Arsenic disulfide synergizes with the phosphoinositide 3-kinase inhibitor PI-103 to eradicate acute myeloid leukemia stem cells by inducing differentiation

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Although dramatic clinical success has been achieved in acute promyelocytic leukemia (APL), the success of differentiating agents has not been reproduced in non-APL leukemia. A key barrier to the clinical success of arsenic is that it is not potent enough to achieve a clinical benefit at physiologically tolerable concentrations by targeting the leukemia cell differentiation pathway alone. We explored a novel combination approach to enhance the eradication of leukemia stem cells (LSCs) by arsenic in non-APL leukemia in the present study. Phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) phosphorylation was strengthened after As2S2 exposure in leukemia cell lines and stem/progenitor cells, but not in cord blood mononuclear cells (CBMCs), propidium iodide-103, the dual PI3K/mTOR inhibitor, effectively inhibited the transient activation of the PI3K/AKT/mTOR pathway by As2S2. The synergistic killing and differentiation induction effects on non-APL leukemia cells were examined both in vitro and in vivo. Eradication of non-APL LSCs was determined using the nonobese diabetic/severe combined immunodeficiency mouse model. We found that a combined As2S2/PI-103 treatment synergized strongly to kill non-APL leukemia cells and promote their differentiation in vitro. Furthermore, the combined As2S2/PI-103 treatment effectively reduced leukemia cell repopulation and eradicated non-APL LSCs partially via induction of differentiation while sparing normal hematopoietic stem cells. Taken together, these findings suggest that induction of the PI3K/AKT/mTOR pathway could provide a protective response to offset the antitumor efficacy of As2S2. Targeting the PI3K/AKT/mTOR pathway in combination with As2S2 could be exploited as a novel strategy to enhance the differentiation and killing of non-APL LSCs.

Introduction

The use of arsenic trioxide (ATO) to induce differentiation has proven to be a revolutionary approach for the treatment of both newly diagnosed and refractory/refractory acute promyelocytic leukemia (APL) (1). However, successful treatment via the use of differentiating agents such as all-trans retinoic acid (ATRA) and ATO has only been achieved in APL (1,2). The presence of the specific chromosomal alteration (t(15;17)(q22;q21) in APL, which encodes the promyelocytic leukemia (PML)/retinoic acid receptor a (RARa) fusion oncogenic protein, is the therapeutic target for ATRA and ATO (3,4). ATRA and ATO act on the PML/RARA fusion protein and reverse the oncogenic protein-induced inhibition of cellular differentiation. The clinical application of ATRA and ATO has turned APL, an AML subtype with very poor prognosis, into a highly curable hematological malignancy. Nevertheless, the success of differentiating agents has not been reproduced in the non-APL AML or other hematological malignancies (1,5). Therefore, there is considerable interest in exploring whether the therapeutic effectiveness of ATO could be extended to non-APL hematological malignancies.

Accumulating evidence supports a potential role for ATO in treating non-APL hematological malignancies. Although ATO alone has demonstrated a less potent efficacy, an objective response has been shown when ATO is combined with other agents in the treatment of non-APL hematological malignancies (5,6). Several clinical trials have shown promising treatment outcomes for AML, myelodysplastic syndromes and multiple myeloma (7–9). In addition to the degradation of the PML/RARA fusion protein, ATO has been demonstrated to induce apoptosis, prevent reactive oxygen species detoxification by inhibiting antioxidant enzymes and inhibit nuclear factor-kappaB (1). Notably, recent research has shed light on the potential ability of ATO to eradicate leukemic stem cells (LSCs) (10,11). In contrast to ATRA treatment, ATO targets APL stem cells partly by inducing the proteasomal degradation of the PML/RARA and PML protein (4). Interestingly, ATO’s ability to eradicate LSCs may extend to non-APL hematological malignancies. In chronic myeloid leukemia (CML), ATO treatment induces the proteasomal degradation of wild-type PML and significantly diminishes the capacity of leukemia cells to reinitiate the disease when transplanted into recipient mice (10). Given the vital role of PML in maintaining stem cell pools and regulating self-renewal in LSCs, it is reasonable to hypothesize that ATO or ATO-based regimens might present a new therapeutic strategy for targeting the ‘stemness’ of non-APL hematological malignancies.

