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Review

Mechanisms of Action of Arsenic Trioxide[1]

Wilson H. Miller, Jr.,[2] Hyman M. Schipper, Janet S. Lee, Jack Singer, and Samuel Waxman

Lady Davis Institute for Medical Research and Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal H3T 1E2, Quebec, Canada [W. H. M., H. M. S.]; Ingenix, Basking Ridge, New Jersey 07920 [J. S. L.]; Cell Therapeutics, Inc., Seattle, Washington 89119 [J. S.]; and Rochelle Belfer Chemotherapy Foundation Laboratory, Mt. Sinai School of Medicine, New York, New York 10029 [S. W.]

Abstract

Arsenic trioxide has shown substantial efficacy in treating both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL). As a single agent, it induces complete remissions, causing few adverse effects and only minimal myelosuppression. These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Substantial data show that arsenic trioxide produces remissions in patients with APL at least in part through a mechanism that results in the degradation of the aberrant PML-retinoic acid receptor α fusion protein. Studies have also investigated concerns about the toxicity and potential carcinogenicity of long-term exposure to environmental arsenic. Arsenic apparently affects numerous intracellular signal transduction pathways and causes many alterations in cellular function. These actions of arsenic may result in the induction of apoptosis, the inhibition of growth and angiogenesis, and the promotion of differentiation. Such effects have been observed in cultured cell lines and animal models, as well as clinical studies. Because arsenic affects so many cellular and physiological pathways, a wide variety of malignancies, including both hematologic cancer and solid tumors derived from several tissue types, may be susceptible to therapy with arsenic trioxide. These multiple actions of arsenic trioxide also highlight the need for additional mechanistic studies to determine which actions mediate the diverse biological effects of this agent. This information will be critical to realizing the potential for synergy between arsenic trioxide and other chemotherapeutic agents, thus providing enhanced benefit in cancer therapy.

Introduction

During the last decade, the efficacy of arsenic trioxide in both newly diagnosed and relapsed patients with APL[3] has been established. Arsenic trioxide can be used as a single agent and induces complete remissions with only minimal myelosuppression (1). Investigators in China introduced arsenic trioxide, alone and in combination chemotherapies, as a cancer therapeutic for patients with APL, achieving notable rates of complete remission. In newly diagnosed patients, complete remission rates have ranged from 70% to 90%, and in relapsed patients, rates of 65% to >90% have been reported (2–5). In one study (2), long-term follow-up revealed that 16 patients (50%) survived >5 years, with survival extending to >17 years. Myelosuppression was minimal. The most common side effects of arsenic trioxide were gastrointestinal. Adverse events subsided spontaneously or with symptom-based interventions and did not require suspension of treatment (1).

Additional studies conducted in the United States confirmed that low doses of arsenic trioxide can induce complete remission in relapsed APL patients. Twelve APL patients who had received extensive prior therapy were treated with arsenic trioxide in doses of 0.06–0.2 mg/kg of body weight until visible leukemic blasts and promyelocytes were eliminated from the bone marrow. Complete remissions occurred in 11 of the 12 patients, with 8 of those 11 patients achieving molecular remissions as well (6). Adverse events associated with arsenic trioxide were mostly mild; they included rash, lightheadedness, fatigue, and musculoskeletal pain. These results were confirmed in a follow-up multicenter trial, in which 34 of 40 patients (85%) achieved complete responses; 31 of those patients also achieved cytogenetic remission (7). Additional trials are under way in patients with APL and in patients with other hematologic malignancies and solid tumors (8, 9).

The success of arsenic trioxide in inducing high rates of complete remission in patients who have relapsed with this aggressive, acute disease provides an impetus for uncovering the specific mechanisms of action underlying these dramatic clinical responses. This review summarizes reports of the activity of arsenic in animal models and in the clinic. We will also address the explosion of literature describing many effects of arsenic on cancer cell growth, differentiation, and apoptosis, including its activation or inhibition of a variety of cellular signaling pathways. Finally, we draw potential links between the observed *in vitro* actions of arsenic and its potential future role in the treatment of human cancer.

Arsenicals and Their Metabolism

Arsenic is a semimetal commonly found in soil, water, and air. Common inorganic and organic forms of arsenic are listed in Table 1. Often, arsenic is complexed with sulfur as red arsenic (As_2S_2), also called realgar or sandaraca, or as yellow arsenic (As_2S_3), also called orpiment or auripigment. White arsenic or arsenic trioxide (As_2O_3) is produced by heating realgar (10). Arsenic is rarely found in a pure state; rather, it exists in both trivalent and pentavalent oxidation states as a chemically unstable sulfide or oxide, or as a salt of sodium, potassium, or calcium (11). Trivalent arsenicals, including sodium arsenite and the more soluble arsenic trioxide, inhibit many enzymes by reacting with biological ligands that possess available sulfur groups. Being pentavalent, arsenic is recognized as an uncoupler of mitochondrial oxidative phosphorylation (12).

Although most inorganic arsenic that is ingested is eliminated fairly rapidly in the urine, a small amount may be modified by methylation to monomethylarsonic acid and to dimethylarsinic acid, a process referred to as biotransformation. Whereas these enzymatic reactions are considered to be detoxifying, some organic arsenic metabolites may actually contribute to the cytotoxicity of arsenic (reviewed in Ref. 13).

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[2] To whom requests for reprints should be addressed, at Lady Davis Institute for Medical Research 3755 Cote Ste Catherine Road, Montreal H3T 1E2, Quebec, Canada.

[3] The abbreviations used are: APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; GADD45, growth arrest- and DNA damage-inducible protein 45; IKK, IκB kinase; JNK, c-Jun NH_2-terminal kinase; Hsp90, 90 K_d heat shock protein; MAP, mitogen-activated protein; NFκB, nuclear factor κB; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKC, protein kinase C; PML, promyelocytic leukemia; RAR, retinoic acid receptor; RA, retinoic acid; IL, interleukin; HO-1, Heme oxygenase-1.

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Table 1 *Arsenic in nature*

Arsenic forms	Chemical formula	Other names
Red arsenic	As_2S_2	Realgar, sandaraca
Yellow arsenic	As_2S_3	Arsenikon, aurum pigmentum, orpiment
White arsenic (created by heating realgar)	As_2O_3	
Phenylarsine oxide	C_6H_5AsO	

History of the Medicinal Use of Arsenic Derivatives

Medicinal use of arsenic and its derivatives dates back more than 2400 years to ancient Greece and Rome. Arsenic was viewed as both a therapeutic agent and a poison (12). Hippocrates administered orpiment (As_2S_3) and realgar (As_2S_2) as an ulcer remedy; Dioscorides used orpiment as a depilatory. Arsenic has also been used to treat the plague, malaria, and cancer and to promote sweating. Physicians prescribed arsenic for both external and internal use throughout the 18th century. Arsenides and arsenic salts were key ingredients in antiseptics, antispasmodics, antiperiodics, caustics, cholagogues, hematinics, sedatives, and tonics. Approximately 60 different arsenic preparations have been developed and distributed during the lengthy history of this agent. More than 20 of these preparations were still in use at the end of the 19th century, including Aiken's Tonic Pills, Andrew's Tonic, and Arsenauro.

