

Review article

From an old remedy to a magic bullet: molecular mechanisms underlying the therapeutic effects of arsenic in fighting leukemia

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Arsenic had been used in treating malignancies from the 18th to mid-20th century. In the past 3 decades, arsenic was revived and shown to be able to induce complete remission and to achieve, when combined with all-*trans* retinoic acid and chemotherapy, a 5-year overall survival of 90% in patients with acute promyelocytic leukemia driven by the t(15;17) translocation-generated promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) fusion. Molecularly, arsenic binds thiol residues and induces the formation

of reactive oxygen species, thus affecting numerous signaling pathways. Interestingly, arsenic directly binds the C3HC4 zinc finger motif in the RBCC domain of PML and PML-RAR α , induces their homodimerization and multimerization, and enhances their interaction with the SUMO E2 conjugase Ubc9, facilitating subsequent sumoylation/ubiquitination and proteasomal degradation. Arsenic-caused intermolecular disulfide formation in PML also contributes to PML-multimerization. All-*trans* retinoic acid, which targets PML-

RAR α for degradation through its RAR α moiety, synergizes with arsenic in eliminating leukemia-initiating cells. Arsenic perturbs a number of proteins involved in other hematologic malignancies, including chronic myeloid leukemia and adult T-cell leukemia/lymphoma, whereby it may bring new therapeutic benefits. The successful revival of arsenic in acute promyelocytic leukemia, together with modern mechanistic studies, has thus allowed a new paradigm to emerge in translational medicine. (*Blood*. 2011;117(24):6425-6437)

Introduction

Arsenic is the 20th most abundant element in the earth's crust.¹ It is a chemical analog of phosphorus and lies directly below P in the periodic table. A unique feature of arsenic is its extremely paradoxical abilities: it is toxic to humans, animals, and plants, but is used instead of phosphorus by a recently isolated bacterium²; it kills people, but saves lives; it can cause some cancers but cures others such as the acute promyelocytic leukemia (APL)³; it is one of the oldest drugs in the world but was progressively revived between the 1970s and 1990s, because of its striking efficacy on APL, which represented the most malignant type of acute leukemia.³ These paradoxical effects of arsenic reflect its unique metabolism and multiple properties.⁴ In this review, we address how arsenic rebuilds its reputation from a notorious poison to a new lease of life by mechanistic molecular and translational medicine studies.

Arsenic in nature

Arsenic is the 33rd element in the periodic table and exists ubiquitously in either inorganic or organic forms, pure metallic arsenic being rarely found in nature. Arsenic occurs in 5 different valence states, +V (arsenate), +III (arsenite), +I (arsonium metal), 0 (arsenic), and -III (arsine).⁵ Most arsenic compounds have no smell or special taste and are white or colorless powders that do not evaporate. Arsenic gets into air when contaminated materials are burned. Inorganic arsenic occurs naturally in soils and

sedimentary rocks such as minerals and ores containing copper or lead, and it occurs in combination with many other elements especially oxygen, chlorine, and sulfur. There are 3 inorganic arsenic forms, namely, red arsenic (As₄S₄, also known as realgar), yellow arsenic (As₂S₃, also known as orpiment), and white arsenic (arsenic trioxide, ATO; As₂O₃). ATO is made by burning realgar or orpiment.⁶ Organic arsenic is arsenic compounds containing carbon that can be found in nature in water, natural gas, and shale oil.⁵ Examples of organic arsenic are methylarsine (CH₃AsH₂), dimethylarsine [(CH₃)₂AsH], trimethylarsine [(CH₃)₃As], monomethylarsonic acid [CH₃AsO(OH)₂, MMA^V], monomethylarsenous acid [CH₃As(OH)₂, MMA^{III}], dimethylarsinic acid [(CH₃)₂AsO(OH), DMA^V], dimethylarsenous acid [(CH₃)₂AsOH, DMA^{III}], trimethylarsinic oxide [(CH₃)₃AsO, TMAO], tetramethylarsonium ion [(CH₃)₄As⁺, TMA⁺], arsenobetaine [(CH₃)₃As⁺CH₂COO⁻, AB], arsenocholine [(CH₃)₃As+CH₂CH₂OH, AC], and others.^{5,7-9} Darnaparsin is an organic arsenic composed of dimethylated arsenic linked to glutathione.¹⁰

Regarding arsenic metabolism, an arsenic methyltransferase, encoded by a gene that was originally annotated as *Cyt19* and subsequently *As3mt*, was identified in rat that catalyzes the methylation of arsenite, with S-adenosyl-L-methionine as the methyl donor.^{11,12} Inorganic arsenic is metabolized by a sequential process involving a 2-electron reduction of pentavalent arsenic to trivalent arsenic, followed by oxidative methylation to pentavalent organic arsenic.^{13,14} The postulated scheme is as follows: iAs^V → iAs^{III} → MAs^V → MAs^{III} → DMAs^V → DMAs^{III} → TMA^V →

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TMA^{III}.¹³⁻¹⁶ Of note, derivatives of MA^{III} and DMA^{III} are more toxic than either iAs^V or iAs^{III}.¹⁶ Methylarsine oxide (MA^{III}O) and to a lesser extent iododimethylarsine are more potent growth inhibitors and apoptotic inducers than iAs^{III} in leukemia cells, and apoptosis is associated with greater hydrogen peroxide accumulation and inhibition of glutathione peroxidase activity. In vivo hepatic methylation of iAs^{III} may contribute to ATO-induced apoptosis but not differentiation of APL cells.¹⁵

Arsenic is toxic to human health in that it can induce skin lesions, hemorrhagic gastroenteritis, cardiac arrhythmia, psychiatric disease, and cancers.^{9,17,18} Generally, inorganic arsenic species are more toxic than organic forms to living organisms, and arsenite is usually more toxic than arsenate. Exposure to ATO by ingestion of 70-80 mg has been reported to be fatal for humans.^{7,19} The World Health Organization set the first International Drinking Water Standard for arsenic concentration at 200 µg/L in 1958, recommended lowering the standard to 50 µg/L in 1963, and further lowered the standard to 10 µg/L in 1993.²⁰ Yet millions of people worldwide ingest drinking water contaminated with arsenic at levels > 100 µg/L.^{5,7,20}

The toxicity of trivalent arsenic is related to its high affinity for the sulfhydryl groups of biomolecules such as glutathione and lipoic acid and the cysteinyl residues of many proteins and enzymes.^{7,21} Arsenic up-regulates glutathione-related genes and enzyme activities and binds to sulfhydryl groups.^{22,23} The formation of As^{III}-sulfur bonds results in various harmful effects by inhibiting the activities of enzymes such as glutathione reductase, glutathione peroxidases, thioredoxin reductase, and thioredoxin peroxidase.^{4,7,21} Because all of these enzymes regulate cellular redox status by providing antioxidant defense, arsenic exposure leads to production of reactive oxygen species (ROS).⁴ Similarly, flavin enzymes such as NAD(P)H oxidase and NO synthase isozymes have been proposed to be involved in the generation of ROS associated with arsenic exposure.⁴ Arsenic also alters global histone H3 methylation.²⁴ Consequently, arsenic affects many signal transduction cascades (eg, activation of the epidermal growth factor receptor [EGFR] signal pathway²⁵) and activates (or inactivates) transcription factors such as activated protein-1²⁶ and nuclear factor-erythroid 2-related factor 2.^{27,28} Biomarkers of arsenic exposure include the total arsenic in urine,²⁹ clastogenicity in peripheral lymphocytes, micronuclei in oral mucosa and bladder cells, and induction of heme oxygenase. A potential susceptibility biomarker is variability in arsenic metabolism, which reflects polymorphisms in the genes that encode the arsenic-metabolizing enzymes.²⁹