Given the less-effective treatment results achieved with ATO as a single agent in non-APL hematological malignancies, investigators have recently begun to evaluate combined approaches for potentiating the antineoplastic activity of ATO. Synergism has been observed in combination with various agents, such as ATRA, proteasome inhibitors, Hsp90 antagonists and phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway inhibitors (12–14). Although some of these combination strategies follow a rational molecular approach, in most instances, they are relatively empirical. Furthermore, few trials have examined the efficacy of combination regimens for targeting the stemness of non-APL hematological malignancies. Therefore, it would be extremely attractive to explore novel combination approaches to enhance the eradication of LSCs by ATO in non-APL hematological malignancies.

Realgar is a traditional Chinese medicine, and its main component is arsenic disulfide (As2S2), which has effects similar to those of ATO in the treatment of APL with fewer side effects (15). As2S2 requires only oral administration and, thus, overcomes a drawback of ATO, which must be administered by intravenous infusion on a daily basis. An effective orally administered agent would thereby contribute to the quality of life and also provide easy access to a consolidation and maintenance therapy in leukemia therapy. The results from this study demonstrate that the treatment of non-APL leukemia cells with As2S2-induced apoptosis in a dose- and time-dependent manner. Interestingly, an unexpected activation of the AKT/mTOR pathway was detected in As2S2-treated non-APL leukemia cells. The AKT/mTOR pathway is well established as a critical survival signaling pathway that is frequently deregulated in cancer (16,17). Notably, the constitutive hyperactivation of the AKT/mTOR pathway was

Abbreviations: APL, acute promyelocytic leukemia; ATO, arsenic trioxide; ATRA, all-trans retinoic acid; CB, cord blood; CBMC, cord blood mononuclear cell; CML, chronic myeloid leukemia; mTOR, mammalian target of rapamycin; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; PE, phycoerythrin; PML, promyelocytic leukemia; RARa, retinoic acid receptor a; STAT3, signal transducer and activator of transcription 3.

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detected in AML stem cells (18,19). Targeting mTOR, on the other hand, appeared to block the growth of leukemia-initiating cells both in mouse leukemia models and in human AML without apparent harm to normal hematopoietic stem cells (20). Based on these findings, we chose to explore if \( \text{As}_2\text{S}_3 \) in combination with the dual PI3K/mTOR inhibitor propidium iodide-103 could kill non-APL leukemia cells more effectively. Our present findings demonstrate that \( \text{As}_2\text{S}_3 \) plus PI-103 preferentially induced the apoptosis of LSCs in vitro. More significantly, \( \text{As}_2\text{S}_3 \) plus PI-103 led to a loss of the potential of primary leukemia cells to regenerate non-APL leukemia in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice and the eradication of LSCs in non-APL hematological malignancies. The eradication of LSCs is, at least in part, caused by the profoundly enhanced induction of AML blast differentiation both in vitro and in vivo. This study provides proof that highlights the attractive potential of LSC eradication by using a combination of \( \text{As}_2\text{S}_3 \) and PI-103 to treat non-APL hematological malignancies.

Materials and methods

**Drugs**

\( \text{As}_2\text{S}_3 \) (Sigma Chemical Co., St Louis, MO) was dissolved in 0.1 M sodium hydroxide to make a stock solution of 1 mM and stored at 4°C. Stock solutions were diluted in RPMI 1640 medium to achieve final concentrations. PI-103 (Cayman Chemical, Ann Arbor, MI) was prepared as a 1 mM stock solution in dimethyl sulfoxide at −20°C.

**Primary sample collection, isolation and culture**

Bone marrow cells were collected from patients with newly diagnosed AML or CML after obtaining informed consent in accordance with institutional guidelines. Individuals were diagnosed with AML in accordance with the standards of French-American-British classification and were diagnosed with CML based on a morphologic examination, the presence of the Philadelphia chromosome and positive BCR-ABL fusion transcripts. Human cord blood (CB) cells were obtained from full-term deliveries. Samples were subjected to density-gradient separation to isolate mononuclear cells, followed by cryopreservation in fetal calf serum (FCS) plus 10% (vol/vol) dimethyl sulfoxide. As needed, the samples were thawed and used immediately. For in vitro studies, cells were cultured in serum-free medium for 1 h (21) before the addition of \( \text{As}_2\text{S}_3 \) and/or PI-103. When \( \text{As}_2\text{S}_3 \) and PI-103 were used in combination, cells were incubated with PI-103 for 1 h prior the addition of \( \text{As}_2\text{S}_3 \). AML (HL-60, THP-1) and CML (K562) cell lines were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were grown in the recombinant human IL-3 (R&D Systems, Minneapolis, MN) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained in a 37°C atmosphere containing 5% CO₂. The dose–effect curves of the single or combined drug treatment were analyzed by the median-effect method (22) using CalcuSyn Software (Biosoft, Cambridge, UK). Morphology was determined using Wright staining of cells centrifuged onto slides by cytospin (100 g, 4 min; Shandon, Runcorn, UK).

