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Drug uptake and pharmacological modulation of drug sensitivity in leukemia by AQP9☆

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Abstract

Leukemia is the most common childhood cancer. Trisenox, the active ingredient of which is trivalent arsenic, is the first line of treatment for acute promyelocytic leukemia. Since drug action usually requires uptake of the drug, it is of importance to determine the transport system responsible for Trisenox uptake. Recently, human aquaglyceroporin 9 (AQP9) has been shown to transport As(III) in *Xenopus* oocytes. In this study we report to show that AQP9 expression modulates the drug sensitivity of leukemic cells. AQP9 was transfected into the chronic myelogenous leukemia cell line K562. The transfectants became hypersensitive to Trisenox and Sb(III). The promyelocytic leukemia cell line HL60 treated with vitamin D showed higher expression of AQP9 and hypersensitivity to Trisenox and Sb(III). This sensitivity was due to higher rates of uptake of the trivalent metalloids by the cell lines overexpressing AQP9. Trisenox hypersensitivity results from increased expression of AQP9 drug uptake system. The possibility of using pharmacological agents to increase expression of AQP9 gene delivers the promise of new therapies for the treatment of leukemia. Thus, drug hypersensitivity can be correlated with increased expression of the drug uptake system. This is the first demonstration that AQP9 can modulate drug sensitivity in cancer.

Keywords: Arsenite; Antimonite; Leukemia; Aquaglyceroporin

Each year in the United States more than 200,000 children and adults are diagnosed with leukemia or lymphoma, and half die of their disease. The identification of arsenic trioxide as a chemotherapeutic agent has led to new optimism in the treatment of acute promyelocytic leukemia (APL). Consequently, arsenic trioxide (Trisenox) was approved for the treatment of relapsed or refractory APL by the US Food and Drug Administration in September 2000. Trisenox is also being successfully used in combination with Gleevec (imatinib mesylate) in vitro against both sensitive and resistant chronic myelogenous leukemia (CML) lines [1]. Arsenic trioxide (As_2O_3) is also undergoing clinical trial in multiple myeloma [2]. Although the exact mechanism of action of As_2O_3 is unknown, a range of in vitro studies suggest that several mechanisms may contribute to its antileukemic effect in vivo. These mechanisms include induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion (PML-RARα) transcripts, antiproliferation, and inhibition of angiogenesis in case of solid tumors [3].

Aquaglyceroporins (AQPs) are members of the major intrinsic protein (MIP) superfamily. The MIP family members fall into two distinct families, the aquaporins or true water channels, and the aquaglyceroporins, which transport glycerol and other neutral solutes [4,5]. GlpF, an aquaglyceroporin homologue in *Esche-*

☆ *Abbreviations:* PCR, polymerase chain reaction; ICP-MS, inductively coupled plasma-mass spectroscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SEM, standard error of the mean.

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richia coli, was first identified as an uptake pathway for As(III)/Sb(III) [6]. Disruption of the *Saccharomyces cerevisiae FPS1* gene, which encodes an AQP homologue, similarly leads to resistance to trivalent metalloids [7] in yeast. In mammals 11 AQPs have been identified. AQP3, AQP7, AQP9, and AQP10 are members of the aquaglyceroporin family. AQP3 is present at the basolateral membrane of kidney, colon, trachea, urinary bladder, skin, and sclera of eye. In contrast, AQP7 is found in testes, adipose tissue, kidney, and heart, and AQP9 transcript is found in liver, lung, spleen, and peripheral leukocytes in humans [8]. Human AQP10 is expressed only in small intestine [9]. AQP9 has the broadest specificity and transports water, glycerol, urea, carbamides, polyols, purines, and pyrimidines in a phloretin- and mercurial-sensitive manner [10].

