Invest New Drugs
DOI 10.1007/s10637-009-9267-z

PRECLINICAL STUDIES

MER1, a novel organic arsenic derivative, has potent *PML-RARα*- independent cytotoxic activity against leukemia cells

Mirna Golemovic · Alfonso Quintás-Cardama · Taghi Manshouri · Nada Orsolic · Hatice Duzkale · Mary Johansen · Emil J. Freireich · Hagop Kantarjian · Ralph A. Zingaro · Srdan Verstovsek

Received: 10 April 2009 / Accepted: 8 May 2009

Summary Arsenic trioxide (ATO) is an inorganic arsenic derivative that is highly effective against *PML-RARα*-positive leukemia but much less against other hematological malignancies. We synthesized an organic arsenic derivative (OAD), S-dimethylarsino-thiosuccinic acid (MER1), which offers a superior toxicity profile and comparable *in vitro* activity relative to ATO. In Swiss Webster mice, maximally-tolerated cumulative dose of MER1 when given IV for 5 days was 100 mg/kg/d. We demonstrated that MER1 induced apoptosis and dose- and time-dependent inhibition of survival and growth in a panel of myeloid leukemia cell lines. Unlike ATO, this activity was independent of *PML-RARα* status and was not associated with induction of myeloid maturation. In NB4 and HL60 cells, MER1 and ATO induced caspase activation and dissipation of mitochondrial transmembrane potential. At the same time, MER1 induced generation of reactive oxygen species (ROS) and cell cycle arrest in G2/M phase and proved to be more potent than ATO at inducing apoptosis. ROS generation and intracellular glutathione levels were key modulators of MER1-induced cytotoxicity as evidenced by abrogation of apoptosis in myeloid leukemia cell lines pretreated with the disulfide bond-reducing agent dithiothreitol or the radical scavenger N-acetyl-L-cysteine. Collectively, these data indicate that MER1 induces apoptosis in *PML-RARα*-positive and -negative myeloid leukemia cells by enhancing oxidative stress. This agent, therefore, combines low *in vivo* toxicity with formidable *in vitro* pro-apoptotic ROS-mediated activity, and may represent a novel OAD suitable for clinical development against a variety of hematological malignancies.

Keywords MER1 · Organic arsenic derivatives · *PML-RARα* · Acute myeloid leukemia

Electronic supplementary material The online version of this article (doi:10.1007/s10637-009-9267-z) contains supplementary material, which is available to authorized users.

M. Golemovic · A. Quintás-Cardama · T. Manshouri · N. Orsolic · H. Duzkale · E. J. Freireich · H. Kantarjian · S. Verstovsek (✉)
Department of Leukemia,
The University of Texas M. D. Anderson Cancer Center,
Unit 428, 1515 Holcombe Blvd.,
Houston, TX 77030, USA
e-mail: sverstov@mdanderson.org

M. Johansen
Department of Experimental Therapeutics,
The University of Texas M. D. Anderson Cancer Center,
Houston, TX, USA

R. A. Zingaro
Department of Chemistry, Texas A&M University,
College Station, TX, USA

Introduction

The vast majority of acute promyelocytic leukemia (APL) cases are associated with the t(15;17) translocation that generates a fusion transcript PML-RARα [1, 2]. PML-RARα oncoprotein arrests maturation at the promyelocytic stage of myeloid development. The inorganic arsenic derivative arsenic trioxide (As_2O_3; ATO) induces apoptosis and partial differentiation of APL cells [3–5]. Down-regulation of the PML-RARα oncoprotein by ATO overcomes the maturation blockade [3]. ATO induces apoptosis in other cancer cell types through a variety of mechanisms, such as inhibition of nuclear factor-κB (NFκB), caspase activation [6] and downregulation of human telomerase *hTERT* transcription [7]. Upon administration, ATO is

Published online: 26 May 2009

biotransformed by methylation reactions, rendering organic arsenic derivatives (OADs) that are less toxic and are readily excreted in the urine [8]. For example, dimethylarsinic acid, the metabolic by-product of ATO, is 50 times less toxic to mice than ATO, but is also significantly less potent as an anti-leukemic agent [9]. Thus, research efforts have been directed towards the synthesis of OADs that optimize arsenic's potential for anticancer activity while reducing its toxicity profile [10]. To this end, we synthesized a novel OAD, S-dimethylarsino-thiosuccinic acid (MER1) that offers a superior toxicity profile *in vivo* and comparable *in vitro* activity relative to ATO [11], as presented in this report.

Mitochondrial transmembrane potential ($\Delta\psi_m$) disruption, activation of caspase-8 and -9, and disruption of the intracellular redox equilibrium are the most commonly observed events relating to ATO-induced apoptosis[5, 6]. Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen [12]. Although it is known that ROS generation is involved in arsenic-induced apoptosis of tumor cells, its precise mechanism of action remains to be elucidated. Preliminary data suggest that ATO induces apoptosis upon decreased levels of reduced glutathione and increased levels of ROS [12]. These effects vary across cell lines owing to their different inherent levels of ROS, which are modulated by the cellular antioxidant enzymatic system [13, 14]. In this study we used two leukemic cell lines, HL60 and NB4, as the main experimental model because they differ in *PML-RARα* expression and activity of the enzymes involved in the maintenance of ROS equilibrium.

Materials and methods

Cells, animals, and reagents

Cell lines included: OCI/AML-3 (acute myeloid leukemia), K562, KBM5 and KBM7 (chronic myeloid leukemia), NB4 (APL), HL60 (acute myelomonocytic leukemia), CAG (multiple myeloma), Jurkat and CEM-1301 (T cell acute lymphoblastic leukemia), Z119 (B cell acute lymphoblastic leukemia), Raji (B cell lymphoma), and U937/PR9. The U937/PR9 acute myeloid leukemia cells express *PML-RARα* in a Zn^{2+}-inducible manner [15]. The OCI/AML3, KBM7, KBM5, CAG, and K562 cells were cultured in Iscove's modified Dulbecco's medium (IMDM). HL60, NB4, U937/PR9, Jurkat, Raji, Z119, and CEM-1301 cells were cultured in RPMI1640 medium. Both mediums were supplemented with 10% fetal bovine serum (FBS, Invitrogen; Carlsbad, CA) and penicillin-streptomycin solution (100 U/mL) (Gemini Bio-Products; Woodland, CA).

ATO (99% arsenous acid) was purchased from Sigma-Aldrich (A1010, St. Louis, MO) and 5 mM stock solution was prepared by dissolving ATO in 100 μl 1 M NaOH before adding water and adjusting to pH 7.0 by addition of 1 M HCl. MER1 was provided by Professor Ralph A. Zingaro (Texas A&M University; College Station, TX). MER1 is water soluble, and was dissolved in saline before its use. N-acetyl-L-cysteine (NAC), L-buthionine-[S,R]-sulfoximine (L-BSO), and dithiothreitol (DTT) were purchased from Sigma Chemical Co. Toxicity testing of MER1 was carried out in 11-week old Swiss Webster mice (Taconic Laboratory Animals and Services: Germantown, NY).

Cytotoxicity and cell proliferation assays

To assess the cytotoxicity induced through escalating concentrations of ATO or MER1, different cell lines were seeded (5×10^4 cells/mL) in 24-well plates and cultured for 72 h. The number of living cells was scored daily by Trypan blue exclusion test.