**Cell staining, sorting and flow cytometry**

Cell cycle and apoptosis analyses were performed as described (23,24). Human cells from treated mice or primary specimens were assessed using a mouse antibody specific to human CD34 (anti-CD34) conjugated to phycoerythrin (PE)–cyanine 5, anti-CD38 conjugated to fluorescein isothiocyanate, anti-CD45–PE, anti-p-Akt–PE (BD Biosciences Pharmingen, San Diego, CA), anti-CD45 conjugated to APC, anti-CD19–APC, anti-CD16–APC, anti-CD11b–APC and anti-CD25–APC (Bio Legend, San Diego, CA). Isotypic controls were used to avoid false-positive signals. For sorting CD34⁺CD38⁻ leukemia cells, leukemia blasts were stained with anti-CD34–PE–cyanine 5 and anti-CD38–fluorescein isothiocyanate and then sorted using a Mo-Flo cell sorter (BD Biosciences Pharmingen). The viability and purity of sorted cells exceeded 96%. Leukemia blast cells were stained in accordance with the protocol provided in the Intracellular staining kit (BD Biosciences Pharmingen), and the expression of p-Akt in the Lin−CD34⁺CD38⁻ cell population was determined by flow cytometry.

**NOD/SCID mouse assays**

NOD/SCID mice purchased from the Beijing animal research institute were raised at the specific pathogen free unit of the animal center at Tongji Hospital. The NOD/SCID mice were exposed to a nonlethal dose of radiation (2.0 Gy) 1 day before transplantation. Leukemia or CB cells (10−15 million) were resuspended in 0.2 ml of phosphate-buffered saline (GBICO) supplemented with 2% albumin and transferred into sublethally irradiated hosts via the tail vein (primary transfer). Two weeks after the transplantation of leukemia cells and CB cells, the mice were treated with 5 mg/kg \( \text{As}_2\text{S}_3 \) and/or 5 mg/kg PI-103. Eight weeks after transplantation, the animals were killed, and the bone marrow or peripheral blood was analyzed for the presence of human engram using flow cytomtery. Serial transplantsations (secondary transfer) were also performed after treatment by intravenous injection as described (23).

**Colony formation assay**

Leukemic blast cells or CB cells (1 × 10⁶/ml) were plated in 1.1 ml of 0.8% methylcellulose (M3231; StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with 50 ng/ml Stem Cell Factor, 10 ng/ml interleukin-6 and 10 ng/ml interleukin-3. Cells were plated in 35 mm culture dishes and placed in a humidified box at 37°C with 5% CO₂. Colonies were counted after 14–21 days using an inverted microscope. Each colony count represents the mean colony number of replicates. A colony was defined as a cluster of >40 cells.

**Apoptosis assay**

Annexin V–fluorescein isothiocyanate and PI staining (BD Biosciences Pharmingen) were used to determine apoptosis according to our previously described method (25).

**Western blot**

Cells were treated with the indicated doses of \( \text{As}_2\text{S}_3 \) for the indicated periods of time and subsequently lysed in phosphorylation lysis buffer for protein analysis. Standard western blot analyses were performed using antibodies for phospho-STAT3 (Y705), phospho-AKT (473), phospho-\( \text{S}_6\)K1, phospho-4EBP1 (Cell Signaling Technology, Danvers, MA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were detected using the Pierce (Pierce Biotechnology, Rockford, IL) enhanced chemiluminescence system.

**Statistical analysis**

Assays were set up in triplicate, and the results are expressed as the mean ± SD. The statistical significance of differences between experimental and control groups was determined using one-way analysis of variance followed by the Student–Newman–Keuls test using SPSS software version 13.0 (SPSS, Chicago, IL). Statistical significance was defined as \( P < 0.05 \).