Until recently the route of entry of As(III)/Sb(III) into mammalian cells, including humans, was unknown. Recently, we showed that mammalian AQP7 and AQP9 transport ^{73}As(III) and ^{125}Sb(III) when expressed in *S. cerevisiae* and oocytes [7], although AQP9 seems to be the most efficient transporter of As(III)/Sb(III). AQP7 also transported As(III) or Sb(III) but poorly when compared to AQP9. AQP3 and AQP10 did not show any significant metalloid uptake when expressed in oocytes [11]. Given that the pK_as for the hydroxides of As(III) and Sb(III) are 9.2 and 11.8, respectively, the anions arsenite and antimonite do not exist in solution at neutral pH. We have shown that the species present in solution is $As(OH)_3$ [12], but minor polymeric species probably also exist [13]. However, the evidence that AQP9 can catalyze metalloid transport in human cells is still lacking.

Drug uptake is usually necessary for drug action. It is reasonable to think that AQP9 would modulate drug sensitivity in leukemic cells. In this study we show that increased expression of AQP9 in leukemic cells K562 and HL60 sensitizes them to As(III) and Sb(III). Addition of vitamin D induced the AQP9 expression in HL60 cells. Sensitization of leukemic cells overexpressing AQP9 to drugs was thought to be due to higher rates of uptake of the trivalent metalloids by AQP9. This is the first report that AQP9 can modulate drug sensitivity in human leukemia cells.

Materials and methods

Reagents and media. All reagents unless otherwise mentioned were purchased from Sigma–Aldrich. As(III) and Sb(III) were purchased in the form of sodium arsenite and potassium antimonyl tartrate. DNA manipulation reagents were obtained from Qiagen and Invitrogen.

Cell lines and cultures. Two leukemia cells lines K562 and HL60 were obtained from ATCC and maintained in Iscove's modified Dulbecco's medium with 10% or 20% serum and 50 μg/ml gentamycin.

Cloning of human AQP9. hAQP9 was cloned from human cDNA by PCR using gene specific primers, as described before [11]. We subcloned hAQP9 into *Eco*RI digested mammalian expression vector pIRESneo3 (Clontech) under the control of pCMV promoter by digesting hAQP9/pGEM-T with *Eco*RI giving rise to pIRES-hAQP9. pIRESneo3 has an internal ribosome entry site of the encephalomyocarditis virus, which permits the translation of two open reading frames from one mRNA. Thus, after selection with G418 (geneticin), almost all colonies stably express the gene of interest.

Transfection of hAQP9 in K562. K562 cells were transfected with pIRES-hAQP9 or pIRESneo3 alone using Lipofectamine 2000 (LF2000) reagent (Invitrogen). Log phase K562 cells were washed once with phosphate-buffered saline, pH 7.4 (PBS), and suspended at 2×10^6 cells/ml in Dulbecco's modified Eagle's medium with 10% serum. DNA was diluted to 30 μg/ml in OPTI-MEM (Invitrogen) and incubated with LF2000 (1:1) at room temperature for 20 min. The DNA-LF2000 conjugate was then diluted 10-fold with K562 cells in the same medium and incubated at 37 °C in a CO_2 incubator for 12–15 h. The cells were then diluted to 10^6 cells/ml in the presence of 1.1 mg/ml G418. Cells resistant to G418 were selected after 3–4 weeks and maintained in 0.7 mg/ml G418. Clonal selection was not necessary as the internal ribosomal entry site (IRES) in the pIRESneo3 vector allows the expression of the gene of interest from each cell that is resistant to G418.

Metalloid sensitivity assays. Log phase K562 transfectants were diluted to 10^5 cells/ml in Iscove's modified Dulbecco's medium with 10% serum containing various concentrations of Trisenox [As(III)] or potassium antimonyl tartrate [Sb(III)]. Cell growth was monitored after 96 hr by counting viable cells using a Nucleocounter (New Brunswick Scientific). In this instrument the cell nucleus is stained with propidium iodide in a NucleoCassette pre-coated with the dye. The nonviable cell count was taken before cell lysis and later subtracted from total cell count to arrive at the actual viable cell number. Each assay was performed three times. Error bars were calculated from standard error of mean (SEM).