Arsenic as an old remedy

Arsenic is one of the oldest drugs in the world. It was first mentioned by Hippocrates (460-370 BC) who used realgar and orpiment pastes to treat ulcers in Western medicine. In China, arsenic pills for the treatment of periodic fever were recorded in the Chinese Nei Jing Treaty (263 BC).³⁰ Si-Miao Sun (581-682 AD) purified a medicine composed of realgar, orpiment, and ATO in treating malaria,³¹ whereas Shi-Zhen Li (1518-1593 AD)³² in the Ming Dynasty described the use of ATO as a remedy for a variety of diseases in his pharmacopedia. Arsenic therapy was introduced to Europe by Avicennes (980-1037 AD) and Paracelsus (1493-1541 AD). In 1774, Lefébure introduced an arsenic-containing paste proposed to be an "established remedy to radically cure all cancers."³⁶ Fowler solution (1% potassium arsenite, KAsO₂) was

first described in 1845 and was used to treat anemia and rheumatism, psoriasis, eczematous eruptions, dermatitis herpetiformis, asthma, cholera, and syphilis. In 1865, Fowler solution was the first chemotherapeutic agent used in the treatment of leukemia that produced some transient improvement.³³⁻³⁵ In 1931 Forkner and Scott, at Boston City Hospital, "rediscovered" Fowler solution for the treatment of chronic myeloid leukemia (CML), and arsenicals and irradiation remained the treatment of choice until busulphan was introduced in 1953.³³⁻³⁵

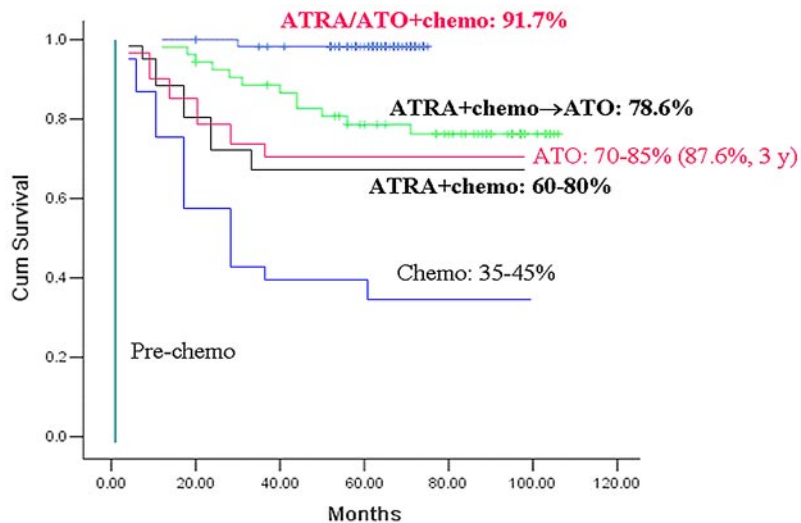
Arsenic in treating APL

APL, the M3 subtype of acute myeloid leukemia (AML M3), is characterized by the accumulation of abnormal promyelocytes in blood and bone marrow, the occurrence of fibrinogenopenia and disseminated intravascular coagulation, and the specific chromosomal translocation t(15;17)(q22;q21).³⁶⁻³⁸ The t(15;17) fuses the retinoic acid receptor α (*RAR* α) gene on chromosome 17 to the promyelocytic leukemia (*PML*) gene on 15q, yielding the PML-*RAR* α fusion protein that is the key driver of APL leukemogenesis.³⁶⁻³⁸ APL was firstly described by Hillestad in 1957³ and was considered at the time the most fatal type of acute leukemia.^{3,36,38} The past 5 decades have witnessed tremendous advances in improving APL outcome from highly fatal to highly curable (Figure 1).^{35,36,38,39} Although chemotherapy (anthracyclines) shines the first light of hope,⁴⁰ all-*trans* retinoic acid (ATRA), which triggers terminal differentiation of APL cells, achieves a complete remission (CR) rate of 90%^{38,41-44} (and references within these articles). Although ATRA alone rarely cures APL, its combination with anthracyclines allowed a significant number of cures. ATO further prolongs survival of patients with APL, especially those with relapsed or refractory disease, and cures a number of them as a single agent. Moreover, combined use of ATRA and ATO not only markedly enhances clearance of PML-*RAR* α transcript, but allows the 5-year overall survival (OS) to reach 91.7%^{38,39,45} (Figure 1). Mechanistically, both ATRA and ATO trigger catabolism of the PML-*RAR* α fusion protein. However, ATO has no effect on APL driven by promyelocytic leukemia zinc finger (PLZF)-*RAR* α , which is generated by t(11;17) and accounts for 1%-2% of patients with APL.⁴⁶⁻⁵¹ Thus, the story of APL can serve as a model for the development of targeted therapies and curative approaches for malignant disease.⁵¹

Clinical efficacy

In the early 1970s, a group from Harbin Medical University in northeastern China tested Ailing-1 containing 1% ATO and a trace amount of mercury chloride in a variety of cancers by intravenous administration. In the 1990s, Sun et al⁵² showed that Ailing-1 induced CR in 21 of 32 patients with APL with an impressive 10-year survival rate of 30%. The efficacy of pure ATO in treating relapsed APL was then reported by Shanghai Institute of Hematology (SIH) in 1996-1999.^{53,54} Shen et al⁵⁴ evaluated the therapeutic effect of ATO in the treatment of 15 patients with APL at relapse after ATRA-induced and chemotherapy-maintained CR. ATO was administered intravenously at the dose of 10 mg/d. They showed that CR was achieved in 9 of 10 patients (90%) treated with ATO alone and in the remaining 5 patients with ATO in combination with low-dose chemotherapeutic drugs or ATRA. No bone marrow depression was encountered during ATO treatment.⁵⁴ Niu et al⁵³ reported that clinical CR was obtained in 8 of 11 newly diagnosed

Figure 1. A historical view of the treatment outcome (represented by 5-year overall survival) of APL.



cases (72.7%) and in 40 of 47 relapsed patients (85.1%). They recommended that ATRA is used as first choice for remission induction in newly diagnosed APL cases, whereas ATO can be either used as a rescue for relapsed cases or included into multidrug consolidation/maintenance clinical trials. Furthermore, after CR achieved with the use of ATO alone, a molecular remission is obtainable in a relatively high proportion of the patients, from 72%⁵⁵ to 91%⁵⁶ in different multicenter studies, showing that ATO is an effective drug for APL. With the use of ATO as a single agent, a good long-term remission can be obtained, as evidenced by a 5-year event-free survival (EFS) of 69%⁵⁷ to 72.7%⁵⁸ in 2 recent reports (Table 1).