When physicians first boiled arsenous acid with an alkali in the late 1700s and produced a water-soluble compound, the administration of medicinal arsenic changed radically from generally external to primarily internal. In 1786, Thomas Fowler, a physician to the General Infirmary of the County of Stafford, England, recommended use of potassium arsenite for the treatment of intermittent fever. Fowler's Solution gained great renown and was used to treat many conditions, including paralytic afflictions, rheumatism, hypochondriasis, epilepsy, hysteria, melancholia, dropsy, rachitis, heart palpitations, convulsions, syphilis, ulcers, cancer, and dyspepsia (14). In 1911, Fowler's Solution was used as a treatment for pernicious anemia, asthma, psoriasis, pemphigus, and eczema. In 1910, additional experimentation with the properties of arsenic led Paul Ehrlich, the German physician and founder of chemotherapy, to the discovery of an organic arsenical, salvarsan (arsphenamine; Ref. 11). Arsphenamine was the standard therapy for syphilis for nearly 40 years before it was replaced by penicillin (15). Arsphenamine was also believed to be an effective treatment for trypanosomiasis (11). As already mentioned, arsenic has proven recently to be highly effective in the treatment of APL; its use induces and maintains complete remissions, with a less toxic profile than traditional chemotherapy (1). The therapeutic history of arsenic is additionally outlined in Table 2.

Arsenic was nicknamed "The Mule" not only for its dependability in many therapeutic regimens but also for the stubborn persistency with which it was used and the inconstant nature of its toxic capacity (14). Although arsenic was found to be beneficial in many disease states, and side effects or later repercussions of therapy were inconsistent from patient to patient, concerns among medical professionals about toxicities associated with arsenic use, especially long-term use, surfaced in later years. The IARC first evaluated the carcinogenicity of arsenic and arsenic compounds in 1973. It found a "causal relationship between skin cancer and heavy exposure to inorganic arsenic in drugs, drinking water with a high arsenic content, or in the occupational environment." However, experimental studies with arsenic in animals were considered inadequate, and the causative role of arsenic remained largely unclear. In 1979, the agency classified arsenic and certain arsenic compounds as Group 1 carcinogens, those that were "carcinogenic to humans," on the basis of epidemiological studies of the relationship between exposure to arsenic compounds via occupation, ingestion, or medical use and skin cancer. In 1980, reevaluation of the data determined that exposure to arsenic and arsenic-containing compounds was a cause of lung cancer in humans. Finally, in 1987, support for arsenic as a cause of human cancers was judged as adequate on the basis of "limited evidence of carcinogenicity in experimental animals" (15).

Dermatological manifestations of arsenic use were commonly observed in patients with prolonged arsenic exposure. Long-term ingestion of Fowler's Solution produces signs of chronic arsenic intoxication. In one study, palmar and plantar keratoses occurred in all of the patients evaluated, although the minimum time to onset was 15 years (median, 24 years) after beginning arsenic treatment. Basal cell carcinoma developed in a majority of these patients, with the same time to onset. Other manifestations included carcinoma *in situ*, squamous cell carcinoma, breast adenocarcinoma, and colon carcinoma, all appearing within a minimum of 28–63 years after treatment. The precise amounts of arsenic these patients received over time is not known, because small amounts of arsenic are present in the environment, and there are any number of possible routes of exposure. Nonetheless, despite the anecdotal nature of the evidence, the keratoses and carcinomas that developed were attributed to past and prolonged arsenic intake (16). Another study retrospectively evaluated 478 patients who had taken Fowler's Solution for periods of 2 weeks to 12 years between 1945 and 1969. A search for evidence of prolonged arsenic ingestion revealed 142 patients with dermatologic manifestations, 45% of whom had keratoses, 14% of whom had hyperpigmentation, and 11% of whom had skin cancer. Arsenic toxicity was related to age and dose: compared with patients who displayed no signs of arsenic toxicity, those who did were on average 7 years older and had received higher median doses (672 mg *versus* 448 mg, a highly significant difference; $P = 0.001$). Overall, internal malignancies attributed to the use of Fowler's Solution were minimal; dermatological diseases attributed to arsenic treatment were more numerous (17).

Potential Targets of Arsenic in Chemotherapy for APL

Via numerous pathways (Fig. 1), arsenic facilitates profound cellular alterations, including induction of apoptosis, inhibition of proliferation, stimulation of differentiation, and inhibition of angiogenesis. The biological effects of arsenic (principally the trivalent forms, arsenite and arsenic trioxide) may be mediated by reactions with closely spaced cysteine residues on critical cell proteins (18). Several proteins with a high cysteine content and accessible thiol groups are

Table 2 *Key events in the medicinal use of arsenic*

Year	Key events
400 BCE	Hippocrates used realgar (As_2S_2) and orpiment (As_2S_3) as remedies for ulcers
50 AD	Dioscorides used orpiment as a depilatory
14th century	Angelus Salva used arsenic against the plague
16th century	Jean de Gorris used arsenic as a sudorific
17th century	Lentilius and Friceius used arsenic as a treatment for malaria
18th century	Arsenic preparations were used therapeutically: Aiken's Tonic Pills, Andrew's Tonic, Arsenauro, Gross' Neuralgia Pills, Cholor-Phosphide of Arsenic, Sulphur Compound Lozenges These were still in circulation at the end of the 19th century
19th century	Arsenic was a mainstay of the materia medica Fowler's Solution (potassium arsenite) was praised for its success in treating asthma, chorea, eczema, Hodgkin's disease, pemphigus, pernicious anemia, and psoriasis
20th century	Ehrlich discovered an organic arsenical (salvarsan) that cured syphilis and was used to treat trypanosomiasis Used in traditional Chinese medicine for hundreds of years arsenic derivatives are still used to devitalize the pulp of diseased teeth and in regimens for psoriasis, rheumatic diseases, and syphilis