**Results**

**Inducing apoptosis in leukemia cells using \( \text{As}_2\text{S}_3 \)**

To determine the kinetics and dosage range within which \( \text{As}_2\text{S}_3 \) would induce apoptosis in different leukemia cell lines, we treated the HL-60, K562 and THP-1 cell lines with various doses of \( \text{As}_2\text{S}_3 \). As shown in Figure 1A, \( \text{As}_2\text{S}_3 \) induced apoptosis in the three leukemia cell lines in a dose- and time-dependent manner. Apparently, a 24 h exposure to 200–1200 nM \( \text{As}_2\text{S}_3 \) resulted in relatively low levels of apoptosis in these leukemia cells. On the other hand, a 48 h exposure to 200–1200 nM \( \text{As}_2\text{S}_3 \) led to much more significant apoptosis in the same leukemia cells. To understand the potential mechanism underlying \( \text{As}_2\text{S}_3 \)-induced apoptosis, we determined the effects of \( \text{As}_2\text{S}_3 \) on the activation of signal transducer and activator of transcription 3 (STAT3) and AKT because STAT3 and AKT inhibition by ATO has been previously reported (14,26). Initially, we chose 400 nM \( \text{As}_2\text{S}_3 \) to treat leukemia cells and cord blood mononuclear cells (CBMCs) for different time periods. This dosage was based on earlier pharmacokinetic research as well as the fact that this concentration is similar to the serum levels of patients undergoing \( \text{As}_2\text{S}_3 \) treatment (15). STAT3 phosphorylation in the three leukemia cell lines was inhibited 48 or 72 h after treatment with 400 nM \( \text{As}_2\text{S}_3 \) (Figure 1B), which is consistent with previous findings (26). However, the immediate activation of the AKT/mTOR pathway became evident upon \( \text{As}_2\text{S}_3 \) treatment in all three leukemia cell lines examined (Figure 1B). \( \text{As}_2\text{S}_3 \) treatment induced significant phosphorylation of AKT as well as its downstream targets \( \text{S}_6\)K1 and 4EBP1. The activation of the AKT/mTOR pathway lasted up to 48 h before dropping to baseline levels. In the same experiments, the expression of apoptosis-related molecule bcl-2 was also inhibited 48 h after \( \text{As}_2\text{S}_3 \) treatment. However, no changes in the expression levels of p-STAT3, the AKT/mTOR pathway or bcl-2 in CBMCs treated with \( \text{As}_2\text{S}_3 \) were observed. When exposed to 1000 nM \( \text{As}_2\text{S}_3 \), which is much higher than the serum levels of \( \text{As}_2\text{S}_3 \) in patients undergoing \( \text{As}_2\text{S}_3 \) treatment (15), the phosphorylation changes of AKT, bcl-2 and the AKT pathway were similar to an
exposure of 400 nM As$_2$S$_2$ (supplementary Figure S2A is available at Carcinogenesis Online). To determine if activation of the AKT/mTOR pathway at 24 h had a protective effect on leukemia cells, HL-60 cells, K562 cells, THP-1 cells and CBMCs were treated with As$_2$S$_2$ for 24 or 48 h before being subjected to the leukemia colony formation assay. Although exposure to As$_2$S$_2$ for 24 h did not significantly impair leukemia colony formation (control group versus As$_2$S$_2$ treatment group at 24 h, $P = 0.1577$ for K562; $P = 0.2321$ for HL-60; $P = 0.0933$ for THP-1), a 48 h exposure substantially reduced the number of leukemia colonies in all three leukemia cell lines (Figure 1C). In the same study, exposure to As$_2$S$_2$ neither for 24 h nor for 48 h obviously reduces CBMC colonies. Therefore, it seems that the activation of the AKT/mTOR pathway at 24 h had a protective effect on leukemia cells.

**Targeting activation of the AKT/mTOR pathway substantially enhances the As$_2$S$_2$-induced killing of leukemia cells**

To further understand the significance of the As$_2$S$_2$-induced transient activation of the AKT/mTOR pathway, we chose PI-103, a potent dual inhibitor of both PI3 kinase and mTOR, to treat leukemia cells in combination with As$_2$S$_2$. The addition of PI-103 completely abolished the 400 nM As$_2$S$_2$-induced activation of AKT/mTOR in K562 and HL-60 cells (Figure 2A). Interestingly, the bcl-2 protein level was also profoundly reduced by the combination of As$_2$S$_2$ and PI-103 as compared with either As$_2$S$_2$ or PI-103 alone (Figure 2A). When exposed to 1000 nM As$_2$S$_2$, the reduction of AKT and 4EBP1 phosphorylation and expression levels of bcl-2 in HL-60 and K562 cells were similar to an exposure of 400 nM As$_2$S$_2$ (supplementary Figure S2B is available at Carcinogenesis Online). When used as a single agent, either As$_2$S$_2$ or PI-103 could induce the apoptosis of K562 and HL-60 cells. As$_2$S$_2$-induced apoptosis was also significantly enhanced by the addition of PI-103 (Figure 2B, $P < 0.05$, the As$_2$S$_2$ plus PI-103 group compared with the As$_2$S$_2$ group). When treated with the combination of As$_2$S$_2$ and PI-103 for 48 h, the rates of apoptosis in K562 and HL-60 cells were different, which might be due to a cell-specific sensitivity to As$_2$S$_2$ and PI-103. Furthermore, we wonder whether As$_2$S$_2$ and PI-103 exert synergistic rather than additive killing effects on the K562 and HL-60 cell lines. Therefore, the dose–effect curves of the single or combined As$_2$S$_2$ and PI-103 treatments were analyzed by the median-effect method (22), in which the combination indices (CI) of less than, equal to or greater than 1 indicate synergistic, additive or antagonistic effects, respectively. Our data demonstrated that PI-103 and As$_2$S$_2$ concentrations ranging from 400 to 1000 nM resulted in combination indices values $<1$, which indicated synergistic rather than additive effects between As$_2$S$_2$ and PI-103 on K562 and HL-60 cells (Figure 2C). To determine whether the synergy of As$_2$S$_2$ and PI-103 also occurred in primary leukemia cells, we observed the effects of As$_2$S$_2$ and PI-103 on primary leukemia cells and CBMCs.
As$_2$S$_2$ synergizes with PI-103 to preferentially induce LSC apoptosis