The efficacy of As₄S₄ in APL was also investigated. In 1995, Huang et al⁶³ reported remarkable results of the Realgar-Indigo Naturalis Formula, in which As₄S₄ is the principle ingredient, in treating 60 patients with APL, including 43 newly diagnosed cases. In 2002, Lu et al⁶⁴ showed that of the 129 patients with APL receiving pure As₄S₄, 103 cases (79.8%) achieved CR. In the newly diagnosed group (19 patients), the estimated disease-free survival rates for 1 and 3 years were 86.1% and 76.6%, respectively, with a median follow-up time of 13.5 months. They further showed that in 114 patients receiving As₄S₄ in combination with ATRA or chemotherapy (mitoxantrone or hydroxyurea or both), the estimated 4-year disease-free survival was 94%.⁶²

Mechanisms of action

PML-RAR α as a direct arsenic target. That ATO exerts drastic therapeutic effects against APL, but not other subtypes of AML (including variant APL driven by the PLZF-RAR α fusion), sug-

gests a crucial link between its mechanism of action and PML-RAR α , a potent transcriptional regulator that alters expression of ATRA or non-ATRA target genes.^{38,65-67} Indeed, it was rapidly shown that arsenic efficiently triggers the degradation of PML-RAR α through its PML moiety.⁶⁸⁻⁷⁰

The wild-type PML and the PML moiety in fusion protein harbor the RBCC domain,^{69,70} which contains 1 RING and 2 B boxes (B box 1 and B box 2) motifs capable of binding metal (physiologically zinc) ions, and a coiled-coil (CC) motif mediating homodimer formation.^{71,72} Figure 2A is a schematic representation of major domains of PML, RAR α , and PML-RAR α . In normal cells, PML proteins are the main components of spherical nuclear organelles designated nuclear bodies (NBs), which play a key role in regulation of apoptosis, epigenetic control of chromatin, and transcriptional expression, as well as storage/modulation of certain nuclear proteins.⁷³⁻⁷⁵ NB structures are disrupted in APL cells, because of formation of PML/PML-RAR α heterocomplex. This yields a much larger number of tiny dots by immunofluorescence analysis, showing the disorganization of this nuclear domain.⁷⁶ The ATRA and arsenic-triggered degradation process is intimately coupled to changes in PML/PML-RAR α localization.^{46,68,70,77-79} Indeed, in ATO-treated APL cells or cells transfected with PML, PML-RAR α ,⁷⁹ both PML-RAR α and wild-type PML are quickly translocated to the nuclear matrix, sumoylated, ubiquitinated, and subsequently degraded by the proteasome. In parallel, in arsenic-treated APL cells, dots of PML-containing proteins are aggregated to form larger particles at the nuclear matrix, before their ultimate disappearance (Figure 2B) and the cells are committed to apoptosis or partial differentiation (Figure 2C).

Table 1. Outcome of patients with APL treated with ATO-based regimens since 2006

| Year | Author | No. | Regimen | CR, % | EFS, %* | DFS, %* | OS, %* |
|------|---------------------------------|-----------------------------|--|-------|------------|------------|------------|
| 2010 | Powell et al ⁵⁹ | 244 (237 standard controls) | Induction: ATRA/CT; consolidation: ATRA/CT/ATO | 90 | 80 (3 y) | 90 (3 y) | 86 (3 y) |
| 2010 | Zhou et al ⁵⁸ | 19 (age \leq 15) | ATO | 89.5 | 72.7 | NR | 83.9 |
| 2010 | Mathews et al ⁵⁷ | 72 | ATO | 86.1 | 69 | 80 | 74.2 |
| 2009 | Dai et al ⁶⁰ | 90 | ATO/ATRA | 93.3 | 92.2 (3 y) | | |
| 2006 | Ghavamzadeh et al ⁶¹ | 111 | ATO | 85.6 | NR | 63.7 (2 y) | 87.6 (3 y) |
| 2008 | Hu et al ³⁹ | 85 | ATO/ATRA/CT | 94.1 | 89.2 | NR | 91.7 |
| 2007 | Wu et al ⁶² | 114 | As ₄ S ₄ /ATRA or As ₄ S ₄ /CT | | | 94 (4 y) | |

NR indicates not reported.

*Five years, unless otherwise indicated.

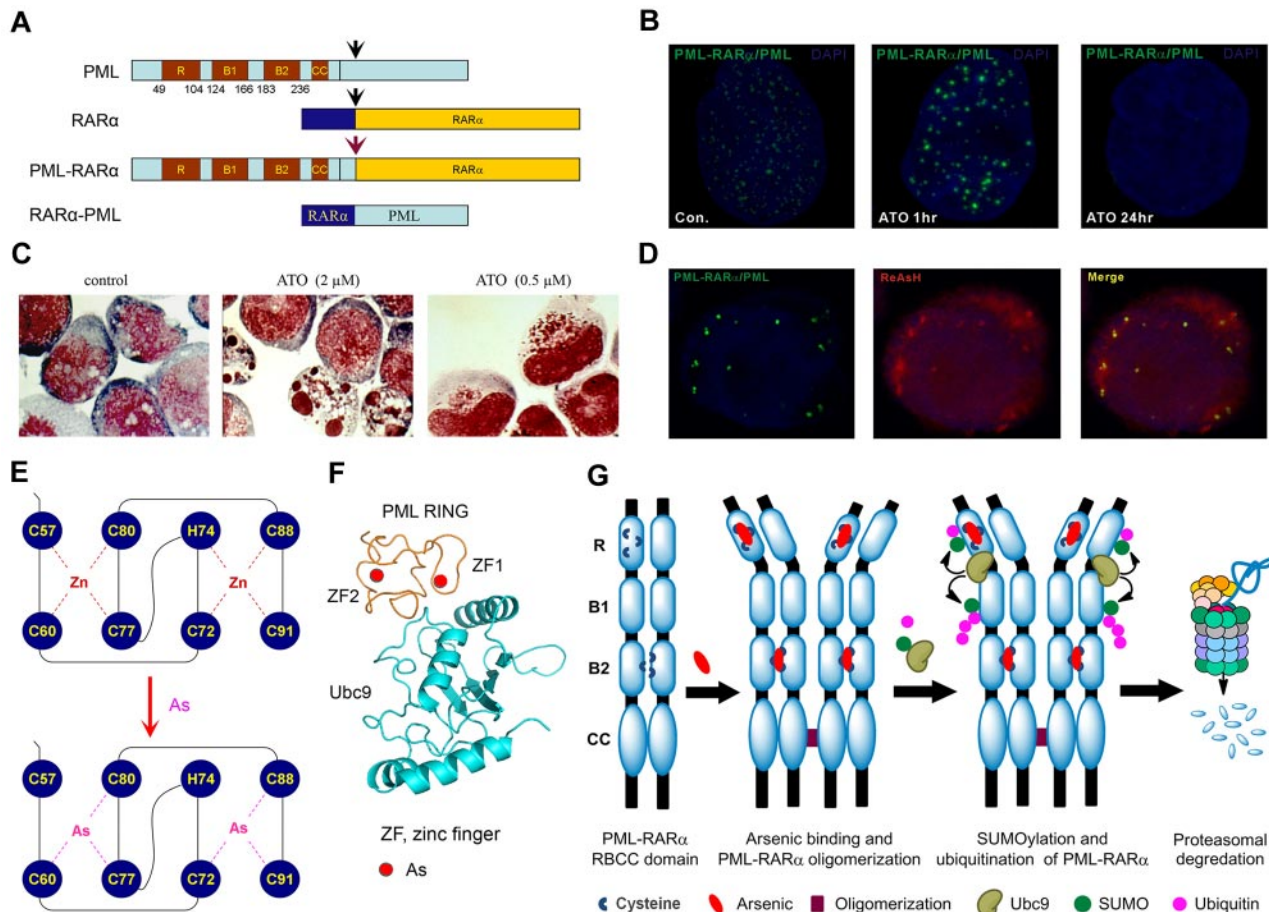


Figure 2. Effects of arsenic on APL cells. (A) Schematic represents the structure of PML, RAR α , and PML-RAR α . (B) The NB4 cells were treated with 1 μ M ATO for indicated time points and were assessed by immunofluorescence staining with an anti-PML antibody (green). Stainings were analyzed using a Leica TCS SP5 confocal microscope equipped with a 63 \times /1.4 NA oil objective (Leica Microsystems). Images were processed using Leica AF lite software. (C) Arsenic induces dual effects on APL cells. The NB4 cells were treated with indicated concentration for 48 hours and stained with the Wright stain. Stainings were analyzed using an Olympus BX51 research microscope equipped with a 100 \times /1.30 NA oil objective (Olympus). Images were processed using Adobe Photoshop CS (Adobe Systems). Original magnification, \times 1000. (D) Colocalization of PML and PML-RAR α with the fluorescent organic arsenical ReAsH in NB4 cells. Images were visualized and processed using the equipment described for panel B. (E) The schematic diagram of the structure of PML RING coordinated with zinc or arsenic. (F) Predicted structure of PML RING/UBC9 complex. (G) A working model of the mechanism by which arsenic controls the fate of PML and PML-RAR α .

