantly, much less cytotoxicity was observed in peripheral blood mononuclear cells (PBMNCs) from healthy volunteers when treated with concentrations of Vitex/casticin showing significant cytotoxicity in both leukemic cell lines [112]. Since recent studies have demonstrated that less differentiated cancer cells, referred to as leukemia stem cells (LSCs), acquired limitless self-renewal through oncogenic transformation and that the incomplete eradication of primary LSCs is closely linked to chemotherapy resistance and consequently contribute to eventual disease relapse [117], our findings thus provide fundamental insight into the clinical application of Vitex/casticin for hematopoietic malignancy in combination with arsenic compounds. We further demonstrated [113, 114] that Vitex/casticin-triggered cytotoxicity in HL-60 cells was implicated in histone H3 phosphorylation through the activation of the p38 MAPK pathway, which is a common signaling pathway involved in the mechanism underlying the cytocidal effects of arsenic compounds [4, 6, 16]. These findings suggest that Vitex/casticin could be promising candidates of adjunct therapeutic reagents for leukemia patients.

Delphinidin (Figure 2), a major anthocyanidin known to be present in pigmented fruits and vegetables, such as pomegranate, berries, dark grapes, eggplant, and red onion, is a diphenylpropane-based polyphenolic ring structure that carries a positive charge in its central ring [118]. Delphinidin has been gaining considerable attention, as it appears to possess a strong antioxidant/oxidant property as well as other potentially beneficial traits, such as anti-inflammatory, antimutagenesis, and antiangiogenesis activities [119, 120]. Furthermore, delphinidin and its glycosides have been demonstrated to trigger apoptosis in HL-60 cells

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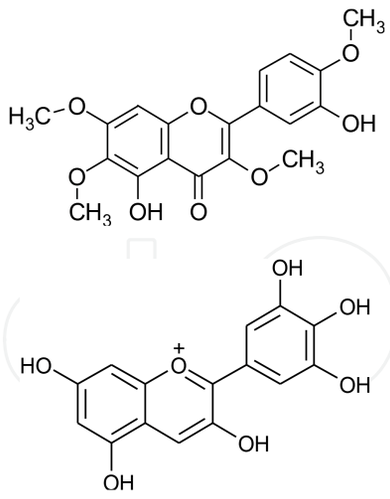

Figure 2. Chemical structures of casticin and delphinidin.

through a ROS/JNK-mediated mitochondrial death pathway [121, 122]. We have recently demonstrated that delphinidin exhibited a dose- and time-dependent cytotoxic effect against NB4 cells, in which intrinsic/extrinsic pathway-mediated apoptosis, but not cell cycle arrest, was involved (Yuan et al, submitted). We further demonstrated that delphinidin exerted more potent cytotoxicity against NB4 cells than normal PBMNCs and that delphinidin in combination with arsenite achieved an enhanced cytocidal effect against NB4 cells, but lesser on PBMNCs (Yuan et al, submitted). These results suggest that delphinidin selectively sensitizes NB4 cells to arsenite, resulting in an enhancement of arsenite cytotoxicity by strengthening intrinsic/extrinsic pathway-mediated apoptosis induction. Our observations may offer a rationale for the use of delphinidin to improve the clinical efficacy of arsenite.

Intriguingly, it has been revealed that flavonoids can inhibit the function of ATP-binding cassette transporters such as multidrug-resistance-associated proteins (MRPs) as well as P-glycoprotein (P-gp) [123, 124], which are known to be responsible for the efflux of arsenic and may consequently contribute to resistance to arsenic therapy [4, 125, 126]. In addition, we have recently demonstrated [101] that MRP2 and AQP9, which belongs to the aquaporin superfamily and is closely associated with arsenic uptake, contribute to the differential sensitivity of primary human-derived normal cells to arsenite using a unique *in vitro* primary cell culture system [79, 127, 128]. Therefore, the influence of naturally derived substances on the expression of transporters associated with intracellular arsenic accumulation should be taken into account during treatment in combination with arsenic compounds. More recently, by using human embryonic kidney epithelial (HEK) 293 cells overexpressing MRP4 cells, we have demonstrated (Yuan et al., manuscript in preparation) that MRP4, another member of ABC transporters, could be one of the major contributors to arsenic resistance, although further investigation into the correlation between the expression status of MRP4 and the treatment outcome of leukemia patients treated with arsenic-based regimens is needed.