Cell growth was assessed by measuring the number of living cells using MTS assay (CellTiter 96® Aqueous One Solution Reagent, Promega Corporation; Madison, WI) as previously described [16]. Results were used to calculate the drug concentration resulting in 50% inhibition of cell proliferation (IC_{50}).

Differentiation analysis

The NB4 APL cells were used to evaluate the effect of ATO and MER1 on maturation. The anti-CD11b-PE monoclonal antibody (BD Pharmingen; San Diego, CA) was used to detect CD11b, an early-expressed myeloid maturation marker. After 72 h of incubation with drugs, cells were washed in PBS and then incubated with monoclonal antibody (dilution 1:10). An isotypic control was prepared in the same manner. Cells were analyzed by flow cytometry (FACScan, Becton Dickinson; San Jose, CA).

Morphology and granulocytic differentiation of NB4 were determined using Diff-Quick® stain set (Baxter Healthcare; Miami, FL) assay. Cells were centrifuged onto slides using a Cytospin® (Shandon; Runcorn, UK. Nitro-blue tetrazolium (NBT; Sigma) reduction was assayed colorimetrically as previously reported [17].

Evaluation of apoptosis and caspase activity

Following treatment with MER1 or ATO for various lengths of time, cells were washed in Ca^{2+}-free PBS and stained with Annexin V-FITC following manufacturer's recommendations (Trevigene; Gaithersburg, MD). PI was added to exclude necrotic cells during analysis. Binding of annexin V to apoptotic cells was analyzed using the FACScan flow cytometer.

To detect and measure caspase 3 and caspase 3-like activities in viable cells, drug treated cells were washed and resuspended in fluorogenic substrate PhiPhiLux G1D2 (OncoImmunin,Inc; Gaithersburg, MD). Following 1 h of incubation at 37°C in the dark, cells were washed and resuspended in PBS. PI was added to exclude necrotic cells during analysis. Prepared cell samples were analyzed by flow cytometry.

Inner transmembrane potential dissipation ($\Delta\varphi_m$)

Upon treatment with arsenic compounds for different periods of time, cells were incubated with submicromolar concentrations of MitoTracker Red CMXRos and MitoTracker Green FM (Molecular Probes; Eugene, OR) in order to evaluate $\Delta\varphi_m$ changes by flow cytometry as previously described [16].

Detection of intracellular hydrogen peroxide production

The intracellular H_2O_2 level was detected by preincubating cells with 10 μM 5,6-carboxy-2′,7′,-dichlorofluorescein-diacetate (CM-H_2DCFDA) (Molecular Probes) for 20 min at 37°C, followed by the addition of MER1 or ATO. At the end of the incubation period, cells were washed in 2 mL of Ca^{2+}-free PBS, resuspended in 0.5 mL of Ca^{2+}-free PBS, and then fluorescence intensity was measured (FACScan).

Effect of MER1 and ATO on intracellular GSH

The *in vitro* growth inhibition effect of MER1 and ATO with or without the addition of BSO or DTT (both from Sigma Aldrich Inc.) was determined on NB4 cells by MTS assay (Promega). After 24 h of preincubation with or without the addition of 1 mM L-BSO or 1 mM DTT, cells were seeded in triplicate in 96-well microtiter plates (Falcon; USA) at a density of 2×10^4 cells/well. MER1 or ATO were added at different concentrations. Following 72 h of incubation, MTS assay was performed as previously described.

Cell cycle analysis

Following treatment with MER1 or ATO, cells were collected, washed in Ca^{2+}-free PBS, and fixed overnight in 70% cold ethanol at −20°C. Next, cells were washed twice in cold PBS and resuspended in hypotonic PI solution for 15 min in the dark at room temperature. Cell cycle contents and subG1 were determined by flow cytometry. Collected data were analyzed using ModFit software (Becton Dickinson).

Western blotting analysis

Following incubation with different concentrations of MER1 or ATO for different time periods Western blot analysis was performed as previously described [16] using the following primary antibodies: anti-β-actin (Sigma Chemical), anti-Bcl2 (Upstate Biotechnology; Waltham, MA), anti-Bcl-x_L, anti-Bax (Santa Cruz Biotechnology; Santa Cruz, CA), anti-caspase-9 and anti-PARP (Cell Signaling, Beverly, MA), and polyclonal anti-PML (H-238; Santa Cruz, CA). Detection of PML-RARα proteins in NB4 cells included immunoprecipitation techniques.

Telomere length measurement

Telomere length was measured by fluorescence *in situ* hybridization and flow cytometry. The cells were cultured over 1 month period of time in the presence of gradually increased concentrations of ATO or MER1. The experiments were performed at the point when the cells achieved stable viability in the presence of 1 μM of drug. DAKO telomere peptide nucleic acid (PNA) kit/fluorescein isothiocyanate (FITC) for flow cytometry (DAKO Cytomation, Forth Collins, CO) was used to determine relative telomere length in NB4 cells. CEM-1301 cell line was used as an internal control (it is tetraploid and has very long telomeres [>30 kb] that allow easy identification of DNA content and telomere signals in flow cytometric dot plots) [18]. Samples were prepared according to the manufacturer's instructions. Analysis was performed on FACScan flow cytometer equipped with CellQuest Pro software. Relative telomere lengths (RTLs) were calculated according to the manufacturer's recommendation.