To assess the effects of As$_2$S$_2$ on AKT phosphorylation at the LSC level, Lin$^{CD34^+CD38^-}$ cells from cases 1–7 (listed in supplementary Table S1, available at Carcinogenesis Online) of newly diagnosed AML or CML were analyzed initially by multiparameter flow cytometry. Similar to the findings obtained from leukemia cell lines, an immediate phosphorylation of AKT was induced in a Lin$^{CD34^+CD38^-}$ subpopulation of primary leukemia cells 6 h after treatment with As$_2$S$_2$ (the positive rate for p-AKT 473 in LSCs treated with As$_2$S$_2$ versus control: 30.71 ± 10.69% versus 16.15 ± 4.04%, $P < 0.05$; Figure 3A). AKT activation dropped to a baseline level 24 h after treatment with As$_2$S$_2$ (Figure 3A). Parallel experiments with CB showed that As$_2$S$_2$ had no active effect on AKT (Figure 3A). The immediate phosphorylation of AKT 6 h after As$_2$S$_2$ treatment could be abolished by adding PI-103 to all the leukemia samples examined (Figure 3B).

To assess the effects of the single or combined As$_2$S$_2$ and PI-103 treatments on leukemia or normal stem cells, Lin$^{CD34^+CD38^-}$ cells were isolated from the bone marrow of patients with newly diagnosed AML and CML and from healthy CB cells using multiparameter flow cytometry. Cell cycle analyses indicated that the Lin$^{CD34^+CD38^-}$ cells from both the normal and leukemic origins were predominantly in G0/G1 (Figure 4A). Lin$^{CD34^+CD38^-}$ cells from leukemic and normal specimens corresponding to cases 1–4, 6, 8 and 9–12 (listed in supplementary Table S1, available at Carcinogenesis Online) were then cultured for 16 h in 1000 nM As$_2$S$_2$ and/or 1.0 μM PI-103 before being subjected to apoptosis and colony assays. Figure 4B depicts representative data for the apoptosis of Lin$^{CD34^+CD38^-}$ cells from both normal and leukemic origins treated by the single or combined As$_2$S$_2$ and PI-103 treatments. As$_2$S$_2$ treatment alone was moderately toxic to leukemic Lin$^{CD34^+CD38^-}$ cells (mean apoptosis rate: 44.8 ± 8.7%). Treatment with PI-103 alone had only a small effect on cell viability (mean apoptosis rate: 18.7 ± 5.3%). In contrast, the combination of As$_2$S$_2$ and PI-103 synergized to induce robust apoptosis (mean apoptosis rate: 72.9 ± 10.1%). In the same experiments, As$_2$S$_2$, PI-103 or the combined As$_2$S$_2$ and PI-103 treatment
induced a significant apoptotic response in Lin-CD34+CD38- cells from normal specimens (P > 0.05, control group compared with all other treatments, Figure 4B). These results were further supported by quantitative colony formation assays. Although As2S2, PI-103 or the combined As2S2 and PI-103 treatment could reduce colony formation in Lin-CD34+CD38- cells from normal specimens, As2S2 synergized with PI-103 to significantly reduce leukemia colonies (P < 0.05, As2S2 plus PI-103 group compared with all other treatments, Figure 4C).

