Relationship of expression of aquaglyceroporin 9 with arsenic uptake and sensitivity in leukemia cells

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Arsenic trioxide (As2O3) is highly efficacious in acute promyelocytic leukemia (APL). Aquaglyceroporin 9 (AQP9) is a transmembrane protein that may be involved in arsenic uptake. In 10 of 11 myeloid and lymphoid leukemia lines, quantitative polymerase chain reaction (Q-PCR) and Western blotting showed that AQP9 expression correlated positively with As2O3-induced cytotoxicity. As a proof-of-principle, transfection of EGFP-tagged AQP9 to the hepatoma line Hep3B, not expressing AQP9 and As2O3 insensitive, led to membrane AQP9 expression and increased As2O3-induced cytotoxicity. Similarly, the chronic myeloid leukemia line K562 expressed low levels of AQP9 and was As2O3 insensitive. The K562EGFP-AQP9 transfectant accumulated significantly higher levels of intracellular arsenic than control K562EGFP when incubated with As2O3, resulting in significantly increased As2O3-induced cytotoxicity. Pretreatment of the myeloid leukemia line HL-60 with all-trans retinoic acid (ATRA) up-regulated AQP9, leading to a significantly increased arsenic uptake and As2O3-induced cytotoxicity on incubation with As2O3, which might explain the synergism between ATRA and As2O3. Therefore, AQP9 controlled arsenic transport and might determine As2O3 sensitivity. Q-PCR showed that primary APL cells expressed AQP9 significantly (2-3 logs) higher than other acute myeloid leukemias (AMls), which might explain their exquisite As2O3 sensitivity. However, APL and AML with maturation expressed comparable AQP9 levels, suggesting that AQP9 expression was related to granulocytic maturation.

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Introduction

Arsenic trioxide (As2O3) is a standard treatment for acute promyelocytic leukemia (APL)1-3 and is showing promise in multiple myeloma and other hematologic malignancies.4,5 The intracellular actions of As2O3 include interaction with proteins possessing high cysteine and histidine contents,6 generation of superoxides and reactive oxygen species,7,8 disruption of the mitochondrial transmembrane potential with release of cytochrome c and caspases activation, and blockade of cell cycle at the G1/S and G2/M phases.9

As2O3 is detoxified by the glutathione redox system, comprising thiol-rich glutathione (GSH) carrier proteins, glutathione peroxidase, and glutathione S-transferase (GST-π).9,10 GSH forms a transient As(GSH)3 complex with As3+, a process catalyzed by GST-π.11 As(GSH)3 binds the multi-drug–resistant protein 1 (MRP1), inducing a conformational change.12,13 This in turn leads to an ATP-driven transmembrane transport of As(GSH)3 to the extracellular space.13,14 Free reduced GSH carrier protein is then replenished by glutathione peroxidase.

The arsenic GSH redox system might be one of the mechanisms that account for the varying sensitivities of different cell types to As2O3. The sensitivities to As2O3 had been shown to be inversely proportional to the cellular GSH content.15 Furthermore, cell lines treated with ascorbic acid and buthionine sulfoxide (BSO), which decreased intracellular GSH levels, had increased sensitivity to As2O3.15 The role of MRP1 in arsenic sensitivity is more controversial. Overexpression of MRP1 had been shown to confer resistance to As2O3 in lung cancer cells and the HeLa cell line.16 However, an HL-60 variant, HL-60/AR, that overexpressed MRP1 did not show increased arsenic sensitivity.17

The transmembrane protein aquaglyceroporin 9 (AQP9) was first identified in adipose tissue and leukocytes.18,19 It is also expressed in the liver, lung, and spleen.20 AQP9 facilitates the passage of water and glycerol, as well as a large variety of small noncharged solutes (caramides, polyols, purine, pyrimidine, and protonated monocarboxylates).20 Recently, AQP9 was shown to restore arsenic sensitivity to a Saccharomyces cerevisiae mutant that lacked FPS1, the homologue of AQP9.21 Furthermore, Xenopus laevis oocytes microinjected with AQP9 cDNA exhibited increased uptake of As+ ions.22 Although these results showed that AQP9 facilitated arsenic transport in yeast and Xenopus, further investigations are required to determine the function of AQP9 in arsenic transport in mammalian cells.

In this study, we tested the hypothesis that AQP9 mediated arsenic uptake in leukemia cells and might be a determining factor in arsenic sensitivity.