Because the wild-type PML and the PML moiety in the fusion protein harbor a number of adjacently located cysteine residues with metal-binding ability in their RBCC domain,^{71,72} we hypothesized that ATO might target PML/PML-RAR α at this domain. We tested this possibility by biotin-arsenic/streptavidin pull-down affinity assay and a red fluorescent organic arsenic compound ReAsH/immunofluorescent analysis. Zhang et al⁷⁹ and Jeanne et al⁴⁶ showed that ReAsH colocalized with PML/PML-RAR α (Figure 2D) and that arsenic could directly bind the wild-type and fusion proteins. Consistently, deletion of the RING domain in PML abolished the ReAsH colocalization signal. The PML RING peptide containing C3HC4 (aa 57-91) zinc finger (ZF) was expressed and purified for refined arsenic-binding analyses. With the use of matrix-assisted laser desorption/ionization–time of flight mass spectrometry, it was found that 1 PML RING molecule without metal ions (apo-PML RING) could bind 2 arsenics at ATO concentrations of 1–2 μ M.⁷⁹ Arsenic bound PML RING through thiol groups of cysteines with the formation of arsenic-sulphur bonds, as evidenced by near-ultraviolet absorbance spectrometry assays. With the use of x-ray absorption spectra assay, including extended x-ray absorption fine structure and x-ray absorption near-edge structure, the local structures of PML RING around metal ions within \sim 6 Å were obtained, and it was found that

trivalent arsenics could coordinate to PML RING each by 3 conserved cysteines, in ZF1 with C60, C77, and C80 and in ZF2 with C72, C88, and C91, compared with the coordination by zinc in ZF1 with C57, C60, C77, C80 and in ZF2 with C72, H74, C88, and C91 (Figure 2E). In addition, the PML B box 2 domain also directly bound arsenic in vitro, whereas its deletion led to a significant reduction of ReAsH colocalization signals in cells.^{46,79} Of note, arsenic was able to competitively replace zinc in PML RING coordinated with zinc (zinc-PML RING) according to the nuclear magnetic resonance heteronuclear single-quantum coherence spectra.⁷⁹ On binding to arsenic, PML RING underwent conformational changes and aggregation, most probably because of the formation of homodimer by intermolecular As-S bonds, followed by oligomerization among these homodimers.^{46,79} In in vitro reconstituted conditions and mammalian 2-hybrid assay in cells, arsenic binding to PML facilitated its interaction with the unique small ubiquitin-like protein (SUMO) E2 conjugase UBC9, leading to sumoylation at K65 and K160.⁸⁰ K160 sumoylation also mediated subsequent recruitment of 11S proteasome.^{78,81} A 3-dimensional structure modeling shows that the 2 ZFs in PML RING motif locate at the interface with UBC9, providing a structural basis for the formation of PML/UBC9 complex in the presence of arsenic (Figure 2F). Recent studies indicate that RNF4, a ubiquitin E3 ligase containing

Table 2. Arsenic binding proteins

| Proteins and references | Disease or cell lines | Category | Functions |
|-------------------------------|---------------------------------|--------------------------------------|--|
| PML ⁷⁹ | APL | Phosphoprotein; scaffold protein | Tumor suppressor; probable transcription factor |
| c-CBL ⁸³ | CML, K562 cells | Ubiquitin E3 ligase | Proteolysis |
| SIAH1 ⁸³ | CML, K562 cells | Ubiquitin E3 ligase | Proteolysis |
| Trx R ⁸⁴ | MCF-7 cells | Oxidoreductase | Redox regulation |
| GSR ⁸⁵ | Arsenic toxication | Oxidoreductase | Redox regulation |
| TPX-2 II ⁸⁶ | Ovary cells | Peroxidase | Redox regulation |
| PDJ ⁸⁷ | Fibrosarcoma cells | Oxidoreductase | Redox regulation |
| MTs ⁸⁸ | Arsenic detoxication | Metallothioneins | Binding heavy metals |
| MTF1 ⁸⁹ | Arsenic detoxication | Transcription factor | Activation of metallothionein transcription |
| Keap1 ²⁷ | hepa1c1c7 cells | Phosphoprotein | Transcription regulation |
| Tubulins ⁹⁰⁻⁹² | K562 cells | Cell skeleton proteins | Structural subunit of microtubules |
| β-Actin ⁹¹⁻⁹³ | K562 and MCF-7 cells | Cell skeleton proteins | Cytoskeleton |
| PPM1D ⁹⁴ | Malignancies | Phosphatase; oncoprotein | Protein serine/threonine phosphatase; regulation of cell cycle |
| JNK phosphatase ²⁶ | Carcinogenesis | Dual specificity protein phosphatase | Cellular signaling |
| IκB ⁹⁵ | Inflammation and carcinogenesis | Protein kinase | Regulation of NFκB pathway |
| Galectin-1 ^{86,96} | Ovary cells | Pyruvate kinase | Regulation of NFκB pathway |
| PKM2 ⁹¹ | MCF-7 cells | Phosphoprotein | Glycolysis |
| Hemoglobin ⁹⁷ | Red blood cells | Globin family | Oxygen transport |

SUMO interaction motifs,^{77,82} could recruit sumoylated PML/PML-RARα and promote their proteasomal degradation.^{77,82} These results show that ATO controls the fate of the PML-RARα by directly binding PML (Figure 2G) and, at least partially, explaining why ATO is effective for APL.⁷⁹

In addition to formation of arsenic-cysteine bonds that favor aggregation, arsenic-induced ROS also initiate intermolecular disulfide formation.⁴⁶ Disulfide-linked PML or PML-RARα multimers become nuclear matrix-associated and form NBs. Thus, PML oxidation regulated NB biogenesis. In that respect, non-arsenical oxidants also elicited PML-RARα multimerization, NB association, degradation, and leukemia response in vivo. Critically, oxidants did not affect PLZF-RARα-driven APL, a genetic demonstration that PML is the key target.⁴⁶ Arsenic can also bind other proteins, including the ubiquitin E3 ligases c-CBL (Casitas B-lineage lymphoma) and SIAH1,⁸³ both harboring RING finger motifs (Table 2).