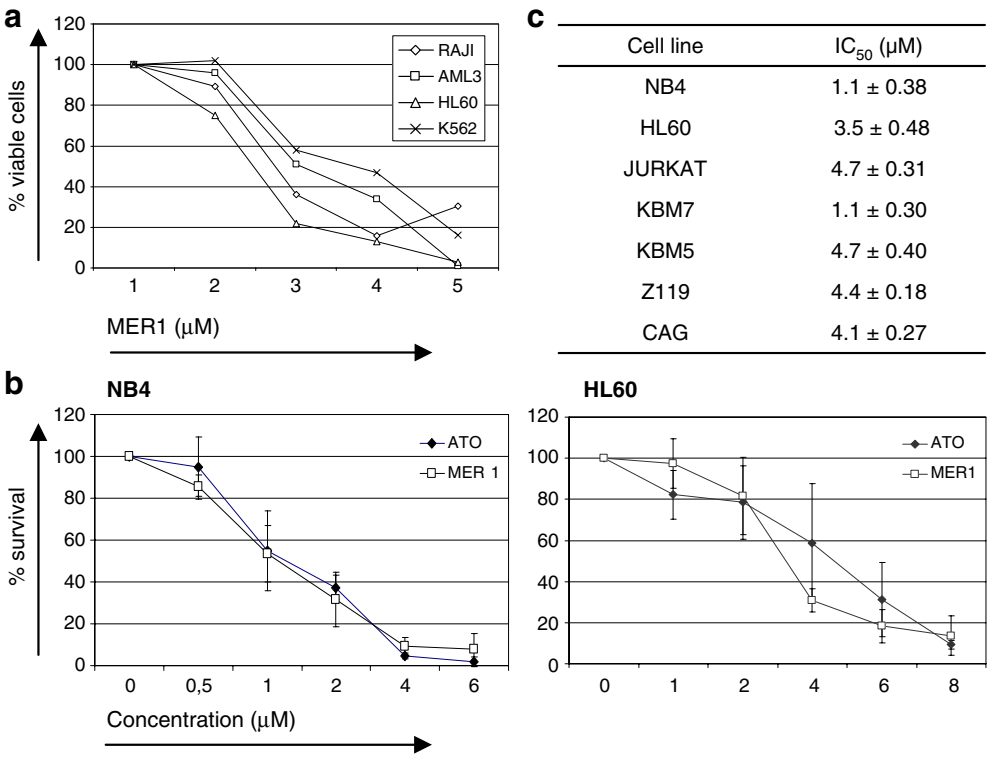
Results

MER1 induces growth inhibition in a variety of cell lines

MER1 was initially proven to have *in vitro* activity at low micromolar concentrations against a panel of 60 cell lines in experiments conducted at the National Cancer Institute (data not shown). As shown by Trypan-blue die exclusion assay (Fig. 1a) and 72-h MTS assay (Fig. 1b), MER1 demonstrated significant activity against a panel of leukemia cell lines. The IC_{50} values for MER1 ranged between 1.1 μM and 4.7 μM (Fig. 1c).

Toxicity studies

Swiss Webster mice were treated with MER1 by tail vein injection on a 10 mg-dose escalating schedule with doses ranging from 0 mg/kg/d to 150 mg/kg/d for 5 consecutive days, in groups of 6 (doses up to 100 mg) or 10 (doses above 100 mg) mice. No deaths were observed at MER1 doses up to 100 mg/kg/d×5 days. The lethal dose at which half the mice in a group died (LD_{50}) was reached at



c

Cell line	IC_{50} (μM)
NB4	1.1 ± 0.38
HL60	3.5 ± 0.48
JURKAT	4.7 ± 0.31
KBM7	1.1 ± 0.30
KBM5	4.7 ± 0.40
Z119	4.4 ± 0.18
CAG	4.1 ± 0.27

Fig. 1 Activity of MER1 against different cell lines. **a** Cell viability after MER1 treatment in 72-h Trypan blue assay. Representative data is shown. **b** Growth inhibition by MER1 of NB4 and HL60 cells in 72-h MTS assay (mean±SD from three independent experiments). **c** Summary of the MER1-induced cell growth inhibition in 72-h MTS assay, against a variety of cell lines

110 mg/kg/d for 5 days (total cumulative dose 550 mg/kg). The maximal tolerated dose (MTD) was established at 100 mg/kg/d for 5 days (total cumulative dose 500 mg/kg). Of note, the MTD of ATO is 5 mg/kg/d IV for 5 days [19] underscoring the high tolerability of MER1 *in vivo*.

MER1 inhibits cell growth independent of PML-RARα expression

U937/PR9 cell line expresses *PML-RARα* in a Zn^{2+} inducible manner. Upon treatment with 0.1 M $ZnSO_4$, *PML-RARα* expression was established in U937/PR9 cells and remained stable for 48 h. *PML-RARα* expression was confirmed by TaqMan PCR and compared to untreated U937/PR9 and NB4 cells (Fig. 2a). The mean increase in the level of *PML-RARα* mRNA in U937/PR9 cells treated with $ZnSO_4$ was 548-fold higher than in untreated U937/PR9 cells, and 26-fold higher than in NB4 cells. $ZnSO_4$—treated and—untreated cells were incubated with increasing concentrations of ATO or MER1 for the next 48 h. Treatment with MER1 equally decreased the proliferation of U937/PR9 cells regardless of their *PML-RARα* expression status (Fig. 2b). MER1 IC_{50} values ranged between 2 μM and 4 μM. As expected, ATO efficiently killed *PML-RARα*-positive cells (IC_{50} 4 μM). Further support of the *PML-RARα*-independent mechanism of action of MER1 was provided by Western blot experiments in which ATO induced degradation of PML-RARα protein in treated NB4 cells, whereas MER1 did not induce degradation (Fig. 2c).

MER1 does not induce cell differentiation in leukemic cells

A significant increase of CD11b expression was seen after treatment with low-dose ATO but not after MER1 treatment (Fig. 2d, left panel) in NB4 cells. The same results obtained by colorimetric NBT assay additionally confirmed these findings (Fig. 2d, right panel).

MER1 induces apoptosis in HL60 cells in a time- and dose-dependent manner

Cells were cultured in the presence of 2 μM and 4 μM of each compound. Apoptosis induction was assessed by flow cytometry by using three markers of apoptosis: compromised cell membrane integrity (Fig. 3a), caspase activation (Fig. 3b), and dissipation of mitochondrial transmembrane potential (Fig. 3c). By all three measures, the proportion of apoptotic HL60 cells was significantly higher after treatment with MER1 than with ATO. Apoptotic changes induced by the treatment with either agent were both concentration and time dependent.

MER1 induces reactive oxygen species production and activates caspase-9

To investigate ROS production in response to MER1 and ATO, we used a CM-H_2DCFDA probe to measure H_2O_2 content in HL60 cells. When HL60 cells were treated with MER1 for 2 h, the production of H_2O_2 relative to untreated