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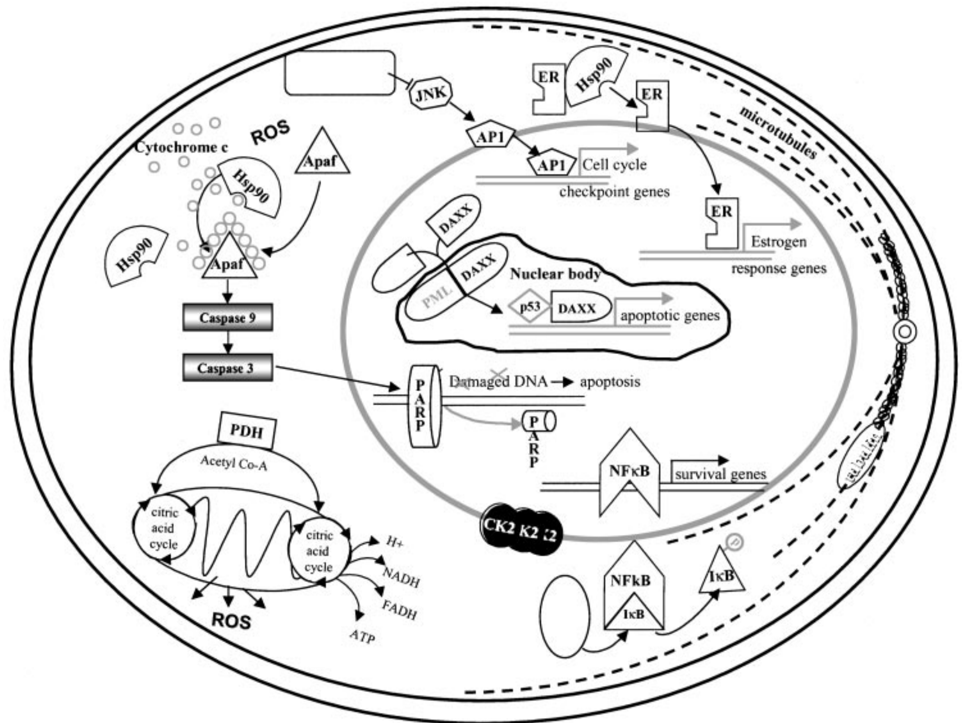

Fig. 1. Cellular targets of arsenic trioxide. Arsenic trioxide targets multiple pathways in malignant cells resulting in apoptosis or in the promotion of the differentiation program. Objects highlighted in *gray* are potential molecular targets for arsenic trioxide and arsenite.

candidates for interactions with arsenic. APL is the clinical setting in which arsenic trioxide has achieved notable success. A substantial body of evidence has accumulated during recent years suggesting the mechanisms by which the drug produces remissions in patients with APL. Unless otherwise noted, studies cited in this review have used concentrations of arsenic that are physiologically achievable (<5 μM).

PML-RARα (Inducer of APL). The vast majority of cases of APL are characterized by the t(15;17) translocation. This translocation generates a fusion between the *PML* gene and the *RARα* gene, which encodes a transcription factor (19, 20). The resulting PML-RARα fusion protein blocks the expression of genes required for normal myeloid differentiation. Sequence analysis of the *PML* gene has indicated the presence of a cysteine-rich region that may be a principal candidate for interaction with trivalent arsenic. The endogenous PML protein in normal cells has been shown to be localized to a novel macromolecular structure in the nucleus, the nuclear body. Expression of the PML-RARα fusion protein in leukemic cells disrupts the nuclear bodies, and the PML protein is dispersed into smaller fragments of these structures (21–23).

The block in myeloid differentiation by PML-RARα can be released by treatment with pharmacologic levels of RA, providing the basis for ATRA therapy for APL. RA-induced differentiation of APL blasts results in degradation of the fusion protein and the relocation of wild-type PML from the deviant nuclear structures to its normal location in nuclear bodies (24–26). In NB4 APL cells, the PML-RARα fusion protein displays a micropunctate distribution in nuclei and cytoplasm; ATRA treatment leads to the typical PML staining pattern of nuclear speckles. Arsenic trioxide also causes degradation of the PML-RARα fusion protein, although probably by a different mechanism than RA, because normal PML structures are not completely restored (24).

Arsenic trioxide has been proposed as an alternative to treatment with ATRA because it can induce complete remissions in both RA-sensitive and RA-resistant APL patients. Although both compounds induce degradation of PML-RARα, several differences in their action on APL cells have been reported. *In vitro*, ATRA induces differentiation of APL cells along a granulocytic pathway, whereas in APL and a variety of malignant cell lines, arsenic induces apoptosis with little evidence of differentiation (24, 25). In APL cells sensitive to ATRA, *in vitro* treatment with arsenic antagonizes ATRA-induced differentiation, whereas treatment with ATRA decreases arsenic-induced apoptosis (25, 27). However, in several models of cells resistant to differentiation by RA alone, subapoptotic concentrations of arsenic can synergize with ATRA to induce differentiation (27, 28).

When arsenic trioxide is used in patients with APL who have relapsed after ATRA treatment, it can induce differentiation (6). In a xenograft mouse model of APL, treatment with either arsenic trioxide or ATRA increased survival by 35–39%. However, when these mice were treated sequentially with either arsenic trioxide or ATRA followed by the other agent, their survival was prolonged by 70–80% (27). Transgenic mouse models of APL have shown a possible synergy between arsenic and ATRA, although whether this is maximal with sequential or coadministration of the two agents is not clear (29, 30). Of note, the APL variant characterized by the fusion between PLZF and RARα (PLZF-RARα) did not respond with improved survival to ATRA, arsenic trioxide, or the combination of agents, despite induced degradation of the fusion protein (30). This is consistent with *in vitro* results reported previously suggesting that APL cell growth arrest and differentiation could occur in the absence of PML-RARα degradation (31), and that preservation of the malignant phenotype may not require detectable expression of the fusion protein in some APL clones (32).

One potentially important difference between the mechanisms by which arsenic trioxide and ATRA induce degradation of the PML-RARα fusion protein is that arsenic trioxide targets the PML portion of the protein, whereas RA targets RARα (26). Within nuclear bodies, PML recruits numerous proteins and antagonizes many of the processes required for the initiation, promotion, and progression of malignancy (33).

Gene Products Interacting with PML. The crucial growth-regulating role of PML was underscored by a recent report demonstrating the interaction and colocalization of PML with the tumor-

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Table 3 *Arsenic trioxide: potential mechanisms of action*