Patients, materials, and methods

Cell lines

One APL (NB4), 2 myeloid leukemia (HL-60, ML-1), 1 chronic myeloid leukemia (K562), 1 B-cell acute lymphoblastic leukemia (ALL; NALM-20), 1 non-B non-T ALL (NALM-16), and 5 T-cell leukemia (HPB-ALL, JM, JURKAT, KE-37, MOLT-4) cell lines were studied. They were cultured...
in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in 5% CO2 at 37°C. Cells were treated with As2O3 (Sigma-Aldrich, St Louis, MO) and with all-trans retinoic acid (ATRA; Sigma-Aldrich) in selected experiments.

### Arsenic cytotoxicity

Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in fresh RPMI-1640 medium supplemented with 10% FBS and various concentration of As2O3 (0, 0.5, 1.0, 2.0, and 4 μM) at 2.5 × 10^5 cells/mL. Quadruplicates of 100 μL of each arsenic/cell suspension were incubated for 48 hours. MTI reagent (100 μL) was then added and incubated for 4 hours, followed by the addition of 100 μL of solubilizing buffer and incubation overnight. The absorbance at 560 nm was then measured.

### Western blotting

Leukemic cells were washed once in PBS supplemented with Complete protease inhibitor (Roche, Manheim, Germany). Washed pellets (4 × 10^9 to 5 × 10^10 cells) were resuspended in 5 mL lysis buffer (7.5 mM sodium phosphate, pH 7.0; 0.25 M sucrose; 5 mM EDTA with Complete protease inhibitor; and 1 mM phenethylsulphonylfluoride), sonicated on ice for 60 s, and centrifuged at 10,000g for 10 minutes at 4°C. The crude membrane fraction was then pelleted by ultracentrifugation (200,000g for 90 minutes at 4°C), resuspended in 150 μL buffer (5.0% SDS; 20 mM Tris, pH 8.0; and 5 mM EDTA with Complete protease inhibitor and 1 mM PMSF), sonicated, and incubated at 37°C for 30 minutes. Protein concentrations of each sample were determined (DC protein assay kit II; Bio-Rad, Hercules, CA) and adjusted to 2 μg/mL in lysis buffer and 5 x same buffer (15% SDS; 50 mM Tris, pH 6.8; 30% glycerol; 0.05% bromophenol blue; and 0.5 M dithiothreitol). Protein samples were boiled at 95°C for 5 minutes and kept at 37°C, and 30 μg was resolved in 12% acrylamide gel with 4 M urea, electrotransfered to PVDF membrane (Amer sham Biosciences, Piscataway, NJ), blocked with 5% low-fat milk for 1 hour, incubated with rabbit anti-AQP9 antibody (3.5 μg/mL) or rabbit anti-enhanced green fluorescent protein (EGFP) antibody (1:10,000; Chemicon, Temecula, CA), and probed with an antirabbit secondary antibody (1:1000; Dako Cytomation, Glostrup, Denmark). Immunoblotting was evaluated after chemiluminescence development (Western Lightning Chemiluminescence Reagent; PE Biosystems, Foster City, CA), followed by PointSau staining (Sigma-Aldrich) for normalization of protein loading.

### Quantification of AQP9 gene expression

AQP9 expression was quantified by quantitative polymerase chain reaction (Q-PCR). Briefly, the AQP9 primers (forward, 5’ AGT TGT TGG GAG CCT TTTG TG 3’; reverse, 5’ GGT CGC CGA AGA TAG ATA CGG AGG 3’) and the TaqMan probe (5’ CAA CAC ACA TTGG CAA CAT ACC 3’) detecting a 155-bp amplicon were designed by Primer Express software (PE Biosystems). The primers (forward, 5’ GAA GGT GAA GGT CGG AGT CA 3’; reverse, 5’ CTT CTA CCA CTA CCC TAA AG 3’) and TaqMan probe (5’ GGT CGA AGG GCA AGA TTC GGC 3’) detecting a 155-bp amplicon were designed by Primer Express software (PE Biosystems). Expression of the multi-drug–resistance 1 (MDR1) gene was quantified as previously reported.22 The expression levels of AQP9 were similarly described.23