Log phase HL60 cells were suspended at 10^5 cells/ml and grown for 72 h at 37 °C in a CO_2 incubator before incubation with 100 nM of 1α, 25-dihydroxyvitamin D3 for 48 h. The vitamin D pre-treated cells were harvested, washed twice in PBS at room temperature, and suspended at 10^5 cells/ml in IMDM plus 10% serum containing different concentrations of Trisenox or potassium antimonyl tartrate. Growth was monitored as described above. Each assay was performed three times. Error bars were calculated from standard error of mean (SEM).

Uptake assays. For As(III) uptake experiments, log phase K562 transfectants were washed twice with PBS and suspended at 5×10^7 cells/ml. 0.05-ml portion of this suspension was diluted into 0.6 ml PBS containing 10 mM glucose and 1 μM of ^{73}As(III) (4000 cpm/pmol). One hundred microliters aliquots were removed at 1, 5, 10, 20, and 30 min intervals, filtered through 0.2 μ-nitrocellulose membranes, and washed with 5 ml cold PBS. The filters were dried, dissolved in scintillation cocktail, and counted in scintillation counter (Perkin–Elmer). ^{73}As was obtained from Los Alamos as As(V) and reduced to As(III), as described previously [14]. The same method was followed for HL60 cells treated with 100 nM vitamin D.

For Sb(III) uptake assays, either the K562 transfectants or HL60 cells pre-treated with vitamin D were washed twice with PBS and suspended at 5×10^7 cells/ml. Three milliliters of this suspension was incubated at 37 °C for 5 min before initiating uptake with 0.1 mM potassium antimonyl tartrate for the indicated times. 0.5 ml portions were withdrawn at each time point and centrifuged at 5000*g* for 1 min at room temperature. The supernatant was aspirated and the cells were washed twice with ice-cold PBS. The final cell pellet was digested in 0.2 ml of 70% HNO_3 for 4 h at 70 °C and diluted 25-fold with HPLC grade water. Total Sb content was determined by inductively coupled plasma mass spectroscopy (ICP-MS).

Western blot. K562 cells transfected with AQP9 or HL60 cells pretreated with vitamin D were suspended in 3 ml buffer containing

7.5 mM sodium phosphate (pH 7.0), 0.25 M sucrose, 5 mM EDTA, and 5 mM EGTA supplemented with Complete Protease inhibitor cocktail tablets (Roche) and homogenized on ice with a Potter–Elvehjem tissue grinder using a drill press. Samples were pelleted by centrifugation at 1000*g* for 10 min at 4 °C. The membrane fraction was obtained by centrifuging the resulting supernatant at 200,000*g* for 45 min at 4 °C and analyzed by 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [15]. Immunoblots were probed with anti-rAQP9 antibody (3.5 μg/ml, Chemicon) and developed with ECL Plus (Amersham).

Results

Expression of hAQP9 confers metalloid sensitivity in leukemic cells

hAQP9 was cloned in the mammalian expression vector pIRESneo3, and the resulting plasmid pIRES-hAQP9 was transfected into a human erythroleukemia line K562. Expression of hAQP9 was confirmed by Western blot analysis of the plasma membrane fraction of K562 cells transfected with hAQP9 (Fig. 1A, inset). The K562 cells transfected with vector alone did not show detectable expression of hAQP9 (Fig. 1A, inset). hAQP9 expressing K562 cells became hypersensitive to either As(III) or Sb(III) when compared to vector alone. The hAQP9 transfectants became 3.8-fold more sensitive to the As(III) containing drug Trisenox (Fig. 1A). K562 cells were found to be more resistant to Sb(III) than As(III). The IC_{50} for Sb(III) of cells with vector alone was more than 10 μM compared to 2 μM for K562 cells expressing AQP9 (Fig. 1B).