Affected process	Mechanism of action	Biological effects
Induction of apoptosis	Elevated intracellular (H_2O_2)	• Cytochrome *c* release and activation of the caspase cascade • Release of soluble intermembrane proteins, which cause DNA fragmentation • Inhibition of glutathione peroxidase (GPx) activity
	Increased *bax* expression	• Down-regulation of bcl-2 family members sensitizing cells toward apoptosis
	Inhibition of NFκB activity	• Interferes with intracellular adhesion molecule and vascular cell adhesion molecule up-regulation, inhibiting IL-6 secretion resulting from adhesion of MM cells to bone marrow stroma
	Inhibits GTP-induced polymerization of tubulin	• Cytoskeletal protein damage
	Altered nuclear distribution of PML	• PML is recruited onto matrix-bound nuclear bodies (NB), for degradation
Antiproliferative activity	Inhibition of STAT3 activity	• Transcription inhibition of IL-6-responsive genes
	Growth arrest in the G_1 phase of the cell cycle	• Enhancement of cyclin dependent protein kinase inhibitors (CDKI) p21 and p27
	Induction of differentiation	• Induced expression of cell surface maturation markers (CD11b)
Angiogenesis inhibition	Inhibition of vascular endothelial growth factor	• Diminished vascular endothelial growth factor expression inhibits endothelial cell proliferation in a dose- and time-dependent manner

suppressor protein p53 (34). p53 induces G_1 arrest in normal fibroblasts in response to DNA damage. If DNA cannot be repaired, accumulation of p53 leads to apoptosis. This occurs only in cells with normally functioning p53; cells with mutated p53 do not undergo cell cycle arrest or become apoptotic. The function of p53 in DNA damage-induced apoptosis, transcriptional activation, and the DNA-binding activity of p53 may depend on intact PML. The biochemical basis of this dependence is provided in recent experiments showing that ras-induced p53 acetylation is lost in cells that do not express PML (35). Therefore, arsenic-induced degradation of PML in non-APL cells might be expected to decrease p53 activity and protect cells with genetic abnormalities from apoptosis. However, the ability of arsenic to stimulate other proapoptotic pathways may counterbalance the effects of PML degradation. In addition, arsenic trioxide promotes the accumulation of Daxx, a nuclear protein that represses transcription and colocalizes with PML in the nuclear bodies. Daxx modulates transcription of death-related genes in Fas-induced apoptosis (36). For this reason, it has been suggested that nuclear bodies are intranuclear sites that function in a PML-dependent manner as reservoirs to sequester or concentrate transcriptional repressors for modulating gene transcription (36). Thus, even a transient increase in the accumulation of PML within nuclear bodies followed by its degradation may be sufficient to trigger Daxx-dependent apoptosis.

Arsenic-containing compounds enlarge PML-containing nuclear bodies in the cell nucleus by increasing covalent modification of PML with SUMO-1, a ubiquitin-like protein involved in proapoptotic signal transduction. In a similar manner, arsenic trioxide treatment increases SUMO-1 modification of PML-RARα. Apoptosis ensues, independent of DNA-binding activities of the RARα part of the fusion protein and of Bcl-2 and caspase 3 (37).

There are numerous reports of proapoptotic actions of arsenic in other malignant cell lines that may be dependent on PML as well as p53. In gastric cancer cells and in MBC-1, a B-cell lymphoma line, arsenic trioxide exposure causes up-regulation of p53 expression, resulting in caspase activation and, ultimately, apoptosis (38, 39). p53 accumulation is also implicated in the mechanism by which arsenic trioxide treatment induces apoptosis and G_1 phase arrest in human T-cell lymphotropic virus type 1-infected cells. In these cells, too, on exposure to arsenic trioxide, levels of Cip1/p21 and $p27^{KIP1}$ were increased, and the retinoblastoma protein was dephosphorylated (40). Finally, in human fibroblasts, arsenite causes DNA strand breaks, which leads to accumulation and phosphorylation of p53. Levels of p53 downstream proteins (p21 and others) were also increased. Dissection of this pathway indicated that a phosphatidylinositol-3-kinase-related protein kinase, within an ataxia-telangiectasia mutated pathway, was involved in mediating p53 accumulation after arsenite exposure (41).

In addition to up-regulating *p53* and other genes associated with growth arrest and apoptosis, exposure to arsenic trioxide down-regulates *Bcl-2* RNA and protein expression in APL and other cell types, suggesting the involvement of Bcl-2 in the regulation of arsenic-mediated apoptosis (24, 42–45). In addition, the proapoptotic Bcl-2 family member, Bax, of which the translocation to the mitochondria induces mitochondrial permeability and activates apoptosis, may be recruited by apoptotic signals to the nuclear body, where it associates with PML (33, 46, 47). The cell cycle inhibitor $p27^{KIP1}$ has also been shown to associate with the nuclear body. Thus, arsenic appears to enhance the targeting of PML, Bax, and $p27^{KIP1}$ to nuclear bodies and, in cells with intact PML, acts synergistically with IFNs, which induce PML, to induce cell death (46).

Effects of Arsenic on Cellular Signaling

Although there has been considerable focus on PML-RARα and PML in the response to arsenic, recent investigation has yielded considerable data on the mechanisms by which arsenic acts on other cellular pathways. These potential targets listed in Table 3 are discussed below.

Protein Kinase Signaling. Lines of evidence obtained from very different model systems point to the ability of arsenic to alter the function of several enzymes and signaling molecules, and markedly influence gene expression (Table 3; Fig. 1). Sodium arsenite activates the transcription factor AP-1, as a consequence of increasing the activity of its mitogenic components, c-fos and c-jun (48). This occurs simultaneously with activation of JNKs, which are important for phosphorylation of many transcription factors that enhance expression of immediate early genes. Arsenite may stimulate JNK activity by inhibiting a constitutive JNK phosphatase, of which the role in unstimulated cells is to maintain low basal JNK activity (48).

The oxidation state of arsenic is reported to influence the signaling pathways that are activated. The pathways that are activated may vary depending on tissue-specific origins or characteristics of particular cells. For example, in human embryonic kidney cells, arsenite and arsenate activate JNK via different profiles of MEKKs. Specifically, activation of JNK by arsenate involves MEKK3 and MEKK4, whereas activation of JNK by arsenite requires MEKK2, as well as MEKK3 and MEKK4 (49). In addition, p21-activated kinase is necessary for arsenite but not arsenate-induced stimulation of JNK. In bronchial epithelial cells, exposure to arsenic activates members of the MAP kinase family, including the extracellular signal-regulated kinase (50). This activation is mediated by epidermal growth factor receptor phosphorylation, which can be inhibited by an epidermal growth factor receptor-specific tyrosine kinase inhibitor. In addition, these cells show low levels of Raf-1 kinase activity, which was not increased by metal exposure (50).

The PKC family includes at least 11 isoenzymes with similar structures; all mediate numerous cellular functions. Three PKC isoenzymes, PKCα, PKCϵ, and PKCδ, were reported to mediate arsenite-

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induced signal transduction in an epidermal cell line, leading to AP-1 activation (51). These isoforms of PKC each function through a different MAP kinase pathway; so additional experimental evidence with specific inhibitors and dominant-negative mutants may help dissect which MAP kinase proteins are important for arsenic-mediated actions in a specific tissue. Overall, it is clear that arsenite exposure induces translocation of several PKC isoforms from the cytosol to the plasma membrane, where these enzymes play key roles in mediating signal transduction and so may contribute to the biological effects of arsenic treatment (51).

Finally, cholesterol-rich, detergent-resistant membrane components known as rafts have been identified recently as playing a critical role in modulating intracellular signal transduction. Several important molecules that mediate signal transduction are localized to rafts. In experimental systems, β-cyclodextrin can sequester cholesterol from rafts, compromising their integrity (52). Such effects make cells resistant to the effects of arsenite, because the signals necessary to initiate the apoptosis cascade cannot be properly transduced. Consequently, elements identified previously of the arsenite-induced pro-apoptotic cascade, including MAP kinase activation, superoxide production, and caspase activation, do not occur (52).