### Transfection of Hep3B with pEGFP-AQP9 for assaying of As2O3 sensitivity

Hep3B cells in MEM cultured to 80% confluence were transfected with pEGFP-C2/pSV-βgal or pEGFP-AQP9/pSV-βgal in Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In order to ensure that cells transfected with pSV-βgal would also express AQP9, a 1:5 ratio of pSV-βgal to pEGFP-AQP9 was used. Hence, β-galactosidase activity could be used as an indicator of viability of cells expressing both β-gal and EGFP-C2 or EGFP-AQP9. The transfectants, designated as Hep3B/pEGFP-AQP9/βgal and Hep3B/pEGFP-C2/βgal, were further incubated for 48 hours. The cells were then treated with As2O3 at various concentrations (0, 0.5, 1.0, and 2.0 μM) for 24 hours. For assaying of β-galactosidase activity, transfected Hep3B cells were washed with PBS, lysed with reporter lysis buffer (Promega) for 15 minutes at room temperature, transferred to Eppendorf tubes on ice, vortexed for 20 seconds, and centrifuged at 1000g at 4°C for 1 minute. Cell lysates were mixed with an equal volume of 2 X assay buffer and incubated at 37°C for 1 to 2 hours, after which the reaction was stopped by 1 M sodium bicarbonate. Absorbance at 420 nm was then recorded. Untransfected cells not treated with As2O3 served as 100% viability control. All samples were tested in triplicates.

### Transfection of K562 cells with pEGFP-AQP9 and fluorescence-activated cell sorting

K562 cells at mid-log phase were transfected with pEGFP-AQP9 or pEGFP-C2 as described for Hep3B cells. The K562 transfectants, designated as K562/pEGFP-AQP9 and K562/pEGFP-C2, were washed once with PBS and then resuspended in RPMI-1640 medium supplemented with 500 μg/mL of G418 (Calbiochem, San Diego, CA) at 1 × 10^6 cells/mL. Cells resistant to G418 were selected for 3 weeks. To further enrich for transfected cells, sorting for green fluorescent cells (K562/pEGFP-AQP9 and K562/pEGFP-C2) was performed by an EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA) until a purity of 80% or higher was achieved.

### Arsenic-uptake analysis

K562/pEGFP-AQP9 and K562/pEGFP cells at 1 × 10^6 cells/mL were washed twice with PBS; resuspended in fresh RPMI-1640 medium supplemented with 10% FBS and 1 μM As2O3; and incubated in 5% CO2 at 37°C for 0, 30, 60, 90, and 120 minutes. Cells were then harvested into 1 mL ice-cold PBS and pelleted. After 2 washes in ice-cold PBS, the cell pellets were lysed in 0.9 mL double-distilled water by sonication for 10 minutes. HNO3 (2%) with 10 parts per billion (ppb) of yttrium (Chem Service, West Chester, PA) was then added as an internal standard. Samples were vortexed and centrifuged at 3000g for 10 minutes, and the supernatants were collected and assayed for arsenic concentration by inductively coupled plasma–mass spectrometry (ICP-MS) as previously described.24

### AQP9 expression in leukemia samples

Archival bone marrow aspirate samples from patients with APL and other subtypes of acute myeloid leukemia (AML) were quantified for AQP9 expression. Leukemic diagnoses were based on the World Health Organization classification system.25 The blast cells were isolated by centrifugation, snap-frozen, and stored at −80°C. For quantification of AQP9 expression,
leukemic samples were thawed, total RNA was extracted, and Q-PCR for AQP9 was performed, with the cell line NB4 used as the calibrator in the ΔCt method, using GAPDH as the internal reference. The procurement of materials was approved by the institutional review board at Queen Mary Hospital (Hong Kong, China). Patients gave informed consent and the study was conducted in accordance with the Declaration of Helsinki.

Statistical analysis
Continuous variables were expressed as mean ± standard error of the mean. The correlation between cell viability and AQP9 expression was tested by Pearson correlation test (SPSS software, version 10.0; Chicago, IL). Comparison between groups was performed by Student t test. P values of less than .05 were considered to be statistically significant.