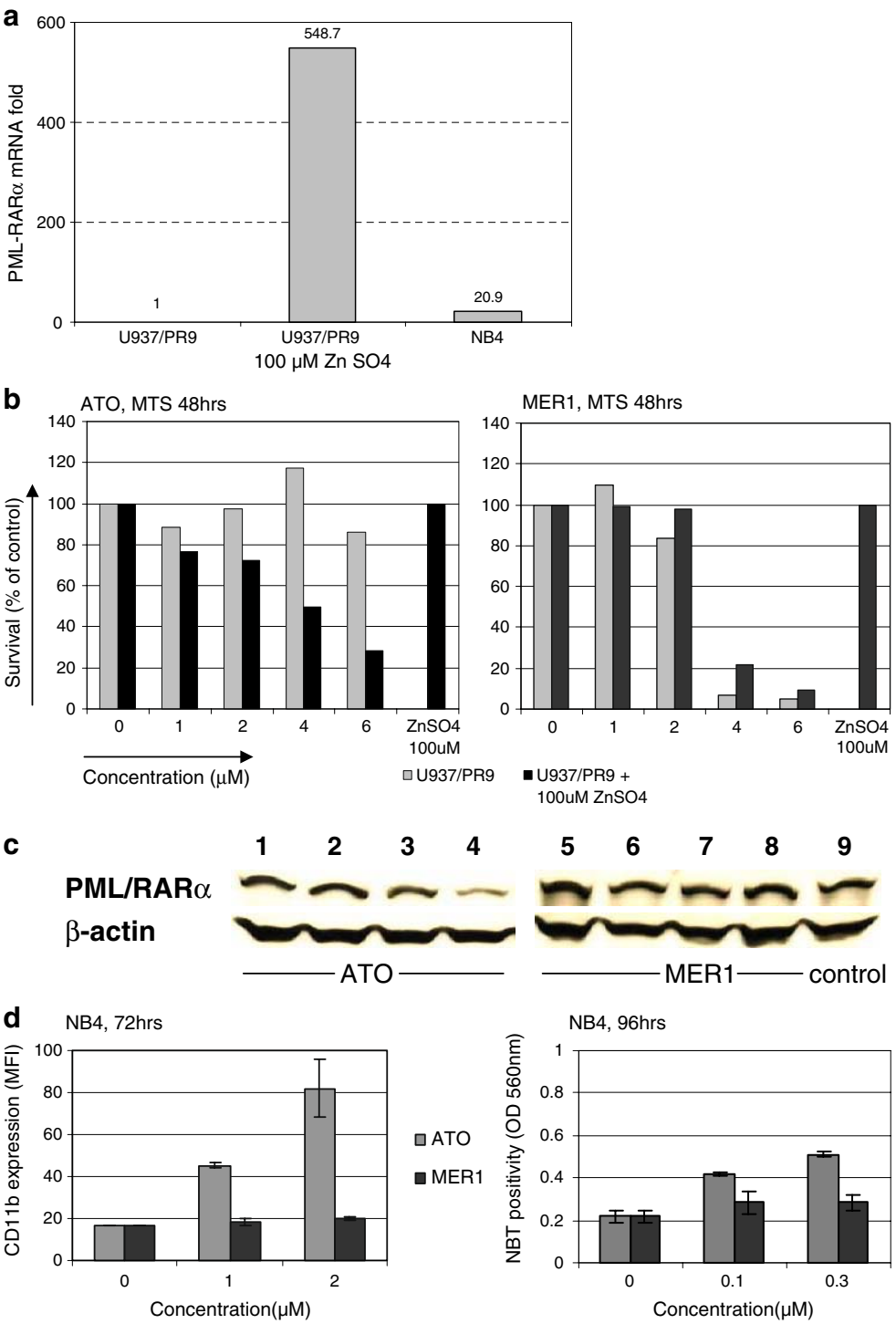


Fig. 2 The antileukemic effect of MER1 is PML-RARα-independent. **a** After incubation with 0.1 M ZnSO4 for 3 h, PML-RARα expression was established in U937/PR9 cells. Stable PML-RARα expression was confirmed by TaqMan PCR and compared to that of NB4 and U937/PR9 cells not treated with $ZnSO_4$. **b** Following $ZnSO_4$-induced PML-RARα expression, U937/PR9 cells were treated with ATO or MER1 for 48 h and their sensitivity to both compounds was tested by MTS assay. Representative experimental data out of 3 is presented. **c** Immunoprecipitation analysis of PML-RARα expression in NB4 cells treated with ATO (lanes 1–4) or MER1 (lanes 5–8) at 0.5 μM, 1 μM, 3 μM, or 6 μM. Lane 9 represents untreated NB4 cells. (D) MER1 does not induce maturation of NB4 cells. On the *left* panel, NB4 cells were treated with ATO or MER1 at 0 μM, 1.0 μM, 2.0 μM for 72 h and the percentage of CD11b-positive NB4 cells was measured by flow cytometry. On the *right* panel, the nitroblue tetrazolium (NBT) reduction assay was performed on NB4 cells treated for 96 h with 0 μM, 0.1 μM, and 0.3 μM of ATO or MER1

control cells was significantly higher (176%) than in those treated with ATO (65%; Fig. 4a). Moreover, after 4 h of ATO treatment, H_2O_2 production tended to diminish significantly, while in MER1-treated cells, H_2O_2 production persisted even after 8 h of treatment, albeit at lower levels (Fig. 4b). The MER1-induced production of O_2^- revealed same kinetics (data not shown) and confirmed H_2O_2 results.

To demonstrate the role of apoptotic pathways in MER1-induced cell death, we cultured HL60 cells with 0 μM, 0.5 μM, 1.0 μM, 5.0 μM, and 10.0 μM of MER1. Cell lysates were then probed with pro-caspase-9 antibody. Western blot analysis after 24 h of treatment revealed cleavage of pro-caspase-9, evidenced by detection of increasing levels of the p35 fragment. Elevated levels of p35 suggest involvement of the apoptotic protease-

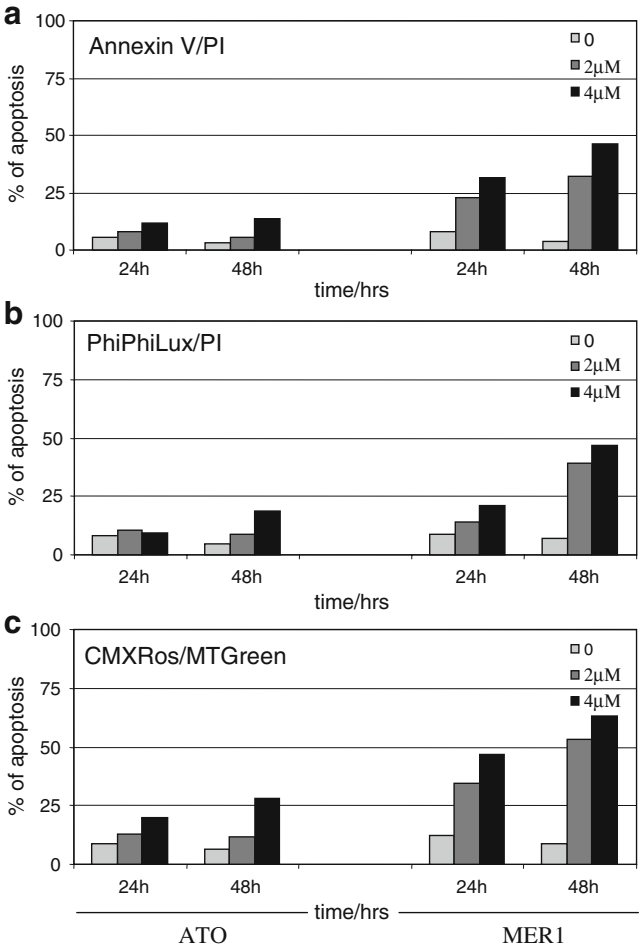


Fig. 3 MER1 induces apoptosis of HL60 cells in a dose- and time-dependent manner. HL60 cells were treated with ATO or MER1 (2 μM or 4 μM) for 24 h and 48 h and apoptosis was evaluated by flow cytometry. Different aspects of the apoptotic process were analyzed. **a** Compromised cell membrane integrity (Annexin V/PI staining). **b** Caspase activation (PhiPhiLux/PI assay). **c** Dissipation of mitochondrial transmembrane potential (CMXRos/MTGreen staining). Representative data is shown, out of two independent experiments

activating factor 1 (Apaf-1) and general mitochondrial involvement in apoptosis induction, whereas the presence of the p37-cleaved fragment indicates subsequent activation of caspase-9 (Fig. 4c). We did not observe any changes in Bcl-2 levels or the Bcl-2-related proteins Bcl-XL and Bax in HL60 cells treated with increasing concentrations of MER1. In keeping with prior reports [20, 21], similar (no effect) results were observed when HL60 cells were treated with ATO at the same concentration range (data not shown). Despite these findings, observed cleavage of PARP (Fig. 4d) confirmed that MER1 did induce apoptosis in treated HL60 cell samples. The treatment with ATO at 5 μM or 10 μM yielded PARP cleavage, manifested as an approximately 89-kDa fragment (Fig. 4d). Previous findings [19] suggested that downregulation of the anti-apoptotic protein Bcl-2 was an important contributor to ATO-induced apoptosis in NB4 cells. We have demonstrated ATO induced degradation of Bcl-2 protein in treated cells while MER1 treatment did not induce the same effect in NB4 cells (Suppl. Fig. 1).