Tyrosine Phosphatases. Tyrosine phosphatases have been proposed as molecular targets for the activity of arsenic. These enzymes play key roles in modulating cellular metabolism; many possess vicinal thiols and, therefore, are a potential site of interaction with arsenic. Studies aimed at defining the role of tyrosine phosphatases in NB4 APL cells demonstrated that phenylarsine oxide, a vicinal thiol binding agent and well-known inhibitor of tyrosine phosphatase (53), did not stimulate nuclear fragmentation, DNA laddering, and caspase activation, whereas both arsenic trioxide and dimethylarsenic did (54). The interpretation of these experiments was that arsenite does not specifically target tyrosine phosphatases at least in this model. Other experiments, using human airway epithelial cells, showed that although arsenic exposure resulted in elevated levels of phosphotyrosine, such exposure caused no change in the level of tyrosine phosphatase activity (55).

IκB Kinase and NFκB Inhibition. Inflammatory signal transduction is also blocked by arsenic. There is evidence that arsenite inhibits IKK, required for activation of the proinflammatory transcription factor NFκB. The mechanism of inhibition appears to be binding to the cysteine residue (Cys-179) in the activation loop of the IKK catalytic subunits, IKKα/β, and mutation of Cys-179 abolished arsenite sensitivity (56). In the experiments reported by Kapahi et al. (56), concentrations of >10 μM were required to achieve significant inhibition of kinase activity. The physiological relevance of this mechanism may be questionable, as the experimental concentrations used were 5–10 times higher than the concentrations achieved in the treatment of patients with leukemia (57). However, high levels of glutathione that are found in the HeLa cells used in these experiments may make them highly resistant to the effects of arsenic (Ref. 58; see role of glutathione system below).

NFκB transcriptional activity plays an important role in the pathogenesis of hematologic malignancies, particularly multiple myeloma, through the regulation of IL-6 expression and the expression of adhesion molecules. Experiments with cultured myeloma cells show that arsenic trioxide prevents tumor necrosis factor α-induced NFκB activation at clinically relevant levels (2–5 μM; Ref. 59). One consequence of the potential inhibition of IKK-mediated phosphorylation and degradation of IκB is the persistent binding of the inhibitor to NFκB preventing its translocation to the nucleus and up-regulation of *IL-6* and other genes. The ultimate effect may diminish IL-6 concentrations and abrogate IL-6-mediated multiple myeloma cell growth, weakening resistance of the multiple myeloma cells to apoptosis (56, 60).

In another recent study, ascorbic acid was also shown to inhibit the activation of IKK, and consequently the functional ability of NFκB in an endothelial cell line and in primary human vascular endothelial cells. In this case, ascorbic acid treatment leads to an increase in p38 MAP kinase activity, providing one explanation for the potentiation of arsenic trioxide-induced apoptosis by ascorbic acid in multiple myeloma and myeloid leukemia cells (61, 62).

The stress sensors NFκB and JNK reciprocally regulate arsenic trioxide-induced, p53-independent expression of the GADD45. Thus, inhibition of NFκB enhances arsenic trioxide-induced JNK activation and p53-independent GADD45 expression. Because GADD45 is responsible for the maintenance of the G_2/M checkpoint to prevent improper mitosis, its rapid induction by extracellular stress signals may be responsible for some of the antiproliferative effects of arsenite. However, IKK-overexpressing cells with normal activation of NFκB showed a transient and less potent increase in GADD45 levels with arsenic trioxide. This suggests that NFκB activation may not be favorable for the induction of cell cycle checkpoint proteins that maintain genomic integrity (63).

Caspases and Apoptosis

Apoptosis is mediated by caspases, a class of cysteine proteases that cleave key cellular proteins to induce morphological features of nuclear condensation and fragmentation. Arsenic exposure has been shown to activate caspases both *in vitro* and *in vivo*. Whether the activation of caspases by arsenic trioxide is a direct or indirect effect of the agent will require additional study. Nonetheless, in their clinical trials with relapsed APL patients, Soignet *et al.* (6) showed that responses to arsenic trioxide were accompanied by enhanced expression of proenzymes for caspases 2 and 3, and activation of caspases 1 and 2. Experiments with cultured myeloma cells show that arsenic trioxide induces apoptosis via caspase-9 activation at clinically relevant levels (2–5 μM), and its effects in combination with dexamethasone are additive in this model (59). Exposure of an adult T-cell leukemia cell line to arsenic trioxide *in vitro* results in activation of caspases 8 and 3, together with poly(ADP-ribose) polymerase degradation, inhibiting DNA repair. Cell cycle arrest occurs as well, with cells accumulating in the G_1 phase (64). Finally, arsenic trioxide exposure promotes apoptosis by activating caspase 3 in neuroblastoma cell lines (65) and in myeloid leukemia cells (66). Additional evidence that caspases mediate the apoptotic response to arsenic is provided by the study of an arsenic trioxide-resistant APL cell line that fails to activate caspase 3 in response to arsenic trioxide. In this line, the PML-RARα continues to undergo degradation demonstrating that fusion protein degradation can be uncoupled with caspase-induced loss of mitochondrial inner transmembrane potential and the apoptotic response (67).

An indirect mechanism by which arsenic may promote apoptosis is the inhibition of telomerase activity. In cultured NB4 cells, treatment with arsenic trioxide resulted in a reduction of telomerase activity related to a decrease in telomerase transcription (68). The decreases in transcription may be related to direct effects of arsenic on transcription factors such as Sp1 and Myc. In any case, decreases in telomerase activity result in an increase in genomic instability and chromosomal abnormalities that directly induce apoptosis.

Effects of Arsenic on Cellular Redox Status and the Cellular Stress Response

Oxidative Stress Pathways. Oxidative damage plays an important role in the effects of arsenic, as it does for other metals, such as iron, copper, nickel, chromium, cadmium, lead, and mercury (69). Arsenic disturbs natural oxidation and reduction equilibria through various

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mechanisms involved in complex redox reactions with endogenous oxidants and cellular antioxidant systems. The redox capacities of many proteins reside in the sulfhydryl groups on cysteines. Most proteins are protected or buffered against oxidation by the highly reduced intracellular milieu. However, proteins with accessible, closely spaced thiol groups with high thiol-disulfide oxidation potentials may be redox-sensitive, and redox regulation clearly mediates important cell functions (70). Both the toxic and the therapeutic effects of arsenic are mediated, at least in part, by redox-sensitive proteins and enzymes. As will be discussed below, the endogenous thioredoxin and glutathione systems play key regulatory roles in redox signaling, potentially protecting cells from the damaging effects of arsenic or other related compounds (70).