Results
A2O3 cytotoxicity to leukemic cell lines
MTT assays were performed on the 11 cell lines tested. The sensitivities of the cell lines differed, although all showed a dose-dependent increase in A2O3 cytotoxicity. Of the 11 cell lines, Jurkat, HL-60, and NALM-20 were most resistant, whereas NB4 was most sensitive (Figure 1; Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

AQP9 expression and sensitivity to A2O3
In the 11 leukemia cell lines tested, the expression of AQP9 as determined by Q-PCR was found to be positively correlated with A2O3-induced cytotoxicity as assessed by MTT (P = .039; Figure 2A). The results were confirmed by Western-blot analysis (Figure 2B), showing a direct relationship between AQP9 protein expression and A2O3 sensitivity (P = .001; Figure 2C). However, the cell line ML-1 was an obvious outlier in both experiments. Finally, there was no relationship between AQP9 and MDR1 gene expression (P = .09, Pearson correlation; Table S1).

AQP9 point mutation in ML-1
As ML-1 showed an apparent high level of AQP9 expression but a low A2O3 sensitivity, the entire coding region of AQP9 in ML-1 was amplified by PCR and sequenced. The results showed a homozygous single base substitution, 835G>A, which resulted in an amino-acid change from alanine to serine at codon 279. Whether this might account for the discordant AQP9 expression and A2O3 sensitivity is under further investigations.

AQP9 transfectants of Hep3B and K562
Fluorescence microscopic examination of the Hep3B and K562 AQP9 transfectants showed a difference in distribution of fluorescence. In Hep3BEGFP-C2 and K562EGFP-C2, the green fluorescence was evenly distributed in the cytosol. However, in Hep3BEGFP-AQP9 and K562EGFP-AQP9, the fluorescence was mainly distributed on the plasma membrane (Figure 3A). The results confirmed selective localization of AQP9 to the plasma membrane. Western blotting was performed to confirm protein overexpression in the transfectants. In K562EGFP-C2 and Hep3BEGFP-C2-gal, an EGFP protein band of around 30 kDa was detected with an anti-GFP antibody (Figure 3B-C). In Hep3BEGFP-AQP9/gal and K562EGFP-AQP9 lysates, the EGFP-AQP9 fusion protein was detected as a 61-kDa band with antibodies specific to GFP or AQP9, representing a summation of the 30-kDa EGFP and the 31-kDa AQP9 protein (Figure 3B-C). An additional band of approximately 30 kDa was also detected in both the anti-GFP and anti-AQP9 blotting of the K562EGFP-AQP9 lysate, which might be due to the degradation of the EGFP-AQP9 fusion protein to EGFP (30 kDa) and AQP9 peptides (31 kDa).

A2O3 sensitivity of Hep3B-AQP9 and K562 transfectants
The Hep3B hepatoma line did not express any detectable amount of AQP9 (Figure 3B) and was insensitive to A2O3. In Hep3BEGFP-gal and Hep3BEGFP-AQP9-gal transfectants, the β-galactosidase activity was used as an indicator of cell viability after treatment with various concentrations of A2O3. As shown in Figure 4, Hep3BEGFP-gal cells, similar to the parental Hep3B cells, were resistant to A2O3. On the other hand, A2O3-treated Hep3BEGFP-AQP9-gal cells demonstrated a significantly lower β-galactosidase activity compared with Hep3BEGFP-gal (Figure 4), showing that overexpression of AQP9 increased A2O3-induced cytotoxicity. Interestingly, the difference of viability of Hep3BEGFP-gal and Hep3BEGFP-AQP9-gal remained constant with increasing concentrations of A2O3.

A2O3 sensitivity and arsenic uptake by K562-AQP9 transfectants
The K562 cells expressed very low levels of AQP9 (Figure 3B) and were insensitive to A2O3. The K562EGFP-C2 transfectant was also insensitive to A2O3 (Figure 5A). However, the K562EGFP-AQP9 transfectants showed a significantly increased sensitivity to A2O3 (Figure 5A). Furthermore, in contrast to Hep3B AQP9 transfectants, the difference of viability of K562EGFP-C2 and K562EGFP-AQP9 widened with increasing concentrations of A2O3. The increase in A2O3 sensitivity could be explained by an increased As³⁺ uptake mediated by the overexpressed AQP9. As shown in Figure 5B, the K562EGFP cells accumulated minimal amounts of arsenic after incubation in 1 μM A2O3 for 2 hours. On the other hand, the K562EGFP-AQP9 showed a time-dependent increase in arsenic accumulation, reaching a significantly higher amount of arsenic (median, 65 pmol/10⁶ cells) after 2 hours.