As2S2 plus PI-103 induces the profound differentiation of leukemia cells

Because a previous study indicated that ATO can reverse the differentiation block of APL (4), we investigated whether this might be part of the mechanism of As2S2 plus PI-103-induced loss of leukemias-forming capacity in vitro. We examined the expression of the CD11b and CD14 cell surface markers after treatment with As2S2, PI-103 or a combination of both agents. CD11b or CD14 expression was induced by As2S2 alone, which indicated that the leukemia cells underwent differentiation in response to the treatment (Figure 5A and B P < 0.05, As2S2 group compared with control group) and that PI-103 treatment alone had no obvious effect, which might have been caused by the low dose of PI-103. The selection of the in vitro dose of PI-103 was based on selecting the smallest dose that could strongly synergize to kill non-APL leukemia cells and promote their differentiation. However, the combined treatment with As2S2 and PI-103 substantially enhanced CD11b or CD14 induction in HL-60 and K562 cells, which suggested that the combined application of the two agents induced a higher level of maturation in these cell lines (P < 0.05 or P < 0.01, As2S2 plus PI-103 group compared with control group).

Notably, induction of CD11b or CD14 appeared to be cell specific in different leukemia cells. For example, As2S2 and PI-103 substantially enhanced CD11b induction in HL-60. However, induction of CD14 expression was not as profound. Nevertheless, either the induction of the granulocytic marker CD11b or monocytic marker CD14 indicated that the leukemia cells underwent differentiation in response to the treatment. We also observed the induction effect of differentiation on primary leukemic specimens treated with As2S2 and/or PI-103. Similar to cell lines, upregulation of the granulocytic and monocytic cell surface markers CD11b and CD14 was also observed in primary blasts from patients with leukemia (Figure 5C). The induction of cell surface markers in treated cells was accompanied by mature morphology changes characteristic of monocyes and granulocytes (i.e. cytoplasm enlargement, loss of cytoplasmic basophilia and increased caryolobism; Figure 5D) as well as the ability to reduce nitroblue tetrazolium (Figure 5E). To understand the potential mechanism underlying As2S2 and/or PI-103-induced differentiation, we sought to determine the effect of the two agents on PML expression. As shown in Figure 5F, the PML expression of HL-60 cells was obviously downregulated after treatment with 400 nM As2S2 for 48 h. However, the addition of 400 nM PI-103 did not further enhance the As2S2-induced degradation of PML protein.

Treatment with As2S2 plus PI-103 leads to the loss of the potential for primary leukemia cells to regenerate leukemia in NOD/SCID mice

Human LSCs in primary leukemia samples were assayed as SCID-leukemia-initiating cells according to their ability to regenerate and maintain a leukemic clone when transplanted into NOD/SCID mice (23). To determine whether the stem cells were impaired after treatment with As2S2 and PI-103, normal and leukemic specimens were
analyzed using transplantation into immunodeficient NOD/SCID mice (Figure 6A). As$_2$S$_2$ and/or PI-103 treatment was started at day 14 after transplant (primary transfer). Eight weeks after transplantation, bone marrow was analyzed for the percentage of donor cells by using a human-specific antibody for CD45. Eight animals were included in each experimental group and treated as indicated. Each data point in Figure 6B represents the percentage of CD45$^+$ cells from one transplanted animal. The data showed that the As$_2$S$_2$ or PI-103 treatment alone moderately impaired the capacity of LSC to proliferate in NOD/SCID mice. The combination treatment with As$_2$S$_2$ and PI-103 impaired this capacity more significantly compared with any other treatment group (P < 0.01, As$_2$S$_2$ plus PI-103 group compared with all other treatments Figure 6B and supplementary Table S2 is available at Carcinogenesis Online). Next, to further determine the potential of established leukemia grafts to regenerate and sustain the leukemia, bone marrow from the experimental groups mentioned above was collected and transplanted into secondary NOD/SCID recipients without any further treatment (secondary transfer). Eight weeks after transplantation, while evidence of leukemia engraftment could be detected in the NOD/SCID recipients treated with either As$_2$S$_2$ or PI-103 alone, CD45$^+$ cells were barely detectable in the marrow of secondary recipients in recipients treated with As$_2$S$_2$ plus PI-103 (Figure 6B and supplementary Table S2 is available at Carcinogenesis Online), which indicated that As$_2$S$_2$ plus PI-103 has a more potent inhibitory effect on the maintenance of human AML in vivo. Considering that the leukemic clone was sustained and regenerated by the AML stem cells, the numbers of SCID–leukemia-initiating cells were determined directly in various experimental groups after the end of the treatment. The proportion of primitive CD34$^+$CD38$^-$/C0 cells within the leukemia graft in the bone marrow was considerably reduced in the As$_2$S$_2$ plus PI-103 treatment group compared with all the other experimental groups (Figure 6C). In contrast, the percentage of CD34$^+$CD38$^-$ cells in CB grafts was not significantly reduced (Figure 6C). Because treatment with As$_2$S$_2$ plus PI-103 induced significant differentiation of human leukemia cells in vitro, we determined whether the induction of differentiation occurred in vivo (Figure 6D). CD11b expression was induced by either As$_2$S$_2$ or PI-103 alone in three primary leukemic specimens, which indicated that the primary leukemia cells underwent differentiation in response to the in vivo treatment. However, the combined treatment of As$_2$S$_2$ and PI-103 substantially enhanced the induction of CD11b, which indicated that combining the two agents induced a much higher percentage of differentiation in vivo.