Redox-sensitive signaling molecules, such as AP-1, NFκB, IκB, p53, $p21^{ras}$, and S-nitrosothiols, are affected by arsenic treatment, which consequently deranges cell signaling and alters gene expression systems (71). Thus, arsenic paradoxically shares many properties of tumor promoters. In addition, after cells are exposed to arsenic, nitric oxide production is enhanced, poly(ADP)ribosylation and NAD^+ depletion occur, DNA strand breaks develop, and micronuclei are formed (13). Cell proliferation is enhanced as well, as was shown in the bladders of mice of which their drinking water was treated with arsenite and in a human bladder epithelial cell line treated with arsenic (72, 73). Arsenite also inhibits activation of extracellular signal-regulated kinase by platelet-derived growth factor-B in a reversible, time-dependent manner; the inhibition is attenuated by antioxidant pretreatment.

Reactive oxygen species generated in response to arsenic exposure lead to accumulation of intracellular hydrogen peroxide by activation of flavoprotein-dependent superoxide-producing enzymes such as $NADT^+$ (NADPH) oxidase (13, 74). The critical effect of hydrogen peroxide in mediating apoptosis by its effects on mitochondria is becoming increasingly understood. For example, in an esophageal carcinoma cell model, the first morphological alteration observed after treatment with arsenic trioxide was aggregation of mitochondria (75).

During apoptosis, the permeability transition pore complex of the mitochondrial membrane can interact with the Bcl-2/Bax complex, leading to progressive permeabilization, with dissipation of mitochondrial inner transmembrane potential (76). Unlike conventional chemotherapy agents, arsenic trioxide acts directly on mitochondria to destroy the mitochondrial inner transmembrane potential to promote apoptosis. These effects of arsenite are illustrated in Fig. 2. By oxidizing thiols, arsenite opens the permeability transition pore complexes; free radical scavengers such as glutathione diminish thiol oxidation (76). Experiments in malignant lymphocytes show that pretreating cells with DTT prevents the arsenic-induced loss of mitochondrial inner transmembrane potential and abrogates apoptosis. In contrast, exposure to a glutathione synthesis inhibitor, buthionine sulfoximine, enhances these effects of arsenic (77). From these studies, it is clear that not one, but multiple oxidative effects of arsenic converge to promote apoptosis.

Ferritin may act as another molecular target of arsenic. Interaction of exogenous methylated arsenic species with ferritin can result in the release of iron from ferritin followed by iron-dependent formation of reactive oxygen species (78). Both dimethylarsinic acid and dimethylarsinous acid caused the release of significant amounts of iron from horse spleen ferritin, and iron release was considerably enhanced in the presence of ascorbic acid. In addition, iron release was greatest under anaerobic conditions, suggesting direct chemical reduction of iron from ferritin by dimethylarsinous acid. This mechanism may augment the production of reactive oxygen species by direct action of arsenic on the proteins of mitochondrial pores (76).

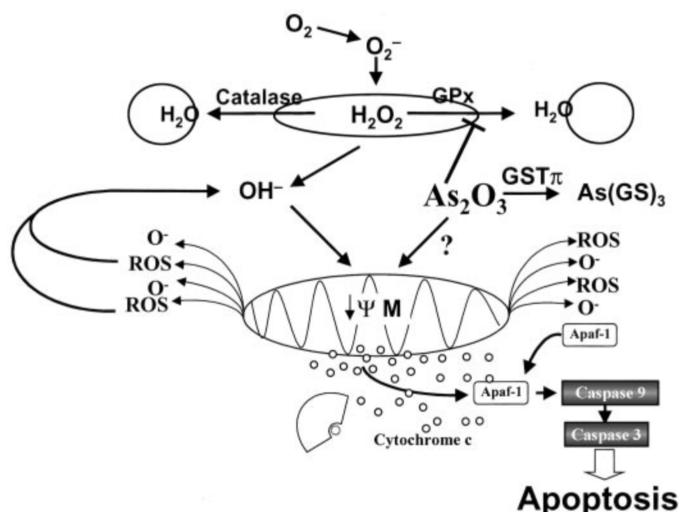

Fig. 2. Arsenic trioxide induces apoptosis via changes in the mitochondrial membrane potential. Arsenic trioxide treatment of malignant cells results in increased intracellular levels of hydrogen peroxide, which lowers mitochondrial membrane potential and leads to cytochrome *c* release and subsequent activation of the caspase pathway (74).

Role of the Glutathione System in Arsenic-induced Apoptosis. In mammalian cells, glutathione peroxidase and catalase are key enzymes regulating the levels of reactive oxygen species and protecting cells from arsenite damage. Fig. 2 illustrates the protective effects of glutathione on ROS-mediated apoptosis. Arsenite-induced apoptosis is inhibited by the thiol-containing antioxidant *N*-acetyl-L-cysteine, a selective scavenger of O^{2-}, and catalase (74). Levels of glutathione peroxidase and catalase were also compared in control and X-ray hypersensitive Chinese hamster ovary cells. The X-ray hypersensitive cell line had 5–6-fold lower glutathione peroxidase and catalase activities as compared with control Chinese hamster ovary cells. With arsenic exposure, micronuclei appeared in X-ray hypersensitive cells, but addition of catalase or glutathione peroxidase decreased micronuclei. In contrast, arsenite damage was enhanced when either 3-aminotriazole, a catalase inhibitor, or mercaptosuccinate, a glutathione peroxidase inhibitor, was added to X-ray hypersensitive cultures (79). In surveys of many malignant cell lines, glutathione peroxidase and catalase were shown to be present at constitutively lower levels in many cells that are very sensitive to arsenic, and lowering of glutathione dramatically increased sensitivity to arsenite in cells with natural or acquired resistance to arsenicals (57). For example, neuroblastoma cells sensitive to arsenic trioxide had <40 nmol reduced glutathione per mg of protein; cells with greater levels of reduced glutathione were protected from arsenic-induced apoptosis. Addition of the antioxidant *N*-acetyl-L-cysteine protected susceptible cells from apoptosis as well (80). Together, these results point to the importance of modulating the intracellular levels of reactive oxygen species to affect the activities of arsenic.