ATRA induced up-regulation of AQP9 and increased arsenic uptake and A2O3 sensitivity
ATRA acts synergistically with A2O3 in vitro and in vivo. The HL-60 cells expressed very low levels of AQP9 (Figure 2A-C) and...
were insensitive to As2O3. However, treatment with ATRA led to a
dose-dependent increase in AQP9 expression (Figure 6A). To assess the biologic significance of
AQP9 up-regulation, HL-60 cells were pretreated with ATRA
and treated with As2O3. ATRA-pretreated HL-60 cells showed an
increased sensitivity to As2O3 compared with untreated HL-60 cells
(Figure 6B). The increase in As2O3 sensitivity was due to increased
arsenic uptake into the cells. Incubation of ATRA-pretreated HL-60
cells in 1 μM As2O3 led to a time-dependent increase in arsenic
accumulation, which was significantly higher than in untreated
HL-60 cells (Figure 6C).

AQP9 expression in leukemic samples
Leukemic samples from 80 patients were studied. These included
19 patients with APL (presentation, 5; relapse, 14), 42 patients with
AML (minimally differentiated, 3; without maturation, 11; with
maturation, 10; myelomonocytic, 5; monoblastic, 1; megakaryoblastic, 1; multilineage dysplasia, 10), and 19 patients with
ALL. The mean level of AQP9 expression was similar (APL 3.07 × 102, AML 9.62 × 102, P = .027; Figure 7A). Interestingly, in subgroup analysis, when
APL was compared with AML with maturation (or AML M2 in the
French-American-British classification system26, n = 10), the AQP9
expression was similar (APL 3.07 × 102 ± 2.08 × 102 versus
AML M2 2.14 × 102 ± 0.72 × 102, P = .75; Figure 7B). Hence,
when APL was compared with AML subtypes other than M2
(n = 32), AQP9 expression was even more significantly different
(3.07 × 102 ± 2.08 × 102 versus 3.93 × 102 ± 1.37 × 102,
P = .009; Figure 7B). However, AQP9 expression in ALL was
comparable with AML (ALL 2.05 × 104 ± 1.94 × 104 versus
AML 9.62 × 104 ± 6.63 × 104, P = .6; Figure 7C).

Discussion
Our findings showed that AQP9 played a critical role in the
transmembrane transport of As2O3. In neutral pH, As2O3 exits
primarily as As(OH)3, which might be recognized by AQP9 as a
small neutral noncharged solute similar to glycerol for facilitated
uptake.27 Accordingly, we showed that in leukemia cells of
different lineages, in vitro sensitivity to As2O3 was directly
proportional to AQP9 expression. The results were confirmed both
at the mRNA and protein level. The ML-1 cell line was an
exception in that although AQP9 was expressed apparently at high
levels, the line was relatively resistant to As2O3. Analysis of the
AQP9 coding sequence showed a point mutation resulting in a base
substitution. Whether this base substitution is of functional signifi-
cance is being investigated. An alternative explanation for the poor
As2O3 sensitivity would be different intracellular constituents or
mechanisms that are distinctive to this cell line. As the MDR1 gene
encoding P-glycoprotein also controlled transmembrane drug traf-
ficking, we investigated if the expression of AQP9 and MDR1
genes might be related. Q-PCR showed that their expressions were
unrelated. This was consistent with previous observations that
although the MDR1 gene might be up-regulated in relapsed APL,
the response to As2O3 was unaffected, suggesting that MDR1
did not contribute to As2O3 sensitivity.22 Lastly, for leukemia cell lines of
different lineages, the APL cell line NB4 showed the highest