Fig. 4. As$_2$S$_2$ and PI-103, both singly and in combination, induce apoptosis and inhibit the colony formation of CD34$^+$CD38$^-$ cells from patients with leukemia. (A) Cell cycle profiles for normal and leukemic CD34$^+$CD38$^-$ specimens. G0 (i.e.Ki67–, 2N DNA content), G1 (i.e.Ki67–, 2N DNA content) and S + G2/M (i.e.Ki67+, >2N DNA content). (B) After a 16 h exposure to 1 μM As$_2$S$_2$ and/or 1 μM PI-103, the apoptotic analyses of Lin$^-$CD34$^+$CD38$^-$ cells from patients with leukemic and normal CB were determined by flow cytometry (cases 2–4, 10 and 12 in supplementary Table S1, available at Carcinogenesis Online). Upper panel: representative FACS profiles are shown. Lower panel: numbers indicate the percentage of apoptotic cells. Data are representative of results obtained with three leukemic samples and two normal samples (means ± SDs). 7-AAD, 7-amino-actinomycin D. (C) Lin$^-$CD34$^+$CD38$^-$ cells from patients with leukemic or normal CB were cultured in a methylcellulose culture system with 400 nM As$_2$S$_2$ and/or 400 nM PI-103 (cases 1–4, 6, 8 and 9–12 in supplementary Table S1, available at Carcinogenesis Online). Colony formation was assessed after 14–21 days. Data are representative of results obtained with six leukemic samples and three normal samples (means ± SDs). *P < 0.05; **P < 0.01.
Finally, we assessed the toxicity of the single or combined As$_2$S$_2$ and PI-103 treatment on nonmalignant cells. The combined As$_2$S$_2$ and PI-103 treatment did not lead to a loss of mouse body weight but decreased the weight of the spleen (supplementary Figure S1A and B is available at Carcinogenesis Online). Additionally, either the single or combined As$_2$S$_2$ and PI-103 treatment did not significantly inhibit human CB engraftment in bone marrow or release to peripheral blood, which indicated that normal hematopoietic grafts were unaffected by either the single or combined As$_2$S$_2$ and PI-103 treatments (supplementary Figure S1C is available at Carcinogenesis Online). Notably, liver function, which was defined as the level of transaminases, was not significantly affected by either the single or combined As$_2$S$_2$ and PI-103 treatments (supplementary Figure S1D is available at Carcinogenesis Online).

Discussion

Our experimental results demonstrate that the therapeutic effectiveness of As$_2$S$_2$ could be extended to non-APL AML by combining As$_2$S$_2$ with PI-103. Two unique mechanisms of action underlying this novel combination might contribute to the potent anti-non-APL leu- kemic activity. Firstly, this novel combination achieved LSC eradication with an efficacy far superior to that of As$_2$S$_2$ alone and led to the loss of the capacity of non-APL AML cells to re-establish and sustain the disease when transplanted into secondary recipient mice. Recent evidence indicates that ATO treatment significantly diminishes the capacity of APL and CML cells to reinitiate leukemia by inducing the proteasomal degradation of wild-type PML (10,11). The novel findings that As$_2$S$_2$ effectively targets non-APL AML LSCs alone
and, more significantly, in combination with PI-103 support the hypothesis that non-APL AML could be eradicated at the LSC level by developing ATO/ATO analog-based regimens that focus on targeting certain critical pathways of non-APL hematological malignancies. As$_2$S$_2$ plus PI-103 also effectively induced non-APL AML differentiation, which contributed at least in part to the eradication of LSCs. Previously, although a number of agents, including ATRA, histone deacetylase inhibitors, demethylating agents and low-dose cytotoxic anticancer drugs, have been shown to induce the terminal differentiation of non-APL AML progenitors in vitro, only ATRA or ATO applications have been successful and only in treating APL rather than in non-APL AML. It is not surprising that many differentiation therapies are successful in in vitro cell culture models but fail to produce meaningful benefits in clinical trials. Unlike in vitro cell culture models, both hematopoietic stem cells and LSCs interact with bone marrow niches in vivo to maintain quiescence and avoid exhaustion by differentiation (23). Whether the differentiation induction of As$_2$S$_2$ in combination with PI-103 could be translated into clinical benefits should be determined clinically in myeloid malignancies. Because treatment with As$_2$S$_2$ plus PI-103 not only induced differentiation of non-APL AML in vitro but also re-established differentiation and led to the exhaustion of LSCs in primary non-APL AML cells in vivo the findings presented here are encouraging enough to warrant a clinical evaluation.