Additional evidence for the importance of oxidative stress in mediating the effects of arsenic comes from studies of purified trypanothione reductase and glutathione reductase demonstrating that these enzymes are direct molecular targets of trivalent arsenicals (81). Trypanothione, analogous to glutathione in mammalian cells, is a novel dithiol molecule in trypanosomes. Although the organic arsenic derivative melarsoprol caused greater inhibition of trypanothione reductase than did sodium arsenite, these trivalent arsenicals appear to exert their inhibition by a similar mechanism. In both trypanothione reductase and glutathione reductase, the arsenic molecule interacts in a disulfide-binding pocket; the sulfides are physically separated by a flavin adenine dinucleotide molecule. Trypanothione forms a stable

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adduct with organic arsenicals that acts as a competitive inhibitor of trypanothione reductase. In this model, free arsenical and previously reduced enzyme (trypanothione reductase or glutathione reductase) equilibrates with an unstable monothioarsane enzyme inhibitor complex. Next, a molecular rearrangement around the arsenic-sulfur bond results in the binding of the arsenic to the cysteine molecule proximal to a flavin adenine dinucleotide, resulting in a more stable dithioarsane complex. Although methylation is required for detoxification of arsenicals, biomethylation of arsenic species can also produce potent glutathione reductase inhibitors that can alter the redox status of cells by inhibiting reduction of glutathione disulfide and preventing elimination of free radicals (82).

The cellular glutathione redox system modulates both the antiproliferative and proapoptotic effects of arsenic trioxide. Therefore, a potential strategy to increase the anticancer activity of arsenicals is to decrease the levels of reduced glutathione in cancer cells. Ascorbic acid is known to reduce intracellular glutathione content. In addition, as ascorbic acid auto-oxidizes, intracellular hydrogen peroxide may increase, potentially enhancing the proapoptotic actions of arsenic trioxide (83). Indeed, in freshly isolated leukemic cells from patients with acute myelogenous leukemia, coadministration of ascorbic acid with arsenic trioxide produced a significant increase in apoptosis in six of eight samples with low intrinsic sensitivity to arsenic trioxide, indicating substantial synergy between these two agents (62). Increased intracellular glutathione concentrations have also been associated with chemoresistance in multiple myeloma, prompting parallel experiments with multiple myeloma cells. Ascorbic acid potentiated arsenic trioxide-induced cell death in both multiple myeloma cell lines and in myeloma cells freshly isolated from human patients but showed little effect on normal bone marrow cells (61, 84). These studies have provided the rationale for ongoing clinical evaluation of arsenic trioxide with ascorbic acid in the treatment of patients with multiple myeloma.

Heat Shock Proteins. The cellular response to arsenic shares many features with the heat shock response as well as oxidative stress. Both heat stress and arsenite exposure induce heat shock proteins, and both of these stressors initiate signal transduction cascades. Likewise, MAP kinase, extracellular signal-regulated kinase, JNK, and p38 are all reported to be induced after arsenite exposure (48, 73), and transcription of immediate early genes *c-fos*, *c-jun*, and *egr-1* is mediated by increased DNA binding of the AP-1 transcription factor (13, 72). In a rat hepatoma cell line, expression of heat shock protein 70, activation of MAP kinase, induction of *c-jun*, and induction of the MAP kinase phosphatase MKP-1 were all related to the development of tolerance and sensitization to arsenite after a priming heat treatment (85). Furthermore, arsenic treatment of malignant lymphocyte cell lines resulted in growth inhibition by increasing the duration of the cell cycle, yet the levels of cell cycle-related proteins c-myc, retinoblastoma protein, CDK4, cyclin D1, and p53 were not altered (77).

The most abundant molecular chaperone, Hsp90, is actively involved in the regulation of redox status in the cell. Redox-regulated interactions between cytochrome *c* and cytoplasmic Hsp90 are involved in apoptosis. These interactions can be blocked by high concentrations of sodium arsenite (1 mM) in a purified system (86). Nonetheless, sulfhydryl groups in the Hsp90 molecule are critical to the reducing activity of Hsp90 on cytochrome *c* and appear to be the substrates for inhibition by arsenite. Interference with the protein folding or chaperone function of Hsp90 by ansamycin antibiotics results in the degradation of key signaling proteins that include Raf serine kinase, met tyrosine kinase, steroid receptors, and HER kinase family members (87, 88). Because many types of malignant cells are dependent on the function of these signaling proteins, ansamycin derivatives suppress proliferative signals and induce growth arrest followed by apoptosis. The antitumor activity of one ansamycin derivative has been documented in cell culture and animal models, and is currently in clinical evaluation (89). Similarly, arsenic-induced growth arrest and apoptosis may be facilitated by targeting Hsp90.

Another member of the stress protein superfamily that mediates oxidative degradation of heme to biliverdin is HO-1 (90, 91). The *HO-1* gene contains heat shock elements and AP1, AP2, and NFκB binding sites in its promoter region, which render it rapidly inducible by metal ions, amino acid analogues, sulfhydryl agents, oxidative stress, hyperthermia, and proinflammatory cytokines (92, 93). Both arsenite and arsenate are potent inducers of *HO-1* in a host of normal and neoplastic tissues both *in vivo* and in cell culture. For example, in HeLa cells, *HO-1* mRNA and protein levels are up-regulated by exposure to 10 μM sodium arsenite, an effect that can be blocked by coadministration of 5 μM sodium selenite (94). *HO-1* mRNA levels are also augmented 5–10-fold in intact mouse liver relative to saline-treated controls within 3 h of s.c. injection of arsenite (95). On the basis of the sensitivity of HO-1 to arsenic and observations made in six individuals, Menzel *et al.* (96) advocated measurement of lymphocyte HO-1 protein as a biological marker of arsenic exposure in humans.

After arsenic exposure, induction of HO-1 may confer cytoprotection by promoting the conversion of pro-oxidant metalloporphyrins, such as heme, to bile pigments (biliverdin and bilirubin) with free radical scavenging capabilities (97, 98). Enhanced resistance of certain cell types (*e.g.,* the human lung adenocarcinoma cell line CL3) to the cytotoxic effects of arsenic appears to be related to relatively high levels of HO-1 in these cells (99). Conversely, in certain tissues, free iron and carbon monoxide (CO) released during HO-1-mediated heme catabolism may exacerbate intracellular oxidative stress, and promote injury to mitochondria and other subcellular compartments (100). In rat astrocyte cultures, enhanced HO-1 expression promotes mitochondrial iron sequestration and membrane damage (93). Thus, it is conceivable that HO-1 induction may mediate, rather than protect against, the cytotoxic effects of arsenic under certain experimental and clinical conditions.

Other Cellular Targets of Arsenic

As discussed above, arsenite can bind closely spaced and accessible thiol groups, and dithiols are common to the binding sites of numerous receptor-binding domains or enzymatic active sites, potentially allowing for arsenite binding (101). At concentrations below 10 μM, arsenite inhibits proper ligand binding to the glucocorticoid receptor in a region that contains a dithiol critical to proper ligand binding (101, 102). In a similar way, arsenite can bind to cysteine residues in the estrogen receptor-α hormone binding site, and binding assays show that arsenite blocks interaction of estradiol with the estrogen receptor-α (103). Surprisingly, arsenite was shown to activate the estrogen receptor-α and enhance transcription of estrogen-dependent genes. Although arsenite can induce apoptosis in MCF-7 cells growing in normal serum containing media,[4] growth of MCF-7 breast cancer cells in the absence of estrogen is stimulated by low concentrations ($\leq$1 μM) of arsenite exposure; this stimulation is also blocked by a complete antiestrogen (103). Together these findings suggest that arsenic may act as an "environmental estrogen."