Transfection of HL60 cells with pIRES-hAQP9 was unsuccessful. For this reason we searched for physiological compounds that would increase expression of the chromosomal AQP9 gene in this cell line. Log phase HL60 cells treated with 100 nM vitamin D for 48 h expressed hAQP9 (Fig. 2A, inset). Although vitamin D is known to induce differentiation in HL60, we did not observe significant differentiation or morphological changes during the course of the experiment. Vitamin D pre-treated cells were 2.5-fold more sensitive to Trisenox (Fig. 2A) and Sb(III) (Fig. 2B) compared to untreated cells.

hAQP9 facilitates uptake of Sb(III) and As(III) in leukemic cells

The ability of hAQP9 to transport As(III) and Sb(III) in leukemic cells was investigated. K562 cells expressing hAQP9 exhibited rapid uptake of As(III) (Fig. 3A) and Sb(III) (Fig. 3B) compared to transfectants with vector alone. Similarly HL60 cells pre-treated with vitamin D also exhibited rapid rates of uptake of As(III) (Fig. 4A) and Sb(III) (Fig. 4B). Even though these channels do not catalyze net accumulation, binding to intracellular sites, metabolic alteration or sequestration in organelles may result in an apparent accumulation of the metalloids. So, for all practical purposes, the uptake assay measures accumulation. Each cell line expressing hAQP9 had much higher rates of uptake and exhibited an apparent steady state level of As(III) and Sb(III) that was 2- to 5-fold more than the vector alone or untreated controls. Although the HL60 and K562 cells do not express AQP9 intrinsically (Figs. 1A and 2A), they take up As(III) and Sb(III). Liu and co-workers [16] have recently shown that hexose permeases in yeast are responsible for As(III) uptake. Both K562 and HL60 cells express glucose transporters like Glut1 [17]. Therefore, it is possible that the residual uptake of the metalloids in these cells is by glucose transporters. Work in these lines is in progress.

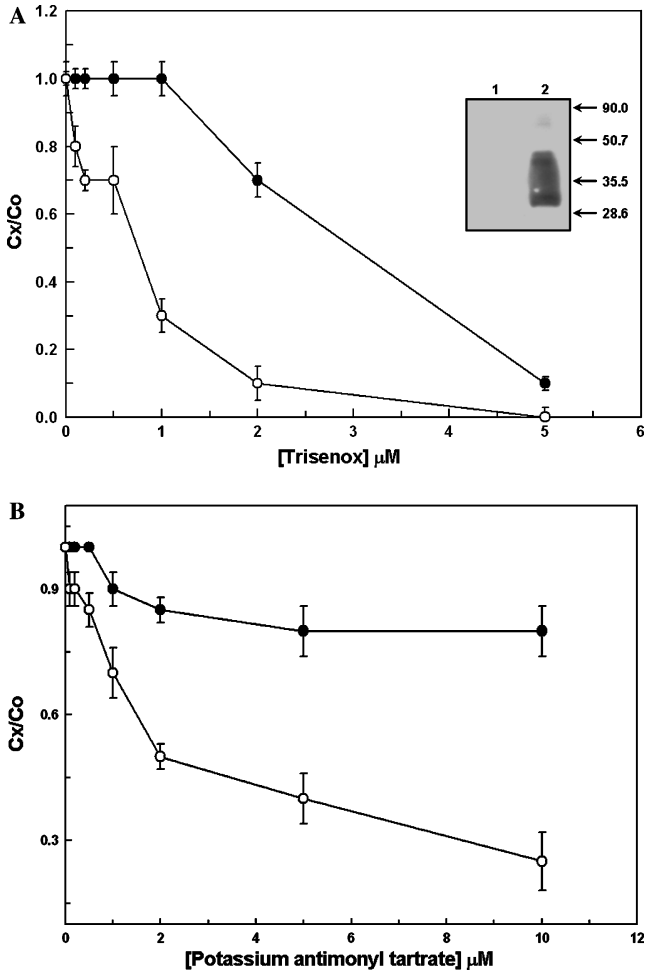

Fig. 1. hAQP9 expression sensitizes K562 cells to As(III) and Sb(III). (A) Trisenox and (B) potassium antimonyl tartrate. (●) Vector alone and (○) transfected with hAQP9. C_x is the cell density after treatment with the metalloid at concentration x and C_0 is the cell density of the untreated control. Each point represents the average of three separate assays. Data are expressed as means ± SEM. (Inset) Western blot analysis of K562 cells transfected with (1) vector alone and (2) hAQP9.

