The Organic Arsenic Derivative GMZ27 Induces PML–RARα-Independent Apoptosis in Myeloid Leukemia Cells

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Abstract

Arsenic trioxide (ATO) is an inorganic arsenic derivative that is very effective against acute promyelocytic leukemia. However, organic arsenic derivatives (OAD) have a more favorable toxicity profile than ATO. We herein characterized dipropyl-S-glycerol arsenic (GMZ27), a novel OAD. GMZ27 had potent antiproliferative activity against human acute myeloid leukemia (AML) cell lines that was higher than that of ATO. In contrast to ATO, GMZ27 only marginally induced maturation of leukemia cells and had no effect on the cell cycle. The anti-leukemia activity of GMZ27 against AML cells was independent of the presence of the PML–RARα fusion protein. GMZ27 dissipates mitochondrial transmembrane potential, and induces cleavage of caspase 9 and activation of caspase 3 without altering the expression levels of (BCL-2), BAX and BCL-xl. GMZ27 induces the formation of intracellular superoxide, a reactive oxygen species (ROS) which plays a major role in the antileukemia activity of this OAD. In addition to ROS generation, GMZ27 concomitantly reduces intracellular glutathione which markedly weakens the cellular antioxidant capacity, thus enhancing the detrimental intracellular effects of ROS production. These results indicate that GMZ27 induces apoptosis in AML cells in a PML–RARα-independent fashion, through the induction of ROS production. This activity provides the rationale for the testing of GMZ27 in patients with AML.

Keywords

GMZ27; arsenic derivative; organic arsenic derivative; PML–RARα; acute myeloid leukemia; NB4; HL60; U937; KBM-5 cells; flow cytometry; mitochondrial membrane potential

Several studies conducted over the past decade established the efficacy of arsenic trioxide (ATO) in patients with both newly diagnosed or relapsed acute promyelocytic leukemia (APL) (1-3). The success of ATO in inducing high rates of complete remission in patients...
who have relapsed with this, once highly lethal, leukemia provided the impetus for uncovering the specific mechanism of action underlying such dramatic clinical activity. In most APL cells, the t(15;17) results in the production of the PML–RARα-Independent fusion protein which blocks the cell maturation process (3). Several studies have shown that ATO acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways and resulting in a wide range of cellular effects which include induction of apoptosis, arrest of cell cycle progression, block of differentiation, reactive oxygen species (ROS) production, and even inhibition of angiogenesis (3). In PML–RARα-positive cells, ATO promotes degradation of the PML–RARα protein by targeting the PML moiety, which results in therapy-induced restoration of nuclear bodies, leading to cell maturation (3-5). The mechanisms involved in ATO-induced nuclear body reformation involve the formation of ROS and the direct binding of ATO to PML cysteines. PML multimerization and ATO binding represent the primary mechanisms of ATO-induced APL cure (3-5). However, clinical use of ATO is limited by cardiovascular, hepatic, gastrointestinal and central nervous system toxicity (1, 2, 6, 7). In contrast, organic arsenic derivatives (OAD) are much less toxic; in fact, hepatic methylation of inorganic arsenic compounds, including ATO, to yield organic derivatives is a mechanism of detoxification which makes them promising candidates for treating leukemia and other malignancies with a better therapeutic index than ATO (7, 8). Research efforts have been directed towards synthesis of OADs with high efficacy towards leukemia cells and low systemic toxicity (8). On this basis, we synthesized GMZ27, a novel OAD derived from dimethylarsinic acid. In spite of the fact that ATO and OAD are both arsenic derivatives, different mechanisms may be utilized by these compounds to exert their antileukemic activity due to important differences in their chemical structures. Unveiling the mechanisms of antileukemic activity of OAD may be exploited in subsequent clinical studies with these compounds.