In traditional medicines, including TCM, formulae consisting of more than one active ingredients are actually much more frequently used, aiming to act on more than one pharmacolog-

ical targets and thus exerting synergistic therapeutic effects. One of the most successful models is the realgar–indigo naturalis formula (RIF), in which arsenic sulfide (A), indirubin (I), and tanshinone IIA (T) are three major components, and has been found effective against APL in China [16]. In this regard, Wang and colleagues performed the dissection of mechanisms of RIF using an APL murine model and APL cells, including NB4, NB4-R2 (ATRA-resistant NB4-derived cell lines), and primary leukemic cells from APL patients [129]. Their results not only indicated the functions of each component, e.g., A acted as a principal component, whereas I and T served as adjuvant ones, but also demonstrated the generation of expected synergistic effects in view of prolongation of the life span of treated mice, the efficiency of terminal differentiation induction, and the upregulation of APQ9 expression associated with increment in intracellular arsenic accumulation, without apparent severe side effects [129]. This study provides new insight into exploring the value of traditional formulae on a larger scale and helping to bridge Western and Eastern medicines in the era of systems biology. Besides, we also focused on the effects of products derived from the human body, such as progesterone (Pg), and demonstrated that Pg induced a dose- and time-dependent cell growth inhibition in A3 and I9.2 cells, both of which are subclones of a T-cell-derived leukemic Jurkat cell line [130]. We further suggested that growth suppression accompanied with the induction of apoptosis by Pg in these cells was mediated through the mitochondrial membrane disruption, followed by the activation of the caspase cascade [130]. These results provide a novel insight into Pg actions toward its use for clinical application in patients with lymphocytic T-cell leukemia and raise the possibility of combination with arsenic compounds.

5. Conclusions

A striking global *in vivo* and *in vitro* study on the treatment with arsenic compounds, alone or in combination, is being explored to understand detailed mechanisms underlying their efficiency in not only APL but also many other malignancies, including arsenic-resistant hematopoietic cancer or even solid tumors. Due to the higher reactivity and instability of trivalent methyl arsenic metabolites such as MAs^{III} and $DMAs^{III}$, a more accurate assessment of these metabolites in biological samples such as blood still need to be improved, although some preservatives, including diethyldithiocarbomate [48] and 2,3-dimercaptopropanesulphonate [131], have been used to stabilize these metabolites in urine samples. As mentioned above, considerable amounts of arsenic species exist in clinical samples as a protein-bound complex. Although analysis of these protein-bound arsenic complexes has been conducted, the losses of these complexes from the sampling to the detection stage have not yet been resolved [131]. Therefore, the development of more applicable contemporary analytical protocols for these unstable arsenic species shall be explored. Furthermore, in order to achieve better therapeutic effects for individual APL patients and reduce side effects of arsenic compounds, not only detailed arsenic biomonitoring but also a predictive molecular biomarker for arsenic therapy is greatly desired. In this regard, we have recently investigated the correlation between the expression levels of AQP9 and sensitivity to ATO using NB4, HT93A, as well as primary APL cells from newly diagnosed and relapsed APL patients and provided

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direct evidence that the expression of AQP9, rather than other biomarkers such as cell surface markers and chromosomal alteration, closely correlate with the sensitivity to ATO in both APL cell lines and primary blasts [132]. Our findings thus suggest that the AQP9 expression status of APL patients is a predictive marker for the successful outcome of ATO treatment. At the same time, we also showed that flow cytometry may be a new convenient and valuable tool for analyzing the AQP9 status of APL patients compared with current methods such as Western blotting [132]. Many naturally derived substances, including TCM, have been shown to be promising candidates endowed with potent antitumor activities in view of combination with arsenic compounds. In fact, many patients in Asian countries, especially those with end-stage cancer, prefer to choose traditional medicine therapy alone or in combination with chemotherapy, radiation therapy, as well as targeted therapy, aimed to palliate symptoms and improve the quality of life. Therefore, besides efforts in exploring the detailed molecular mechanism underlying their cytocidal effects against cancerous cells, systematic analyses aimed at understanding the pharmacology and toxicology profiles are eagerly awaited when used alone or in combination with other clinical drugs such arsenic compounds.

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