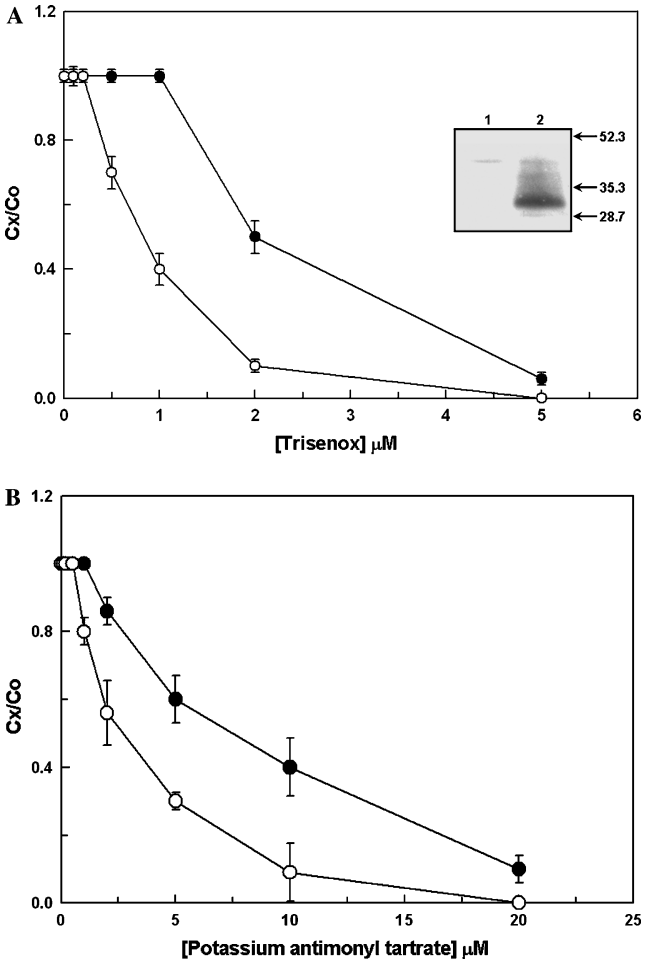

Fig. 2. Vitamin D pretreatment sensitizes HL60 cells to As(III) and Sb(III). (A) Trisenox and (B) potassium antimonyl tartrate. (●) No treatment and (○) pretreated with 100 nM vitamin D. C_x is the cell density after treatment with the metalloid at concentration x and C_0 is the cell density of the untreated control. Each point represents the average of three separate assays. Data are expressed as means ± SEM. (Inset) Western blot analysis of (1) untreated HL60 cells and (2) pretreated with 100 nM vitamin D.