Materials and Methods

Cells and chemicals

Human cell lines used in this study included the following: acute promyelocytic leukemia NB4 cells, acute myelomonocytic leukemia HL60 cells (both provided by Miloslav Beran MD, Ph.D., DVM, The University of Texas M.D. Anderson Cancer Center); human leukemic monocyte lymphoma cell line U937 and human chronic myeloid leukemia KBM-5 cell line, provided by Michael Andreeff, MD, Ph.D., The University of Texas M.D. Anderson Cancer Center. HL60, NB4, and U937 cell lines were cultured in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin solution (100 U/ml; Gemini Bio-products, Sacramento, CA, USA). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. ATO was purchased from Sigma Aldrich (Saint Louis, MO, USA) and dissolved in 1 M HCl; this stock solution was then diluted as requested. GMZ27 (provided by Ralph A. Zingaro, Ph.D., Texas A&M University, College Station, Texas) was dissolved in methanol to a stock concentration of 10 mM. Further dilution was achieved by addition of (PBS). N-Acetyl-L-cysteine (NAC), L-buthionine-[S,R]-sulfoximine (L-BSO), and dithiothreitol (DTT) were purchased from Sigma Chemical Co. Toxicity testing of GMZ27 was carried
out in 11-week-old Swiss Webster mice (Taconic Laboratory Animals and Services, Germantown, NY, USA).

**Determination of cell growth**

Cell growth was assessed by measuring the number of living cells using the 3-(4,5-dimethylthia-zol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The MTS assay was performed according to the manufacturer’s recommendations (CellTiter 96 Aqueous One Solution Reagent; Promega, Madison, WI, USA). Briefly, cells were seeded in triplicate in 96-well microtiter plates (BD Falcon, Franklin Lakes, NJ, USA) and were incubated in the presence of increasing drug concentrations for up to 72 hours. The seeding density utilized depended on the cell line used and varied from 0.05-0.4×10^6/well. Upon drug exposure, the level of growth inhibition as evidenced by the dye color change was quantified by measuring the absorbance at 490 nm and was expressed as a percentage of the control growth (no drug in the sample). The half maximal inhibitory concentration (IC_{50}) value was then determined. The number of live cells was determined by the Trypan blue assay. Briefly, cells were plated at 0.5×10^6/ml and cultured for up to 72 hours in 24-well plates at different concentrations of ATO or GMZ27. Cells were then washed in PBS and were mixed with an equal volume of saline containing 0.4% Trypan blue dye (GIBCO-BRL, Gaithersburg, MD, USA). Dead cells fail to exclude the dye and can be counted by discrimination from unstained, live cells. Survival was calculated in terms of total live cell numbers in each well and was then expressed as a percentage of the survival in control wells.

**Annexin V and propidium iodide (PI) staining**

After treatment with arsenic derivatives for different periods of time, cells were washed in Ca^{2+}-free PBS and resuspended in 100 μl of binding buffer (10 mM 4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid, pH 7.4; 0.15 M NaCl; 5 mM KCl; 1 mM MgCl_2; 1.8 mM CaCl_2) to which annexin V- fluoroisothiocyanate (Travigene) had been added. Cells were then incubated for 15 minutes in the dark at room temperature. After incubation, cells were washed in 2 ml PBS and then resuspended in 0.5 ml of binding buffer. After the addition of PI, the binding of annexin V to apoptotic cells was analyzed on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) by using the program CellQuest (Becton Dickinson).

**In vivo toxicity**

Normal female Swiss-Webster mice were divided into treatment groups which received different single injections on a 10 mg-dose escalating schedule with doses ranging from 0 (control) to 150 mg/kg with each group consisting of five mice receiving the same single dose injection. Drug toxicity was measured as the LD_{50}, which was the dosage at which 50% of mice in the group were killed.

**Detection of caspase activity**

To monitor caspase activity by flow cytometry, the fluorogenic substrate PhiPhiLux G1D2 (Oncoimmunin, Gaithersburg, MD, USA) was used. PhiPhiLux G1D2 is a substrate for the
detection and measurement of caspase-3 and caspase3-like activities in living cells. After treatment with arsenic derivatives for different lengths of time, cells were washed in Ca$^{2+}$-free PBS, resuspended in 25 μl of substrate solution, and were incubated for 1 hour at 37°C in the dark. After incubation, cells were washed and resuspended in PBS. PI was added to exclude necrotic cells during analysis. Cell samples were then run on a flow cytometer (FACScan; Becton Dickinson) and data obtained were analyzed with CellQuest software (Becton Dickinson).