Figure 3. Transfection of AQP9 into Hep3B and K562 cells. (A) K562EGFP-C2 cells examined under fluorescent microscopy showed even cellular distribution of green
fluorescence; however, K562EGFP-AQP9 cells showed selective localization of green fluorescence to the plasma membrane. Images were visualized using an Olympus IX70
microscope equipped with a C plan semi-apochromat 60×/0.7 numerical aperture lens (Olympus, Tokyo, Japan). A Nikon Coolpix 4500 camera (Nikon, Tokyo, Japan) was used to capture
the images. (B) Western-blot analysis of Hep3B cells. In anti-GFP immunoblotting, EGFP (30 kDa) and EGFP-AQP9 (61 kDa) were detected in Hep3BEGFP and Hep3BEGFP-AQP9 cells,
respectively. With anti-AQP9 immunoblotting, only the protein band of EGFP-AQP9 (61 kDa) was detected in Hep3BEGFP-AQP9 cells, showing that Hep3BEGFP cells did not express
detectable levels of AQP9. (C) Western-blot analysis of K562 cells. In anti-GFP immunoblotting, protein bands of EGFP (30 kDa) and EGFP-AQP9 (61 kDa) were detected in K562EGFP
and K562EGFP-AQP9 cells. An additional band (30-31 kDa) was observed in the K562EGFP-AQP9 sample. This might be due to degradation of EGFP-AQP9 fusion protein into EGFP and AQP9
fractions. In anti-AQP9 immunoblotting, a predominant band of EGFP-AQP9 (61 kDa) was detected in K562EGFP-AQP9 cells. At the anti-AQP9 antibody concentration and
exposure time used to avoid overexposure of the K562EGFP-AQP9 lysate, K562EGFP lysate did not show detectable AQP9, as was shown previously in Figure 2.

Figure 2. Correlation of AQP9 expression with As2O3 sensitivity. (A) The quantity of AQP9 mRNA, as determined by Q-PCR, was inversely related to cell survival after
treatment in 2 μM As2O3, for 48 hours. ML-1 was an outlier. (B) Western-blot analysis of AQP9 expression. NB4 expressed the highest amount of AQP9 and was used as the standard for comparison in panel C. (C) AQP9 protein expression, as determined by Western-blot analysis using NB4 as the reference, showed an inverse relationship to cell survival. ML-1 was also an outlier.

R² = 0.7406
P = .001

R² = 6.3531
P = .009
expression level of AQP9 and the most exquisite sensitivity to As$_2$O$_3$. Interestingly, APL is also clinically the leukemic subtype that responds best to As$_2$O$_3$ therapy.

To confirm the role of AQP9 in As$_2$O$_3$ entry and sensitivity, a pertinent investigation will be to express AQP9 in an AQP9-negative cell line and to study the response to As$_2$O$_3$ before and after AQP9 expression. Furthermore, transient transfection may be preferable to avoid inadvertent introduction of additional properties acquired unexpectedly during the selection of permanent transfectants. However, a leukemic line with undetectable AQP9 expression was not available. Moreover, the very low transfection-efficiency characteristic of leukemia cells in suspension would make transient transfection and subsequent experiments difficult. We therefore had elected to choose the Hep3B line, which were adherent cells and did not express any detectable AQP9, for this proof-of-principle experiment. In Hep3B cells, the transfected EGFP-AQP9 fusion protein showed the appropriate membrane localization. The selective cell-membrane partition of AQP9 may be due to the hydrophobicity of the AQP9 moiety or the presence of a membrane-localizing motif in AQP9. Besides accurate localization, the function of AQP9 in facilitating As$_2$O$_3$ uptake was also preserved. This was reflected in an increase in sensitivity to As$_2$O$_3$ in the Hep3B$_{EGFP-AQP9}$ transfectant. The intracellular arsenic was not measured because harvesting the Hep3B cells required trypsinization, which partly permeabilized the cell membrane and resulted in a substantial loss of arsenic.

To further substantiate these observations in leukemia cells, the K562 line was chosen, as it had very low endogenous AQP9 expression and hence was insensitive to As$_2$O$_3$. Due to the low transfection efficiency, stable transfection coupled with flow sorting of fluorescent cells was needed to enrich for AQP9-transfected cells. The stable K562$_{EGFP-AQP9}$ transfectants showed results similar to those observed in transient transfection of Hep3B, in that K562$_{EGFP-AQP9}$ also demonstrated an increased As$_2$O$_3$ sensitivity. Moreover, we were able to show that the higher As$_2$O$_3$ sensitivity was due to increased intracellular arsenic accumulation. These observations confirmed that AQP9 facilitated transmembrane transport of arsenic.

Both Hep3B$_{EGFP-AQP9/β-gal}$ and K562$_{EGFP-AQP9}$ exhibited a higher arsenic sensitivity than Hep3B$_{EGFP/β-gal}$ and K562$_{EGFP-C2}$. However, the difference in viability of K562$_{EGFP-AQP9}$ and K562$_{EGFP}$ cells increased progressively with higher concentrations of As$_2$O$_3$, whereas the difference of viability of Hep3B$_{EGFP-AQP9/β-gal}$ and Hep3B$_{EGFP/β-gal}$ remained comparable at different concentrations of As$_2$O$_3$. Therefore, it might be possible that AQP9-mediated arsenic transmembrane transport could be saturable, depending on the amount of AQP9 expressed. Alternatively, in addition to the amount of intracellular arsenic, other intrinsic properties of the cell may also determine its sensitivity to As$_2$O$_3$.