Elimination of leukemia-initiating cells. Leukemia-initiating cells (LICs) are pluripotent, self-renewing, phenotypically primitive and mitotically quiescent cells that have been identified in acute and chronic myeloid and lymphoid leukemia subtypes. Their noncycling status and inherent or acquired drug resistance mechanisms allow them to escape conventional and targeted therapies that effectively kill proliferating leukemia cells.⁹⁸ In APL, PML-RARα is required, and even a minute amount of the oncoprotein allows LICs self-renewal in vivo.⁹⁹ Zheng et al¹⁰⁰ showed that in *Sca1⁺/lin⁻* murine hematopoietic stem cells retrovirally transduced with PML-RARα and LICs from PML-RARα mice, ex vivo treatment with arsenic overcomes the aberrant stem cell capacity of PML-RARα⁺ LICs. Whereas transcriptional activation of PML-RARα on effect of ATRA probably controls differentiation, only the catabolism of the fusion protein triggers LIC eradication and long-term remission of mouse APL.⁹⁹ ATRA induces differentiation of PLZF-RARα-driven mouse APL, but neither LIC clearance nor disease remission was achieved, explaining the clinical ATRA resistance of this rare APL subtype. Importantly, the ATRA/ATO combination rapidly clears PML-RARα⁺ LICs, resulting in APL eradication in murine models and patients.⁹⁹ Because anthracyclines¹⁰¹ produce ROS, the ATRA and idarubicin regimen¹⁰² may

induce PML-RARα degradation and hence promote LIC clearance, resulting in dramatically prolonged survival.¹⁰²

Arsenic targeting of PML may also be important in the non-APL setting. Indeed, Ito et al¹⁰³ showed that PML was required for hematopoietic stem cell maintenance, and in CML it appeared to be the factor that enabled LICs to maintain their quiescence—the inert state that prevented them from being destroyed by cancer therapies. Interestingly, ATO could reversibly decrease PML expression in LICs, suggesting that this agent may be of broader interest than previously thought. In gliomas, ATO seemed able to inhibit the Notch pathway and deplete the cancer stem-like cell population.¹⁰⁴ ATO was shown to antagonize the Hedgehog pathway by preventing ciliary accumulation and reducing stability of the Gli2 transcriptional effector.¹⁰⁵ ATO also represses NFκB¹⁰⁶ and β-catenin,¹⁰⁷ facilitating elimination of LICs (Figure 3).

Cell differentiation, apoptosis, and autophagy. At the cellular level, ATO exerts dose-dependent dual effects on APL cells⁷⁰ in that it induces apoptosis through activating the mitochondria-mediated intrinsic apoptotic pathway at high concentrations (1–2 μM) and promotes cell differentiation at low concentrations (0.25–0.5 μM; Figure 2C). The mechanisms of proapoptotic activity of ATO were scrutinized by many groups at gene/protein levels,^{38,108} and a large body of information has been gathered, including histone H3 phosphoacetylation at the *CASP-10*¹⁰⁹; the involvement of JNK signaling¹¹⁰; anion exchanger 2¹¹¹ and GSTP1-1¹¹²; the suppression of human telomerase reverse transcriptase (*hTERT*), *C17*, and *C-MYC* through Sp1 oxidation¹¹³; the repression of NFκB activation¹⁰⁶; and the down-regulation of the *WT1* gene.¹¹⁴ A pathway composed of ataxia telangiectasia mutated and Rad3-related, PML, CHK2, and p53 has been proposed to mediate ATO-induced apoptosis.¹¹⁵ ATO up-regulates a set of genes such as *NADPH* oxidase¹¹⁶ and 8-hydroxy-2'-deoxyguanosine (*8-OHdG*),¹¹⁷ and causes generation of ROS, which play a role as a mediator to induce apoptosis through release of cytochrome c to cytosol and activation of caspases. However, although all those mechanisms probably contribute to chronic or acute arsenic toxicity, it is unclear what is their contribution to arsenic response in APL, because they are unlikely to be specific for APL cells.

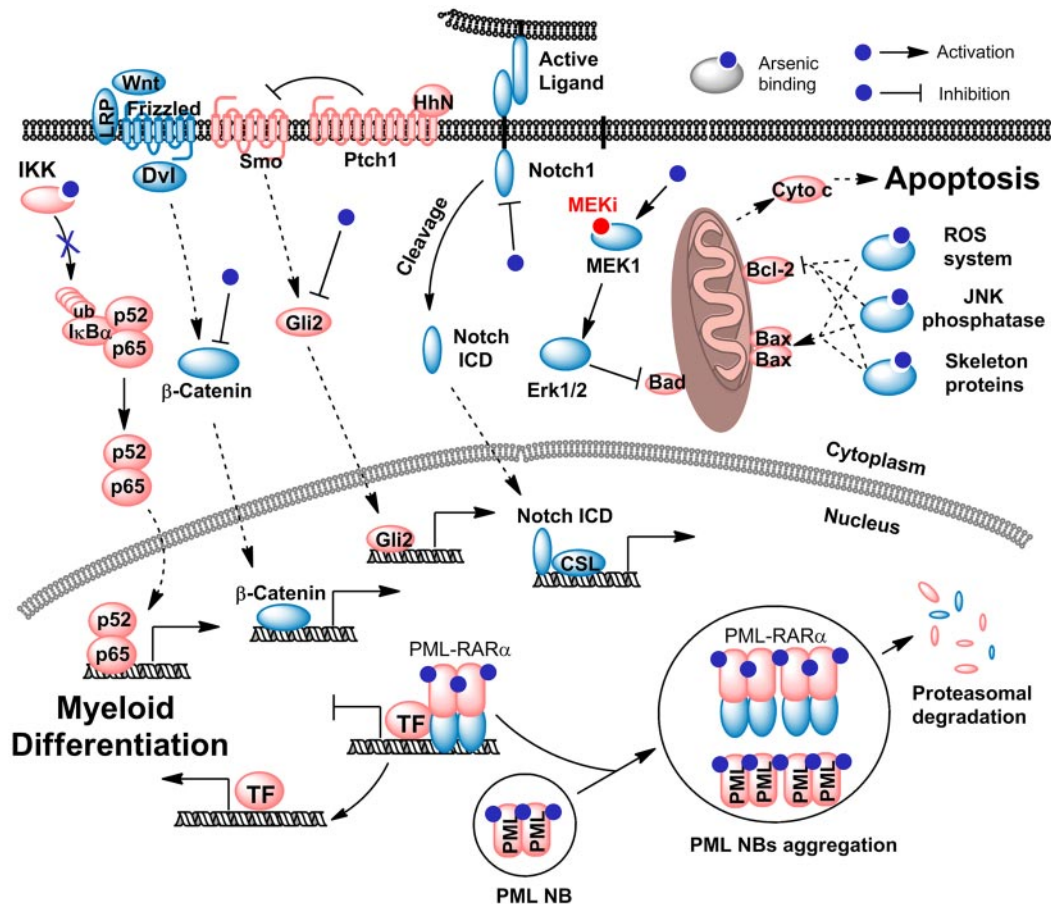


Figure 3. Arsenic targets critical pathways for the leukemia-initiating cells. Arsenic induces generation of ROS, perturbation of some signal pathways, and modulation of transcriptional factors. Arsenic also activates MEK1/ERK pathway, whereas combination of MEK1 inhibitor (MEKi, red filled circle) and arsenic results in synergistic antitumor effects.