Arsenic trioxide may additionally affect tumor cell growth by inhibiting angiogenesis. Treatment of human umbilical vein endothelial cells with arsenic trioxide results in dose- and time-dependent characteristic alterations in morphology and physiology, culminating in apoptosis. Down-regulation of vascular endothelial growth factor

[4] W. H. Miller, unpublished observations.

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production was thought to be responsible. Evidence supporting this idea was suggested by arsenic trioxide treatment of the leukemia line HEL (104). Together, these results suggest that arsenic trioxide exerts antiangiogenic effects by interrupting a reciprocal stimulatory loop between leukemic cells and endothelial cells. Furthermore, in mice with methylcholanthrene-induced fibrosarcomas, a single dose of arsenic trioxide was sufficient to induce preferential vascular shutdown in tumor tissue and the central portion of the tumor demonstrated massive necrosis, whereas skin, muscle, and kidney vasculature remained relatively unaffected (105).

The cytoskeleton has also been suggested as a potential cellular target for arsenic because its major constituent, tubulin, has a relatively high sulfhydryl content. Disruption of microtubule assembly and spindle formation during mitosis can promote apoptosis. Experiments in myeloid leukemia cells show that arsenic trioxide markedly inhibits GTP-induced polymerization of monomeric tubulin for microtubule formation (106). Additional suggestive evidence is provided in experiments using prostate and ovarian cancer cell lines. Although arsenic trioxide effectively induced apoptosis, addition of the strong antioxidant butylated hydroxyanisole did not rescue cells from arsenic trioxide-induced apoptosis. This indicates that the cytotoxic effects of arsenic trioxide were not mediated by superoxide generation alone in this experimental system. Investigators have proposed that apoptosis is induced in these cells via an alternate mechanism, perhaps involving interactions with tubulin or other cytoskeletal elements (107).

For many years, pyruvate dehydrogenase was considered an important molecular target of arsenic, with trivalent arsenic causing decreased gluconeogenesis and hypoglycemia. Physiologically relevant concentrations of arsenite ($<5\ \mu$M) inhibit activity of purified pyruvate dehydrogenase *in vitro*, suggesting that the enzyme may be a direct target (108–110). Additional effects of arsenic on the nuclear matrix and DNA protein crosslinks that have been reported may be related to its effects on cellular redox levels (111–113).

Synergy of Arsenic with Other Agents

Because of the many pathways involved in mediating the effects of arsenic, the potential exists for synergism with other agents to provide enhanced therapeutic benefits. As mentioned earlier, arsenic trioxide shows effects distinct from those of ATRA in APL cells and has clinical efficacy in patients with ATRA-resistant APL. The combination of ATRA and arsenic trioxide may be synergistic or antagonistic *in vitro*, whereas *in vivo* the combination or sequential use of the agents has been reported to accelerate tumor regression by enhancing both differentiation and apoptosis in some but not all of the models. Furthermore, combination therapy may allow for administration of lower doses of arsenic trioxide, minimizing toxicity and potential drug antagonism (27, 29).

In human myeloid leukemia cells that express bcr-abl, arsenic trioxide treatment reduces bcr-abl levels and induces apoptosis. These effects appear to be independent of bcr-abl kinase activity, because they occur even when cells are pretreated with STI-571, a tyrosine kinase inhibitor specific for the bcr-abl tyrosine kinase. Furthermore, treatment with arsenic trioxide decreases proliferation of chronic myelogenous leukemia blasts but does not affect growth of peripheral CD34+ progenitor cells (114). In contrast to these results, concurrent treatment with the combination of arsenic trioxide and STI-571 causes a greater apoptotic response than does treatment with either agent alone. Among the critical consequences of arsenic trioxide treatment was inhibition of the Akt kinase, important for growth and survival, and even greater inhibition of Akt in the presence of both agents (115).

Observations that perturbations in cellular methyl metabolism modulate the cytotoxicity of arsenic have led to the suggestion that methotrexate may act synergistically with arsenic trioxide. Folic acid supplementation abrogates the cytotoxic effects of arsenite. By causing a relative methyl insufficiency, methotrexate may potentiate the effects of arsenite in the presence of excess folic acid (116).

The combination of arsenic trioxide with IFN-α has activity in adult T-cell leukemia cell lines, as well as clinically in patients (117, 118). Synergy between arsenic trioxide and IFN-α was demonstrated using a colony forming unit-granulocyte/macrophage assay with mononuclear cells from six patients in the chronic phase of chronic myelogenous leukemia. Positive results from this study prompted additional investigation of these agents in combination, although there are few results to date (119).

Potential synergy of arsenic trioxide and vitamin C has been shown *in vitro* and *in vivo* by several groups (61, 62, 83, 84), and additional studies of the mechanism both *in vitro* and in the clinic appear warranted. Indeed, evidence for the potentiation by ascorbic acid of arsenic trioxide-induced cell death in multiple myeloma cells has been reported recently (61, 84). These results provided a basis for clinical trials under way to test the combined use of arsenic trioxide and ascorbic acid in refractory multiple myeloma. Furthermore, they suggest that ascorbic acid may be a successful adjuvant in other reduced glutathione-sensitive therapies. Ascorbic acid is not the only agent affecting intracellular redox to synergize with arsenic *in vitro*. The profound increases in arsenic sensitivity *in vitro* associated with glutathione depletion by buthionine sulfoximine suggest a need for experiments in animal models using this or related agents, as well.

Summary and Conclusions

Arsenic acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways and resulting in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion or inhibition of differentiation, and angiogenesis inhibition. Responses vary depending on cell type and the form of arsenic. Investigations using many different experimental systems suggest that arsenic may offer significant therapeutic benefits to patients across many neoplasms, and numerous clinical trials are under way in hematopoietic malignancies and solid tumors.

Chronic exposure to arsenic and arsenic derivatives can have toxic effects. Our molecular understanding to date suggests that similar mechanisms mediate both the therapeutic activities and the toxicities of arsenic. However, several types of neoplastic cells are particularly sensitive to arsenic, and at low doses the therapeutic benefits outweigh the toxicities. Indeed, low doses of arsenic are dramatically effective in APL and show considerable promise in preclinical models of other tumor types. In addition, use of arsenic trioxide generally does not lead to myelosuppression, providing a potential advantage over use of conventional cytotoxic agents. This feature, together with the multiple molecular targets affected by arsenic, suggests the potential for additive or even synergistic effects when arsenic is administered with other cytostatic or cytotoxic agents. Such combinations may result in enhanced antitumor activity with acceptable tolerability, but their rational development will require considerable additional efforts to understand the many molecular actions of arsenic in the cell.

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