MER1 induces G2/M arrest in NB4 and HL60 cells

We treated NB4 (Fig. 5a) and HL60 (Fig. 5b) cells with both drugs and analyzed the percentage of cells in G2/M cell cycle arrest by flow cytometry using PI staining. NB4 cells were treated with MER1 or ATO at 1 μM, while HL60 cells were treated with both agents at 5 μM; amounts were based on *in vitro* sensitivities of the cell lines to these drugs (Fig. 1). After 12 h of MER1 treatment, 55% of NB4 cells were in G2/M phase in contrast to only 14% of control cells and 19% of ATO-treated cells (Fig. 5a). These proportions decreased slightly after 24 h of treatment, when 36% of MER1-treated cells were in G2/M, compared with 13% of untreated control cells and 11% of ATO-treated cells, which is likely a consequence of the increasing fraction of apoptotic cells and dead cells (data not shown). When HL60 cells were treated with 5 μM MER1, within 12 h 42% of cells transitioned to G2/M phase, in contrast with only 20% of cells treated with 5 μM ATO and 12% of untreated cells. After 24 h of treatment these differences were more extreme: 75%, 17%, and 12%, respectively (Fig. 5b). These findings were further confirmed on treated HL60 cells by BrdU incorporation assay (data not shown).

NAC inhibits MER1-induced apoptosis and G2/M cell cycle arrest in HL60 cells

To support the theory that ROS are important mediators in MER1-induced apoptosis, we added 10 mM NAC (provides cellular reduction) to HL60 cells that had been treated with 5 μM ATO. Results indicated that NAC did inhibit ATO-induced apoptosis (data not shown). Next, we incubated HL60 cells with or without the addition of 10 mM NAC in combination with 5 μM MER1 (Fig. 5c). Co-treatment with NAC blocked MER1-induced apoptosis and G2/M arrest in treated cells at each time point (Fig. 5c), whereas treatment with 5 μM MER1 alone induced apoptosis and G2/M cell cycle arrest in a time-dependent fashion (Suppl. Fig. 2). These data imply a level of importance of cellular target proteins with thiol groups in MER1 induction of cell cycle disturbance and apoptosis.

Changes in cellular GSH levels correlate with response of NB4 to MER1

To determine whether changes in intracellular GSH content modulate cell sensitivity to MER1, we pretreated NB4 cells

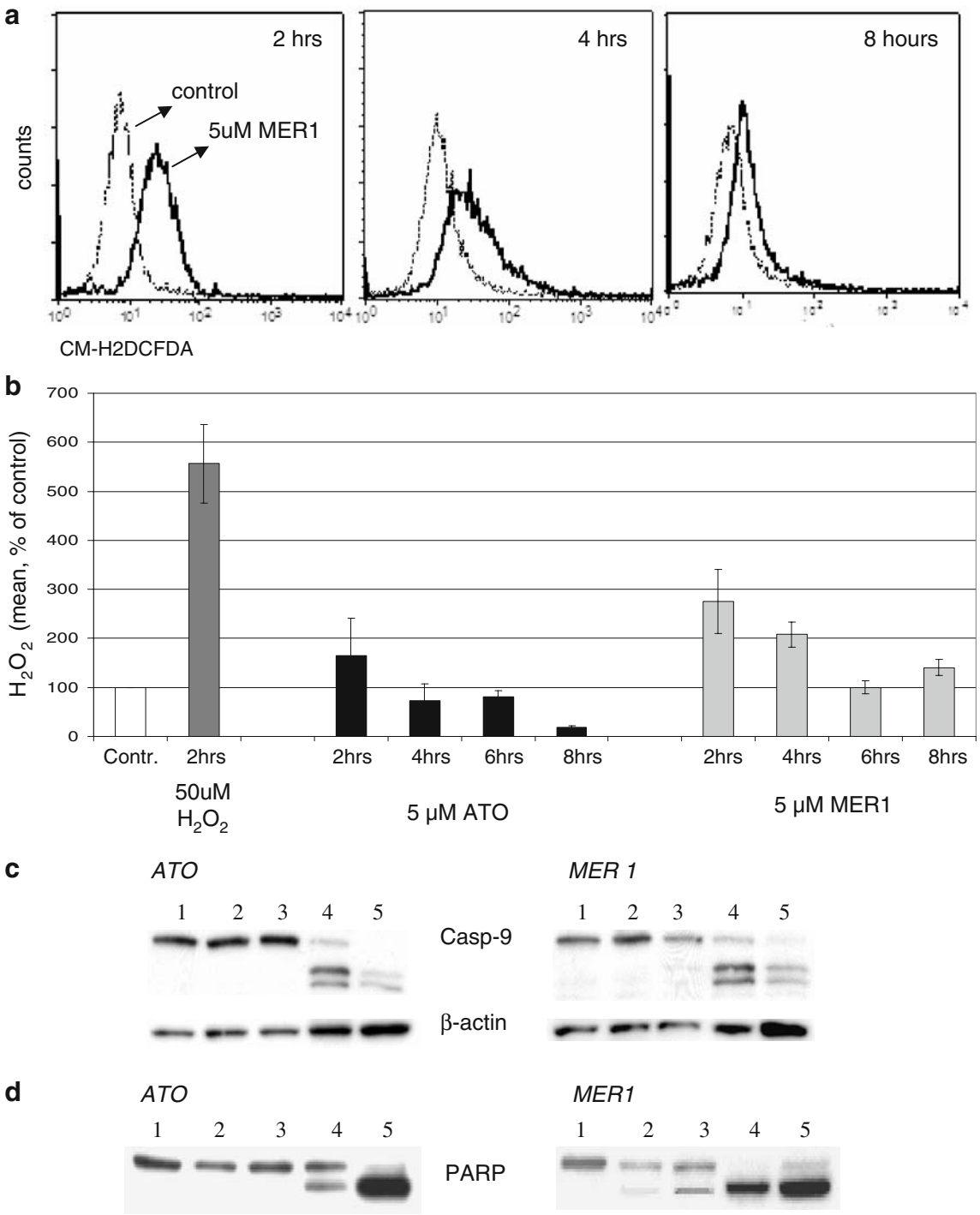


Fig. 4 MER1 treatment results in ROS production and cleavage of pro-caspase-8, -9, and PARP. HL60 cells were treated with MER1 (5 μM) or ATO (5 μM) for 2 h, 4 h, or 8 h. Hydrogen peroxide production was assessed using the CM-H_2DCFDA probe (**a**). Each experimental point represents the mean ±SD from three independent experiments (**b**). **c**–**d** HL60 cells were treated for 24 h with MER1 or ATO at 0 μM, 0.5 μM, 1.0 μM, 5.0 μM, 10.0 μM, respectively (lanes 1–5 for each drug). Whole cell lysates were used for evaluation of expression of selected proteins by Western blotting. **c** Detection of full-length pro-caspase-9 (47 kDa) and cleaved 37 kDa and 35 kDa fragments after treatment with MER1 or ATO. **d** Both MER1 and ATO induced PARP cleavage in HL60 cells, which resulted in 89 kDa fragments