An important prerequisite for putting these observations into therapeutic applications is finding clinically feasible methods for up-regulation of AQP9 in leukemia cells. A synergistic interaction between ATRA and As$_2$O$_3$ has been observed in vitro. Moreover, patients with APL at presentation showed a more efficient leukemic load reduction if treated concomitantly with ATRA and As$_2$O$_3$. Patients with APL at presentation or relapse also responded better to combined ATRA and As$_2$O$_3$ treatment. In the myeloid leukemia line HL-60 we showed that ATRA pretreatment up-regulated AQP9 expression, which was translated into an increase in arsenic uptake and hence sensitivity to As$_2$O$_3$ treatment. As the concentration of ATRA used was as low as 100 nM, which would not induce any significant apoptotic effect on HL-60, the increased cytotoxicity after As$_2$O$_3$ treatment could be attributable to the demonstrable increase in arsenic uptake. These observations provided another mechanistic explanation for the synergism between ATRA and As$_2$O$_3$. We had also conducted similar experiments with the APL cell line NB4. Unfortunately, NB4 was not a good in vitro model. This is because NB4 cells already express very high levels of AQP9 and are extremely sensitive to ATRA and As$_2$O$_3$, even at very low concentrations. Therefore, the exogenous overexpression of AQP9 did not give consistent results of further increases in arsenic uptake or cytotoxicity (data not shown).

To verify the relevance of AQP9 expression in primary leukemia samples, we quantified AQP9 in a series of AML samples. We showed that APL expressed AQP9 significantly (about 2-3 log) more than other subtypes of AML. This observation was consistent with the high sensitivity of APL to As$_2$O$_3$ compared with other

![Figure 4. β-galactosidase enzyme assay on Hep3B$_{EGFP-AQP9/β-gal}$ cells.](image-url)

![Figure 5. Transfection of AQP9 in K562 cells. (A) In MTT assay, K562$_{EGFP}$ showed an increase in As$_2$O$_3$ sensitivity compared with K562$_{EGFP-C2}$ cells (Student t test for each of the points analyzed). Note that the difference of As$_2$O$_3$ sensitivity between K562$_{EGFP-AQP9}$ and K562$_{EGFP}$ cells widened with an increasing concentration of As$_2$O$_3$. NB indicates not significant. (B) In arsenic-uptake assay, K562$_{EGFP-AQP9}$ cells accumulated significantly more arsenic than K562$_{EGFP-C2}$ cells (Student t test for each of the points analyzed). Both lines showed a time-dependent increase in arsenic uptake.](image-url)
studies have shown that steroid hormones and vitamin D3 may particularly in AML without granulocytic maturation. Previous national regulation of the AQP9 gene will be clinically relevant, AQP9 expression. Secondly, investigations of the transcription factors. In fact, the AQP9 promoter contains binding sites for C/EBP, NF-H9260 and AP-1. It is intriguing to note that C/EBP is inducible by retinoids such as ATRA. Therefore, the definition of agents able to induce AQP9 expression in different cancer cell types may hold promise for increasing the uptake of As2O3 and hence its efficacy in these malignancies.

Moreover, the potential contribution of AQP9 dysregulation or mutation to As2O3 resistance needs further investigation. Finally, the organ toxicity of arsenic may also be related to AQP9 expression. In fact, AQP9 is expressed normally at high concentrations in the liver and brain, organs that are most affected by arsenic therapy. Agents that may selectively down-regulate AQP9 in these organs may ameliorate arsenic toxicity, which is an important limiting factor in the therapeutic use of As2O3. These propositions will require future validations.

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Authorship

Contribution: J.L. performed the experiments and wrote and approved the manuscript; A.P. performed the experiments and approved the manuscript; W.-H.Y. performed the experiments and approved the manuscript; Y.-L.K. designed and supervised the study and wrote and approved the manuscript; and E.W.C.T. designed and supervised the study and amended and approved the manuscript.

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References


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