Recent studies further showed that in APL cells both ATRA and ATO induce autophagy by the mammalian target of rapamycin (mTOR)¹¹⁸ or MEK/ERK pathway,¹¹⁹ and autophagic degradation contributes significantly to proteolysis of PML-RAR α . Inhibitors of autophagy or molecular targeting of BECN1 or ATG7 results in reversal of the suppressive effects of ATO on leukemic cells.¹¹⁹

Arsenic-based combinatory therapeutic regimens

ATO/ATRA combination. Rationales. Combination treatment regimens have been carefully designed to conquer APL.³⁸ In a PML-RAR α mouse model and a human NB4 APL cell line–based ascites/leukemia mouse model, ATRA/ATO combination dramatically prolongs survival or even eradicates the disease.^{120,121} Both ATO^{99,100} and ATRA⁹⁹ induce LIC loss in PML-RAR α mouse APL and the combination of the two agents results in synergistic clearance of LICs through cooperative PML-RAR α degradation. Moreover, ATRA was shown to be able to increase the expression of the cell membrane arsenic transporter AQP9, which facilitated arsenic uptake.¹²²

Although ATO alone induces partial differentiation,⁷⁰ it synergizes with cAMP analog 8-CPT-cAMP in inducing full maturation of ATRA-sensitive and ATRA-resistance cells.^{123,124} However, activation of cAMP signaling was shown to enhance LIC loss by ATRA.^{99,125,126} Interestingly, ATRA could rapidly trigger a marked increase in the intracellular cAMP level and cAMP-dependent

protein kinase (PKA) activity.¹²⁷ Therefore, a cross talk may exist between ATO and ATRA signaling pathways through the cAMP/PKA node.

Moreover, ATRA potentiates ATO-induced RXR α phosphorylation and cooperates with ATO to induce apoptosis,¹²⁸ and it was shown that ATRA induced degradation, whereas ATO antagonized catabolism of I κ B.¹⁰⁶ In the maturation-resistant NB4-R1 cells, ATRA exhibited antiproliferative properties through downregulation of telomerase,¹²⁹ and ATO enhanced this effect.¹³⁰ When transcriptome/proteome approaches were used, Zheng et al¹³¹ reported that, although ATRA mainly caused transcriptional remodeling, ATO induced a deeper change of proteome pattern. ATRA/ATO combination amplified RA signaling, as highlighted by molecules involving IFN, calcium, cAMP/PKA, MAPK/JNK/p38, G-CSF, and TNF pathways. ATRA/ATO combination strongly activated the ubiquitin-proteasome pathway and significantly repressed genes/proteins promoting cell cycle or enhancing cell proliferation. Interestingly, the ATRA/ATO combination did not enhance the expression of stress response–related genes, including HSPA8, HSPCA, and AHSAL.¹³¹ Taken together, these results suggest that the ATRA/ATO combination may cause a synergy in therapeutic efficacy but not adverse effects.

Clinical efficacy. A randomized clinical trial comparing ATRA/ATO combination and monotherapy was conducted in SIH from April 2001 to February 2003.^{38,45,132} Sixty-one APL cases were randomly assigned into 3 groups treated, respectively with ATRA,

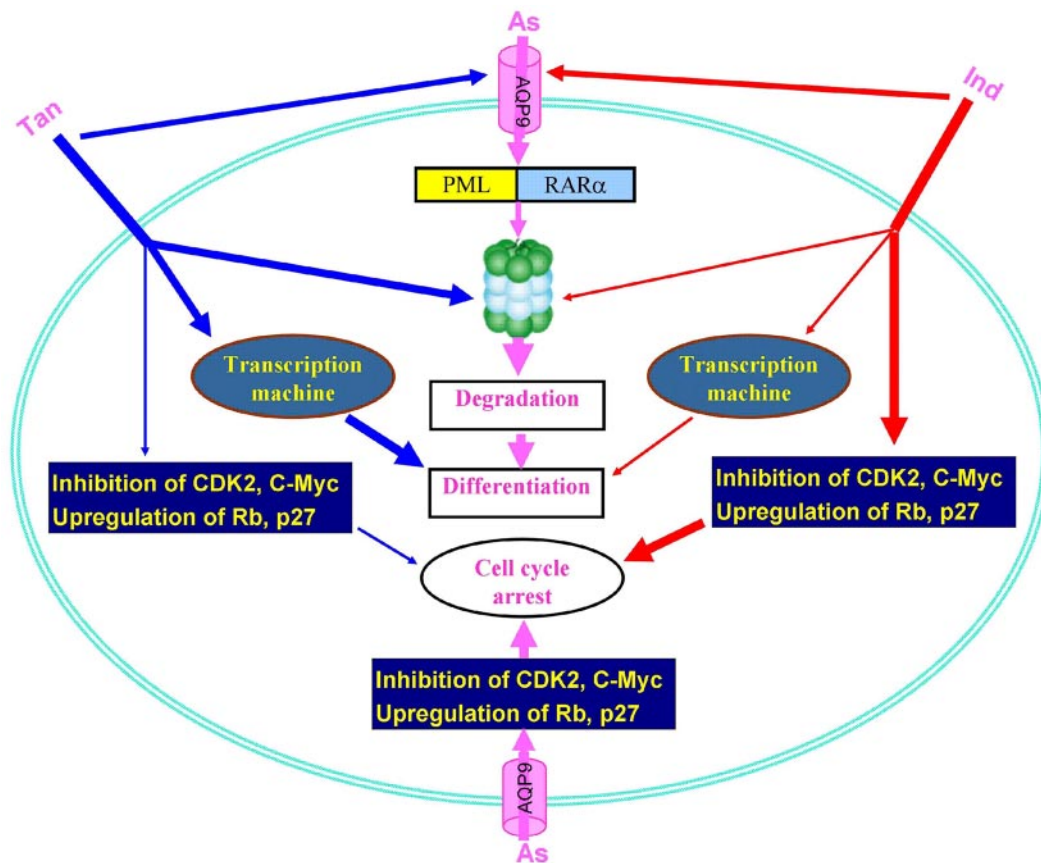


Figure 4. Mechanisms of action of representative components of RIF in treating APL. As, As₄S₄; Ind, indirubin; Tan, tanshinone IIA.

ATO, and the combination of the 2. It was reported in 2004 that the 3 groups achieved the same results in terms of CR rates ($\geq 90\%$), although the combination therapy group needed the shortest time duration for remission induction. An obvious advantage of the combination therapy was that it generated much less minimal residual disease after consolidation than the 2 other therapeutic approaches as measured with real-time quantitative RT-PCR for PML-RAR α . After a follow-up of 8-30 months all patients in the combination therapy group were in good clinical remission, whereas 7 of 37 cases in the 2 monotherapy groups relapsed ($P < .05$), showing the superiority of the combination therapy.⁴⁵ Under this circumstance, the investigators from the SIH decided, from the ethical point of view, a termination of the randomized grouping and only the arm of combination therapy should be extended. In 2009, SIH reported the results of 85 patients administered ATRA/ATO with a median follow-up of 70 months. Eighty patients (94.1%) entered CR.³⁹ Kaplan-Meier estimates of the 5-year EFS and OS for all patients were $89.2\% \pm 3.4\%$ and $91.7\% \pm 3.0\%$, respectively, and the 5-year relapse-free survival and OS for patients who achieved CR ($n = 80$) were $94.8\% \pm 2.5\%$ and $97.4\% \pm 1.8\%$, respectively. On ATRA/ATO, prognosis was not influenced by initial white blood cell count, distinct PML-RAR α types, or FLT3 mutations. The toxicity profile was mild and reversible (see "Adverse effects of arsenic"). The results were confirmed by recent long-term follow-up studies.^{133,134} Powell et al⁵⁹ reported that of the 244 patients who received ATRA/chemotherapy as induction and ATRA/chemotherapy plus ATO as consolidation therapies, 195 cases (80%) achieved a 3-year EFS. Compared with the SIH trial that used ATRA/ATO/chemotherapy as induction therapy,³⁹ the slightly lower EFS rate of this study

might be because of the multicenter nature of the trial or could reflect the advantage of incorporating ATO into induction remedy for newly diagnosed APL. Taken together, ATRA/ATO/chemotherapy combinatory regimen transforms APL from a highly fatal to a highly curable disease.