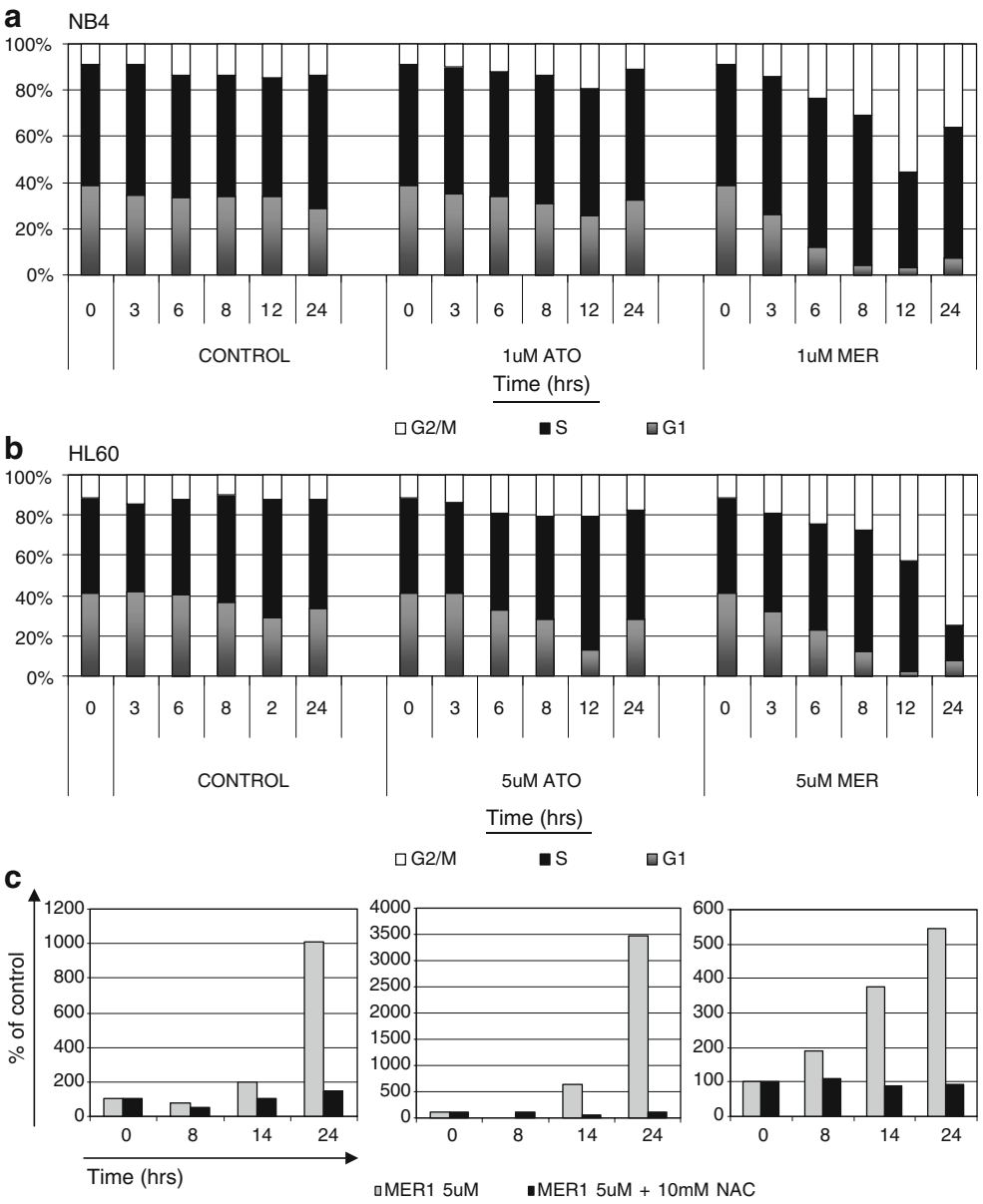


Fig. 5 MER1-induced cell cycle arrest in G2/M phase happens prior to induction of apoptosis and is modulated by intracellular GSH levels. Cell cycle analysis after treatment with MER1 of NB4 (**a**) and HL60 (**b**) cells showed arrest in G2/M phase in both cell lines. (**c**) HL60 cells were treated with MER1 (5 μM) alone or in combination with NAC (10 mM) for 8 h, 14 h, or 24 h. The percentage of apoptotic cells (Annexin-V positive cells or in sub-G_1 phase) and cells in G2/M phase were detected by flow cytometry

for 24 h with 1 mM DTT or 1 mM BSO and incubated them for 72 h with increasing concentrations of MER1 or ATO. DTT is a disulfide bond-reducing agent and BSO is a selective inhibitor of γ-glutamyl cysteine synthetase [22], which is required for GSH biosynthesis. As shown in Fig. 6a, inhibition of ATO-mediated NB4 cell proliferation was significantly enhanced after the addition of BSO, whereas DTT treatment significantly antagonized ATO-mediated NB4 cell proliferation inhibition. BSO-induced GSH depletion resulted in enhancement of sensitivity of NB4 cells to MER1 when compared to cells treated only with MER1 (Fig. 6b). Moreover, treatment with the thiol donor DTT markedly protected NB4 cells from MER1-mediated antiproliferative effects.

MER1-induced NB4 apoptosis is not mediated by telomere shortening

Downregulation of human telomerase *hTERT* transcription has been directly linked to the activity of ATO against the APL cell line NB4[7]. Accordingly, we evaluated the telomere length in NB4 cells by cytofluorimetric analysis to ascertain whether telomere shortening also contributed to the activity of MER1 against NB4 cells. The values obtained from the telomere-specific FITC-conjugated PNA probe were standardized with those of the internal control of the analysis, represented by the CEM-1301 leukemic cell line, which is characterized by a stable telomere length. In this way, RTL was calculated and expressed as a percentage