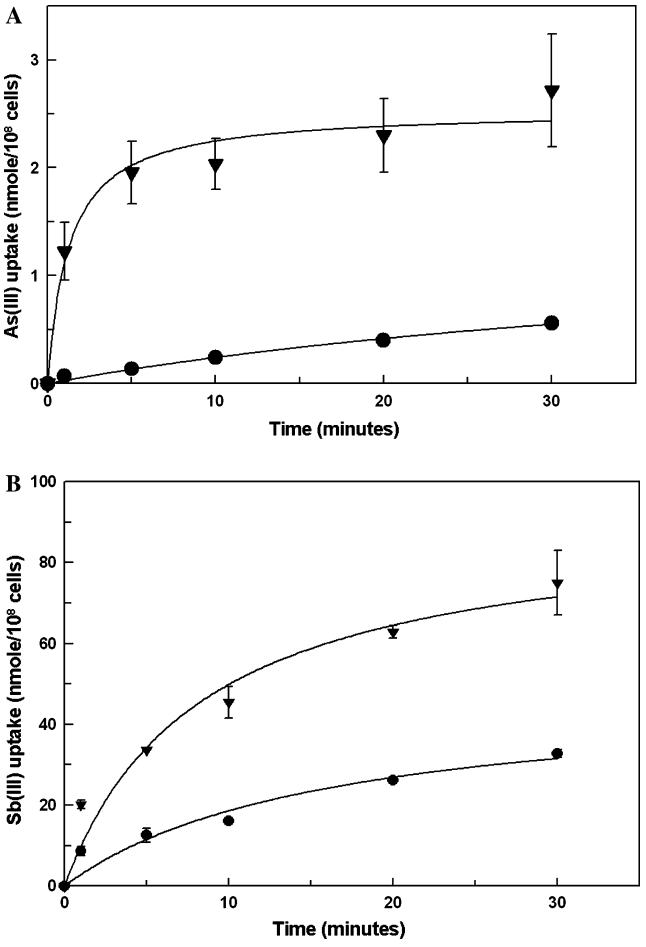

Fig. 3. hAQP9 catalyzes metalloid transport in K562 cells. (A) As(III) and (B) Sb(III). (●) Vector alone and (▼) transfected with pIRES-hAQP9. Each point represents the average of four separate assays. Data are expressed as means ± SEM.

Discussion

The physiological function of AQP9 is still unknown although broad substrate specificity has been elucidated for the channel. It has been proposed that adipocytes release glycerol through AQP7 which is then taken up by AQP9 in liver for gluconeogenesis [18]. It has also been observed that mRNA levels of AQP7 and AQP9 are coordinately regulated during fasting and type 1 diabetes mellitus [18]. It was subsequently shown that expression of AQP9 is altered in rats during fasting and insulin deficiency [10]. However, involvement of AQP9 in cancer or modulation of cancer drug sensitivity by AQP9 has not been demonstrated. In this study we show that overexpression of hAQP9 in a leukemic cell line K562 leads to hypersensitivity to the trivalent arsenic drug Trisenox and trivalent antimony as a result of higher rates of accumulation of the metalloids. Therefore monitoring AQP9 expression in APL patients before or during Trisenox treatment may predict their response to As(III) therapy.

Arsenic often produces significant toxicity during treatment. Depending on the dose, it has deleterious effects on liver, kidney, and other organs [19]. Fatal events at therapeutic doses from hepatic toxicity [20] and sudden death have been reported [21]. Prolonged corrected QT interval (QTc) and ventricular tachycardia (Torsades de pointes) has also been reported [22]. Thus reducing the toxicity of As_2O_3 during treatment would be beneficial. Moreover, individual variability to As(III) toxicity have also been observed [23]. This may be due to variability of AQP9 expression among individuals. Therefore, patients who already have higher AQP9 levels may be more prone to As(III) toxicity but may respond better to lower dosage of chemotherapy.

On the other hand, if AQP9 expression can be induced in patients, then lowering the concentration of the drug and/or reducing the duration of treatment