Realgar-Indigo Naturalis Formula. ATRA/As₄S₄ combination also showed enhanced therapeutic efficacy in APL.⁶² In traditional Chinese medicine, combination therapy containing multiple drugs with distinct but related mechanisms has been advocated for > 2500 years by prescriptions called formulas to amplify therapeutic efficacies of each agent and to minimize adverse effects.^{30,135} On the basis of traditional Chinese medicine theories, a patented Realgar-Indigo Naturalis Formula (RIF) was designed in the 1980s,⁶³ in which a mined ore realgar was the principle element, whereas *indigo naturalis*, *Salvia miltiorrhiza*, and *radix pseudostellariae* were adjuvant components to assist the effects of realgar. Multicenter clinical trials showed that a CR rate of 96.7% ¹³⁶ to 98% ⁶³ and a 5-year OS rate of 86.88% ¹³⁷ were achieved in patients with APL receiving RIF, with moderate gastrointestinal discomfort and rash as the main adverse effects. Realgar in combination with indigo also exhibited an extent of anti-APL activity.¹³⁸ The mechanisms of action of RIF were carefully dissected with the use of As₄S₄ (A), indirubin (I), and tanshinone IIA (T) as representatives of realgar, indigo naturalis, and *Salvia miltiorrhiza*, respectively,¹³⁹ and it was shown that ATI combination yielded enhanced therapeutic efficacies against APL in murine model. ATI combination caused synergic effects and resulted in a much more profound differentiation of APL cells, potentiated ubiquitination and degradation of PML-RAR α oncoprotein, stronger reprogramming of myeloid differentiation regulators,

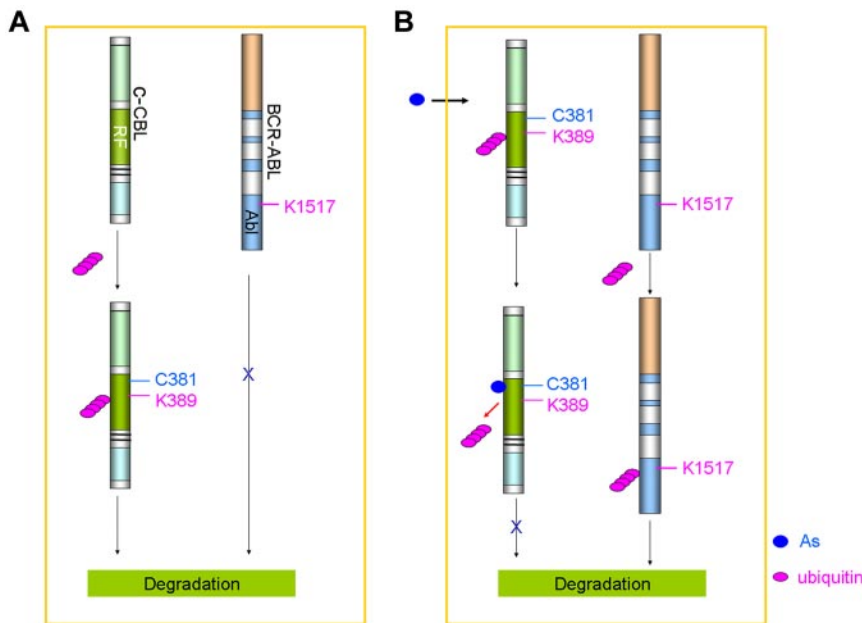


Figure 5. Effects of arsenic on c-CBL and BCR-ABL in CML cells. (A) Without arsenic treatment, c-CBL is self-ubiquitinated and degraded in proteasome. (B) Arsenic treatment inhibits c-CBL self-ubiquitination and proteasomal degradation, and triggers ubiquitination of BCR-ABL at K1517 followed by degradation in the proteasome.

and enhanced G_1/G_0 arrest compared with cells treated with monoagents or biagents. Furthermore, T and I up-regulated AQP9 and facilitated transportation of arsenic into malignant promyelocytes, which in turn intensified arsenic-mediated PML-RAR α degradation and therapeutic efficacies (Figure 4). These results open a new window for a better understanding of the therapeutic strategies of other traditional formulas.

ATO in combination with MEK1 inhibition. Studies have shown that activation of ERK1/2 as well as of the kinases immediately upstream of ERK, known as MAP/ERK kinases or MEKs can confer a drug-resistant phenotype to cancer cells. For example, rapamycin and its analogs activate the MAPK pathway in solid tumor,¹⁴⁰ imatinib increases the activity of p42/44 MAPK in CML CD34⁺ cells that contributes to incomplete elimination of CML progenitors,¹⁴¹ and FLT3 inhibitor-resistant cells show continued activation of PI3K/AKT and/or RAS/MEK/MAPK signaling pathways.¹⁴² Accordingly, MAPK/MEK inhibitors may be helpful to overcome drug resistance in leukemic cells. Altman et al¹⁴³ showed that in leukemia cells on ATO treatment, the AKT kinase is phosphorylated/activated to regulate downstream engagement of mTOR and its effectors. Targeted disruption of AKT1/AKT2 genes or inhibition of mTOR strongly enhances ATO's effects on leukemia cells.¹⁴³ Treatment with ATO induces a MAPK-mediated PML phosphorylation¹⁴⁴ that is associated with subsequent ubiquitination and proteasomal degradation.⁴⁶ Lunghi et al¹⁴⁵ reported that APL cells exploited the RAS-MAPK pathway to inactivate the proapoptotic protein BAD by phosphorylation at Ser112 and to delay ATO-induced apoptosis. MEK1 inhibitors suppressed ERK1/2, dephosphorylated BAD, and inhibited the ATO-induced increase of Bcl-xL, resulting in enhanced apoptosis and overcoming drug resistance.¹⁴⁵ Combined use of ATO and MEK1 inhibitors leads to induction of the p53AIP1 (p53-regulated apoptosis-inducing protein 1) in NB4 and K562 cell lines¹⁴⁶ and primary cells from patients with AML,¹³⁷ and inhibition of tumor growth and elongation of survival in a human xenograft multiple myeloma model. These studies provide the framework for testing MEK1 inhibitor/ATO combination in patients with hematologic malignancies.¹⁴⁸

Arsenic in treating CML

CML, a malignant myeloproliferative disease originated from pluripotential hematopoietic stem cells, is characterized by the Philadelphia chromosome formed by translocation t(9;22) (q34;q11) that generates a chimeric fusion protein BCR-ABL with constitutively activated tyrosine kinase activity.¹⁴⁹ Imatinib mesylate (IM; or Gleevec, Glivec, or STI571), a rationally designed BCR-ABL inhibitor, has shown remarkable clinical efficacy that achieved an estimated 5-year OS of 89% in 553 patients with CML.¹⁵⁰ However, IM^{151,152} and dasatinib^{152,153} do not deplete LICs, whereas a proportion of patients develop IM resistance,^{152,154-156} and patients with advanced stage disease respond initially but then relapse.^{152,157} Moreover, cardiotoxicity of IM was also reported.^{158,159}