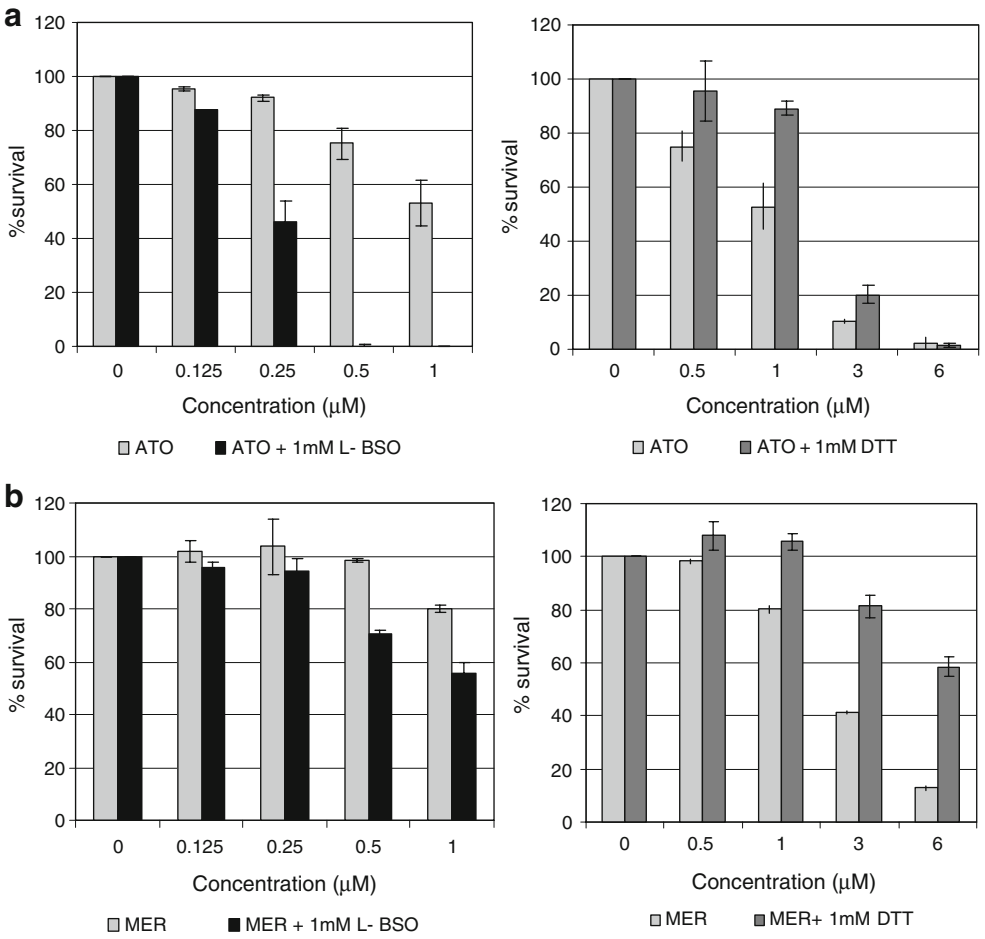


Fig. 6 Effect of intracellular GSH levels on MER1 antileukemic action. NB4 cells were pretreated with L-BSO 1 mM (**b**) or DTT 1 mM (**c**) for 24 h. Then, NB4 cells were cultured in the presence of increasing concentrations of ATO (**a**) or MER1 (**b**) for 72 h. Cell growth inhibition was assessed by MTS assay. Results are expressed as percentage of maximal proliferation (control cells cultured in the absence of MER1). Each experimental point represents the mean ± SD from three independent experiments

of the length of the CEM-1301 cells. The mean RTL in parental NB4 cells was 21% (Suppl. Fig. 3). Long-term exposure to gradually increased concentration of ATO (until reaching 1 μM) resulted in a 25% shortening of telomeres (RTL=16%) relative to results obtained with parental NB4 cells. Notably, no significant telomere shortening was observed when NB4 cells were cultured in the presence of MER1 at the same concentration (RTL=20%).

Discussion

In this study, we showed that the novel OAD MER1 induced time- and dose-dependent growth arrest and apoptosis in leukemic cells independent from *PML-RARα* status. Immunoprecipitation data obtained from NB4 *PML-RARα*-expressing leukemic cells treated with MER1 or ATO demonstrated that the latter, as expected, induced PML-RARα protein degradation, while MER1-mediated cytotoxic effects did not correlate with decreased levels of PML-RARα. Furthermore, unlike ATO, MER1 did not induce differentiation in treated NB4 cells. Final confirmation that MER1-induced cytotoxicity is *PML-RARα*-independent was provided by experiments performed using the U937/PR9 cell line. Collectively, these findings are in accord with those previously reported by Chen et al. [8] that showed methylated trivalent metabolites of arsenic induced *PML-RARα*-independent cytotoxic effects that were not followed by induction of differentiation in treated cells.

The differences between the mechanisms of action of MER1 and ATO on *PML-RARα*-expressing leukemic cells prompted us to evaluate the impact of MER1 at different cellular levels in leukemic cells not carrying *PML-RARα*. Treatment with MER1 induced apoptosis in HL60 cells in a dose- and time-dependent manner, and was associated with $\Delta\varphi_m$ dissipation and caspase activation. The level of apoptosis was higher in cells treated with MER1 than in those cultured with similar concentrations of ATO. Like ATO, treatment with MER1 did not downregulate Bcl-2 family proteins in HL60 cells (data not shown). However, in NB4 cells ATO did downregulate Bcl-2 family proteins, as previously described [4], while MER1 did not (indicating again its *PML-RARα*-independent mode of action).

Molecular events that occur during apoptosis upon cellular exposure to arsenic compounds are not entirely

understood; however two main apoptotic pathways have been described. In one, Fas/APO-1/CD95 and tumor necrosis factor-α death receptors are activated, resulting in cleavage of pro-caspase-8 [23]. The other pathway is mitochondrial and involves Bcl-2-controlled cytochrome *c* release into the cytosol [24], which binds to Apaf-1 and in turn allows for binding to pro-caspase-9 [25]. There is evidence to suggest that ATO predominantly induces apoptosis through the mitochondrial pathway, which is also the case with MER1. ATO has been shown to induce ROS generation in a wide variety of cancer cells [26]. ROS are constantly generated by the mitochondrial respiratory chain through the metabolism of molecular oxygen [27]. Since they can be potentially cytotoxic at high levels, they are promptly scavenged by a highly regulated anti-oxidant system that includes enzymes such as superoxide dismutase, GSH peroxidase, and catalase. Cell susceptibility to arsenic-induced apoptosis depends upon the inherent balance between its specific ROS generation and antioxidant enzymatic activities [12, 14]. Treatment of HL60 cells with MER1 triggered H_2O_2 production that was more abundant and was maintained over a longer time than during ATO treatment, suggesting that MER1-induced apoptosis is modulated at least to some extent by ROS production. Furthermore, ATO (As_2O_3) breaks down to free H^+ and arsenite (AsO_3^{3-}) and the latter targets -SH enzymes due to its sulfhydryl affinity. GSH serves as the largest source of intracellular nonprotein thiol groups and therefore plays an important role in the maintenance of a normal redox state by regulating GSH peroxidase and GSH S-transferase activity, which is crucial for ROS scavenging [28]. The role of GSH as a modulator of MER1-induced ROS generation and apoptosis was supported by the fact that pretreatment with the disulfide bond-reducing agent DTT or the radical scavenger NAC protected cells against free radical generation and increased cell resistance against apoptosis. This observation suggests that GSH is an important determinant of MER1 sensitivity and that ROS generation mediates MER1 pro-apoptotic activity.

It has been shown that NB4 cells vary their ROS content throughout the cell cycle, with higher levels in G2/M phase than in G1 and S phases [29]. Notably, treatment with ATO results in apoptosis of NB4 cells prevailingly in G2/M phase, suggesting that cells in that phase are more susceptible to apoptosis induced by ROS-generating agents [29]. This study showed that MER1 caused G2/M arrest in both NB4 and HL60 cells followed by more abundant apoptosis than upon ATO treatment. Since these effects were reverted upon NAC treatment, it could be hypothesized that G2/M arrest caused by MER1 leads to ROS accumulation above a certain threshold, which then leads to unacceptable oxidative damage and in turn triggers apoptosis. Unlike ATO [7], MER1-induced apoptosis was independent of telomere shortening, and given that MER1 treatment results in cell cycle arrest and induction of apoptosis in both *p53*-deficient (HL60) and wild-type *p53* (NB4) cell lines, the activity of MER1 is also independent of *p53* status.