In this study, we find that As$_2$S$_2$ exhibited a relatively low antileukemic potency when used at concentrations similar to the serum levels of As$_2$S$_2$ in patients undergoing As$_2$S$_2$ treatment. An unexpected transient activation of the PI3K/AKT/mTOR pathway was induced by As$_2$S$_2$ treatment and acted as a protective response to offset the antileukemic potency of As$_2$S$_2$. Targeting the activation of the AKT/mTOR pathway, on the other hand, substantially enhanced the As$_2$S$_2$-induced killing of leukemia cells. The apoptotic action of ATO has been previously reported to be potentiated by PI3K or AKT inhibitors via glutathione depletion and peroxide accumulation in myeloid leukemia cells (14). In contrast to previous uses of PI3K or AKT inhibitors, PI-103 is a dual PI3K/mTOR inhibitor against PI3K and mTOR complexes 1 and 2 and is, therefore, a more potent inhibitor of the PI3K/AKT/mTOR pathway (27). However, the
selective eradication of LSCs partially via the induction of differentiation by As$_2$S$_3$ plus PI-103 is a novel finding. It is well established that two categories of molecular genetic abnormalities synergistically cause the development of AML: impaired differentiation and enhanced proliferation/survival pathways (2). Therefore, it is reasonable that a combination therapy may inhibit both leukemic cell differentiation and proliferation/survival more effectively than a monotherapy. A key barrier to the clinical success of arsenic is that it is not potent enough to achieve a clinical benefit at physiologically tolerable concentrations by only targeting leukemic cell differentiation (28). Interestingly, some tyrosine kinase inhibitors, such as erlotinib, have recently been shown to induce apoptosis and differentiation in non-APL AML cells and have unexpectedly resulted in the complete remission of a case of non-APL AML (29). In comparison with tyrosine kinase inhibitors, PI3K/AKT/mTOR pathway inhibitors should be of clinical relevance to AML therapy. This pathway is considered a critical survival signaling pathway whose deregulation may contribute to tumorigenesis, metastasis and resistance to chemotherapy (16,17). There is evidence for the constitutive activation of PI3K/AKT/mTOR pathway signaling in AML stem cells (18,19). Further research has indicated that this pathway plays a key role in the maintenance of stem cell self-renewal (30). A separate study demonstrated that inhibition of PI3 kinase reduced the growth of AML stem cells (19). Similarly, inhibition of the downstream PI3 kinase mTOR appeared to block the growth of leukemia-initiating cells in a mouse model of AML without apparent harm to normal hematopoietic stem cells (20). In our present study, the As$_2$S$_3$-induced degradation of wild-type PML was not enhanced by the addition of PI-103, which indicates that the mechanisms of action of As$_2$S$_3$ and PI-103 do not overlap. Rather, the enhanced induction of differentiation and killing of LSCs appears to result from interactions with different focal targets of the LSC maintenance pathways. Notably, the pharmaceutical industry has recently seen a shift from the search for ‘magic bullets’ that specifically target a single disease-causing molecule to the pursuit of combination therapies that comprise more than one active ingredient (31). Our current combination strongly suggests that the attractive potential of As$_2$S$_3$ might be exploited as a new therapeutic strategy for targeting the stemness of non-APL hematological malignancies in combination with certain agents.

The persistence of minimal residual disease and LSCs after initial complete remission in AML is the leading cause of treatment failure (32). The minimal residual disease and LSCs that persist after initial treatment are the most drug-resistant cell populations in the context of AML. Whether As$_2$S$_3$ plus PI-103 or other arsenic-based regimens could be exploited to eradicate LSCs after induction chemotherapy needs to be proven in clinical trials. Given the feasibility of As$_2$S$_3$ and PI3K/AKT/mTOR pathway inhibitors, the potential therapeutic implications of this combination are substantial and attractive in a clinical setting.

Supplementary material

Supplementary Tables S1 and S2 and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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References


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