Historically, ATO therapy was the first chemotherapeutic intervention for CML. Fowler solution was used to treat CML in the 19th century and became the mainstream therapeutic reagent for leukemia.¹⁶⁰ In the 1930s, the efficacy of arsenic in the treatment of CML established it as a primary therapeutic agent for this disease.¹⁶⁰ Until the advent of modern chemotherapy, arsenic and radiation were the mainstays of treatment for patients with CML. Arsenic was shown to be able to target PML and to eradicate quiescent LICs in CML.¹⁰³ ATO inhibited translation of mRNA of *BCR/ABL*, resulting in attenuation of BCR/ABL levels and apoptosis of human leukemia cells.¹⁶¹ Zhang et al¹⁶² reported that arsenic targets BCR-ABL by ubiquitination of key lysine residues, leading to its proteasomal degradation. Recently, Mao et al⁸³ showed that arsenic could directly bind c-CBL, the E3 ligase of BCR-ABL, by the conserved cysteines, including C381 at RING finger domain (Figure 5), resulting in inhibition of c-CBL's self-ubiquitination at K389 and subsequent proteasomal degradation. Consistent with these results, substitution of cysteine at 381 or lysine at 389 by alanine abrogated arsenic binding and c-CBL's self-ubiquitination, respectively. Consequently, elevated c-CBL promoted ubiquitination of BCR-ABL at K1517, leading to degradation of the aberrant kinase (Figure 5).

Table 3. Clinical studies of arsenic in treating other malignancies

| Disease | Year | Authors | No. | Regimen | Response |
|-----------------------|------|---------------------------------|-------------------------|--------------------------|--------------------|
| MM | 2006 | Berenson et al ¹⁶⁶ | 65 | ATO + AA + melphan | 2 CR; 15 PR; 14 MR |
| | 2006 | Abou-Jawde et al ¹⁶⁷ | 20 | ATO + AA + dexamethasone | 6 PR |
| | 2006 | Wu et al ¹⁶⁸ | 20 | ATO + AA + dexamethasone | 2 PR; 6 MR |
| | 2007 | Berenson et al ¹⁶⁹ | 22 | ATO + AA + bortezomib | 2 PR; 4 MR |
| ATL | 2004 | Hermine et al ¹⁷⁰ | 7 (Relapsed/refractory) | ATO + IFN | 1 CR; 3 PR |
| | 2009 | Khour et al ¹⁷¹ | 10 (Newly diagnosed) | ATO + IFN + zidovudine | 9 CR; 1 PR |
| MDS | 2006 | Schiller et al ¹⁷² | 76 | ATO | 1 CR; 13 HI |
| | 2006 | Vey et al ¹⁷³ | 115 | ATO | 1 CR; 1 PR; 22 HI |
| | 2008 | Zheng et al ¹⁷⁴ | 21 | ATO + RA + thalidomide | 1 CR; 1 PR; 3 HI |
| Lymphoid malignancies | 2009 | Chang et al ¹⁷⁵ | 16 | ATO + AA | 1 response |

MM indicates multiple myeloma; AA, ascorbic acid; CR, complete response; PR, partial response; MR, minor response; ATL, adult T-cell leukemia/lymphoma; MDS, myelodysplasia syndrome; HI, hematologic improvement; and RA, retinoic acid.

Arsenic exerts synergistic effects with IM in inducing apoptosis of CML cells and in prolonging survival of mice inoculated with CML cells.¹⁶²⁻¹⁶⁴ It was shown that arsenic and IM induce cell cycle arrest at G₂/M and G₁ phases, respectively. Arsenic and IM synergistically activate the endogenous and exogenous endoplasmic reticulum (ER) stress, leading to enhanced cell apoptosis.^{162,163,165} These discoveries provide rationales for a clinical trial to test the arsenic/IM combination therapy in CML.

Arsenic in treating other malignancies

Arsenic has been used in treating multiple myeloma, myelodysplasia syndrome, and lymphoid malignancies, including non-Hodgkin lymphoma, and has displayed beneficial effects in some cases (Table 3). In adult T-cell leukemia/lymphoma (ATL)-derived cells, ATO reportedly synergized with IFN α to induce cell-cycle arrest and apoptosis¹⁷⁶ through down-regulation of the HTLV-1 oncoprotein Tax and inactivation of NF- κ B.^{177,178} Clinically, arsenic/IFN therapy exhibited some efficacy in 7 patients with refractory aggressive ATL,¹⁷⁰ whereas in 10 cases of newly diagnosed chronic ATL arsenic/IFN/zidovudine combination showed an impressive 100% response rate.¹⁷¹ Recent animal studies in Lck-Tax transgenics that develop an ATL-like disease have recapitulated the therapeutic action of the arsenic/IFN α association, strongly suggesting that the latter is actually targeting Tax for degradation.¹⁷⁹ Moreover, transplantation studies have shown that Tax degradation is accompanied by loss of leukemia-initiating activity, but not short-term growth, providing a striking parallel with APL and suggesting that arsenic may promote catabolism of specific classes of oncoproteins.

There are 111 recently completed or ongoing clinical trials listed on www.clinicaltrials.gov that evaluate ATO alone or in combination with other agents for treatment of cancers, excluding APL. ATO is under investigation as treatment for a variety of solid tumors, including lung cancer, hepatocellular carcinoma, and colorectal cancer. Limited clinical activity as a single agent has been reported in a small number of patients with hepatocellular carcinoma, melanoma, and renal cell carcinoma; ATO in combination with chemotherapy has shown promising activity in osteosarcoma and Ewing sarcoma¹⁸⁰ (and references in this review article).

Adverse effects of arsenic

Although arsenic seems to be synonymous with poison, nearly all recent clinical trial results suggest that arsenic at therapeutic

concentrations is generally well tolerated. No bone marrow depression and chemotherapy-associated secondary malignancy were observed with arsenic treatment.^{52,54}

Sudden death was recorded in one study,¹⁸¹ and severe liver impairment was documented.⁵³ These toxicities might be because of a genetic basis with exceptional susceptibilities to arsenic toxicity in rare patients, exposure to anthracyclines or other cardiotoxic agents before ATO therapy, and abnormal electrolyte levels, or other unidentified factors.^{53,57,181,182} Hyperleukocytosis, a retinoic acid syndrome (or differentiation syndrome)-like clinical entity was also reported and was shown to be driven by chemokine production induced by ATO or ATRA as a single agent or in combination.¹⁸³ In long-term studies,^{39,57,58,182} the toxicity profile of arsenic was mild, and 24 months after the last dose of ATRA/ATO patients had urine arsenic concentrations well below the safety limit. Mathews et al⁵⁷ reported that despite counseling against pregnancy after therapy, in view of the absence of data on effect of prior ATO therapy and teratogenicity, 7 patients (4 women and 3 men) have had 8 normal babies.

Perspectives

As a traditional poison, inappropriate use of arsenic may kill people; as one of the oldest drugs in the world, its appropriate application cures some cancer types and saves lives. These facts clearly suggest that when considering how to control an emerging "bad factor," one might try to find out its other side and the safe translation.

Arsenic is the most potent single agent against APL. The revival of arsenic by its application in treating APL is a unique story in cancer research. It also highlights some of the essential concepts in pharmacology, such as the key importance of the therapeutic ratio between normal cells and the target. It illustrates the power of combinations. Indeed, in APL, ATO/ATRA combination has exhibited drastically enhanced therapeutic efficacy compared with either single agent, transforming the fate of an otherwise highly fatal disease. In treating other types of malignancies, including solid tumors, rational combinatory regimens could be designed to improve clinical outcome. For example, because arsenic binds and activates c-CBL that controls signaling of EGFR, the therapeutic efficacy of ATO in combination with EGFR inhibitor could be tested in non-small cell lung cancer and other related human malignancies.

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