In summary, MER1 inhibits the proliferation and induces apoptosis of the AML cell lines NB4 and HL60, indicating that its activity is independent of *PML-RAR*α and *p53* status. Instead, the activity of MER1 appears to be mediated through ROS generation and accumulation, leading to cell cycle arrest in G2/M phase and apoptosis. This novel OAD, endowed with comparable antitumor activity *in vitro* and a more favorable toxicity profile *in vivo* than ATO, therefore, holds promise as an antileukemic agent and its further development is warranted. Therapeutic evaluation of MER-1 in mouse models of leukemia are planned.

Acknowledgement Robert A. Welch Foundation sponsored work done by RAZ.

References

1. de The H, Chomienne C, Lanotte M, Degos L, Dejean A (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. Nature 347(6293):558–561. doi:10.1038/347558a0
2. Borrow J, Goddard AD, Sheer D, Solomon E (1990) Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 249(4976):1577–1580. doi:10.1126/science.2218500
3. Shao W, Fanelli M, Ferrara FF, Riccioni R, Rosenauer A, Davison K, Lamph WW, Waxman S, Pelicci PG, Lo Coco F et al (1998) Arsenic trioxide as an inducer of apoptosis and loss of PML/RAR alpha protein in acute promyelocytic leukemia cells. J Natl Cancer Inst 90(2):124–133. doi:10.1093/jnci/90.2.124
4. Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM et al (1996) In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML. Blood 88(3):1052–1061
5. Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA et al (1998) Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 339(19):1341–1348. doi:10.1056/NEJM199811053391901
6. Miller WH Jr, Schipper HM, Lee JS, Singer J, Waxman S (2002) Mechanisms of action of arsenic trioxide. Cancer Res 62 (14):3893–3903
7. Chou WC, Hawkins AL, Barrett JF, Griffin CA, Dang CV (2001) Arsenic inhibition of telomerase transcription leads to genetic instability. J Clin Invest 108(10):1541–1547
8. Chen GQ, Zhou L, Styblo M, Walton F, Jing Y, Weinberg R, Chen Z, Waxman S (2003) Methylated metabolites of arsenic trioxide are more potent than arsenic trioxide as apoptotic but not differentiation inducers in leukemia and lymphoma cells. Cancer Res 63(8):1853–1859
9. Duzkale H, Jilani I, Orsolic N, Zingaro RA, Golemovic M, Giles FJ, Kantarjian H, Albitar M, Freireich EJ, Verstovsek S (2003) In

vitro activity of dimethylarsinic acid against human leukemia and multiple myeloma cell lines. Cancer Chemother Pharmacol 51 (5):427–432
10. Verstovsek S, Giles F, Quintas-Cardama A, Perez N, Ravandi-Kashani F, Beran M, Freireich E, Kantarjian H (2006) Arsenic derivatives in hematologic malignancies: a role beyond acute promyelocytic leukemia? Hematol Oncol 24(4):181–188. doi:10.1002/hon.787
11. Verstovsek S, Duzkale, H, Orsolic, N et al (2002) S-dimethylarsino-thiosuccinic acid, an organic arsenic derivative, is comparable in in vitro antileukemic activity to arsenic trioxide but possesses significantly less toxicity. Blood 100(11):(abstr 4614)
12. Yi J, Gao F, Shi G, Li H, Wang Z, Shi X, Tang X (2002) The inherent cellular level of reactive oxygen species: one of the mechanisms determining apoptotic susceptibility of leukemic cells to arsenic trioxide. Apoptosis 7(3):209–215. doi:10.1023/A:1015331229263
13. Rojkind M, Dominguez-Rosales JA, Nieto N, Greenwel P (2002) Role of hydrogen peroxide and oxidative stress in healing responses. Cell Mol Life Sci 59(11):1872–1891. doi:10.1007/PL00012511
14. Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, Waxman S (1999) Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood 94(6):2102–2111
15. Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Peschle C, Nicoletti I et al (1993) The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 74(3):423–431. doi:10.1016/0092-8674(93)80044-F
16. Pan J, Quintas-Cardama A, Kantarjian HM, Akin C, Manshouri T, Lamb P, Cortes JE, Tefferi A, Giles FJ, Verstovsek S (2007) EXEL-0862, a novel tyrosine kinase inhibitor, induces apoptosis in vitro and ex vivo in human mast cells expressing the KIT D816V mutation. Blood 109(1):315–322. doi:10.1182/blood-2006-04-013805
17. Takuma T, Takeda K, Konno K (1987) Synergism of tumor necrosis factor and interferon-gamma in induction of differentiation of human myeloblastic leukemic ML-1 cells. Biochem Biophys Res Commun 145(1):514–521. doi:10.1016/0006-291X(87)91351-9
18. Hultdin M, Gronlund E, Norrback K, Eriksson-Lindstrom E, Just T, Roos G (1998) Telomere analysis by fluorescence in situ hybridization and flow cytometry. Nucleic Acids Res 26 (16):3651–3656. doi:10.1093/nar/26.16.3651
19. Rego EM, He LZ, Warrell RP Jr, Wang ZG, Pandolfi PP (2000) Retinoic acid (RA) and As2O3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARalpha and PLZF-RARalpha oncoproteins. Proc Natl Acad Sci USA 97 (18):10173–10178. doi:10.1073/pnas.180290497
20. Zhang W, Ohnishi K, Shigeno K, Fujisawa S, Naito K, Nakamura S, Takeshita K, Takeshita A, Ohno R (1998) The induction of apoptosis and cell cycle arrest by arsenic trioxide in lymphoid neoplasms. Leukemia 12(9):1383–1391. doi:10.1038/sj.leu.2401112
21. Park JW, Choi YJ, Jang MA, Baek SH, Lim JH, Passaniti T, Kwon TK (2001) Arsenic trioxide induces G2/M growth arrest and apoptosis after caspase-3 activation and bcl-2 phosphorylation in promonocytic U937 cells. Biochem Biophys Res Commun 286 (4):726–734. doi:10.1006/bbrc.2001.5416
22. Griffith OW, Meister A (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem 254(16):7558–7560
23. Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85(6):803–815. doi:10.1016/S0092-8674(00) 81265-9
24. Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281(5381):1309–1312. doi:10.1126/science.281.5381.1309
25. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91(4):479–489. doi:10.1016/S0092-8674(00)80434-1
26. Qian Y, Castranova V, Shi X (2003) New perspectives in arsenic-induced cell signal transduction. J Inorg Biochem 96(2–3):271–278. doi:10.1016/S0162-0134(03)00235-6
27. Halliwell B (1999) Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: Measurement, mechanism and the effects of nutrition. Mutat Res 443(1–2):37–52
28. Davison K, Cote S, Mader S, Miller WH (2003) Glutathione depletion overcomes resistance to arsenic trioxide in arsenic-resistant cell lines. Leukemia 17(5):931–940. doi:10.1038/sj.leu.2402876
29. Gao F, Yi J, Yuan JQ, Shi GY, Tang XM (2004) The cell cycle related apoptotic susceptibility to arsenic trioxide is associated with the level of reactive oxygen species. Cell Res 14(1):81–85. doi:10.1038/sj.cr.7290206