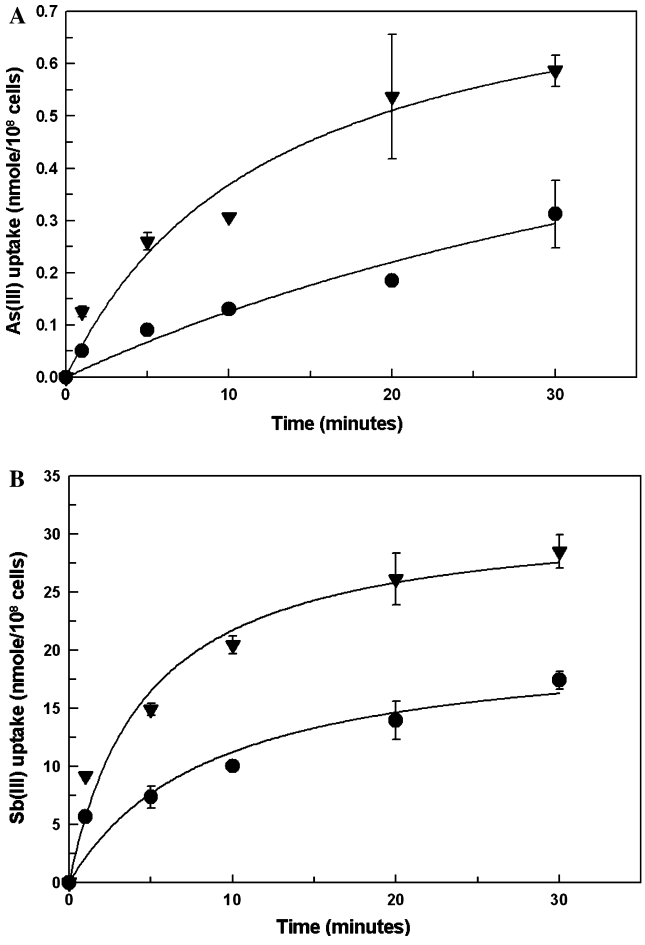

Fig. 4. Vitamin D pre-treatment leads to higher rates of metalloid transport in HL60 cells. (A) As(III) and (B) Sb(III). (●) Vector alone and (▼) transfected with pIRES-hAQP9. Each point represents the average of four separate assays. Data are expressed as means ± SEM.

may also lead to effective therapeutic regimen, avoiding drug-induced toxicity. Co-administration of vitamins and As_2O_3 has been reported to be beneficial for cancer treatment. Ascorbic acid enhances the activity of As_2O_3 in vitro against drug resistant multiple myeloma by depleting cells of glutathione (GSH). The combination is also proven to be effective in a Phase I clinical trial with relapsed/refractory multiple myeloma [24]. Vitamin C has also been reported to augment the effect of As_2O_3 in clinical acute myeloid leukemia samples [25] and T-cell lymphoma cells [26]. Vitamin D is a steroid hormone that is primarily metabolized in the liver and subsequently in the kidney into 1,25-dihydroxyvitamin D (calcitrol), the most biologically active form of vitamin D [27]. The antiproliferative effects of vitamin D have been shown in a wide range of cell types, including carcinomas of the breast, prostate, colon, skin, brain, and myeloid leukemia cells [23]. Recently, vitamin D has also been shown to induce apoptosis and to inhibit angiogenesis, tumor invasion, and metastasis [23]. Transcriptional activation of cyclin dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Waf1}$ in HL60 promyelocytic leukemia cells and U937 myelomonocytic leukemia cells, respectively, has been implicated as the mechanism responsible for cell cycle arrest in response to vitamin D [28,29]. Vitamin D is also known to cause differentiation in HL60 cells [30,31]. Since HL60 cells do not exhibit a significant amount of AQP9 in their plasma membrane (Fig. 2A), vitamin D was added to induce AQP9 expression. We find that addition of 100 nM vitamin D in the log phase of growth of HL60 cells induces high level AQP9 expression with no growth inhibition and differentiation (data not shown). We chose these conditions because it most likely resembles the physiological status of a leukemia patient. We have shown that, under such conditions, vitamin D pre-treatment lowers the IC_{50} of Trisenox significantly. This result implies that in combination with vitamin D low dosages of As_2O_3 would be equally effective as a chemotherapeutic regimen avoiding toxicity.

In this study we also demonstrated that Sb(III) could be equally effective as a chemotherapeutic agent at physiologically achievable dosages when AQP9 is expressed in leukemic cells. It is especially significant for a chronic myelogenous type leukemic cell K562 where the cells are highly resistant to Sb(III) (Fig. 1B), but they become as sensitive as to As(III) when expressing AQP9. Thus, Sb(III) could be an alternative therapy in patients who show higher expression of AQP9 or in patients where expression of AQP9 could be induced by a natural agent like vitamin D.

Acknowledgments

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