**Mitochondrial inner transmembrane potential dissipation**

After treatment with arsenic compounds for the indicated time, cells were incubated with submicromolar concentrations of MitoTracker probes to evaluate the changes in the potential of the mitochondrial membrane. MitoTracker probes passively diffuse across the plasma membrane and accumulate in mitochondria. Treated cells were stained with two probes: MitoTracker Red (CMXRos; Molecular Probes, Eugene, OR, USA) and MitoTracker Green FM (MTGreen; Molecular Probes). Briefly, cells were washed in Ca$^{2+}$-free PBS, stained with MitoTracker dyes and incubated for 1 hour at 37°C in the dark. CMXRos is incorporated into mitochondria by force of the mitochondrial membrane potential and reacts with thiol residues to form covalent thiol ester bonds. MTGreen FM preferentially accumulates in mitochondria regardless of mitochondrial membrane potential, making it a useful tool for determining mitochondrial mass. Samples were run on a flow cytometer (FACScan, Becton Dickinson) and were analyzed by the program CellQuest (Becton Dickinson).

**Detection of intracellular ROS production**

To evaluate superoxide production, cells were treated with arsenic compounds for different times. Twenty minutes before the cells were harvested, 10 μM dihydroethidium (Molecular Probes) was added to cultured cells. The cells were then washed in 2 ml Ca$^{2+}$-free PBS, resuspended in 0.5 ml Ca$^{2+}$-free PBS, and analyzed immediately for ethidium fluorescence intensity by flow cytometry (FACScan; Becton Dickinson).

**Effects of intracellular redox status on the activity of GMZ27**

The *in vitro* growth inhibitory effect of GMZ27 on myeloid cells was determined by the MTS assay (CellTiter 96® Aqueous One Solution Reagent; Promega) with or without the addition of L-BSO (Sigma Aldrich), which depletes GSH, or NAC (Sigma Aldrich), which augments cellular reduction. After a 24-hour preincubation with or without the addition of 100 μM L-BSO or 10 mM NAC, cells were seeded in triplicate in 96-well microtiter plates at a density of 4×104 cells/ml. GMZ27 was added at increasing concentrations. After 72-hour incubation, 20 μl of MTS solution were added to each well. The cells were then incubated for an additional 3 hours at 37°C, and the absorbance was then measured at 490 nm.

**Analysis of cell cycle activity and subG1 DNA fragmentation status**

After treatment with GMZ27, cells were collected, washed in Ca$^{2+}$-free PBS, and were fixed overnight in 70% cold ethanol at −20°C. The cells were then washed twice in cold PBS.
and were resuspended in Hypotonic PI solution [25 μg/ml PI, 0.1% TritonX- 100, 30 mg/ml (PEG), and 3600 U/ml RNAse, all dissolved in 4 mM sodium citrate buffer] for 15 minutes in the dark at room temperature. Collected data were analyzed by means of ModFit software (Becton Dickinson).

Analysis of cell maturation

The NB4 cell line was used to test the effect of ATO and GMZ27 on maturation of promyelocytic leukemia cells. Phycoerythrin-conjugated anti-CD11b monoclonal antibody (BD Pharmingen, San Diego, CA, USA) was used as a marker of leukocyte maturation. After 72 hours of incubation with drugs, cells (1×10⁶ cells/ml) were washed in PBS and were incubated with anti-CD11b (dilution 1:10), in the dark at room temperature for 15 minutes. After incubation, cells were washed and resuspended in 500 μl PBS. To exclude nonspecific binding, appropriate isotype control antibody-stained cells were prepared in the same manner. Cells were sorted by using a flow cytometer (FACScan; Becton Dickinson) and were analyzed with CellQuest software (Becton Dickinson).

Degradation of PML–RARα fusion protein

NB4 cells were treated with different concentrations of GMZ27 for 24 hours; whole cell lysate was then obtained and subjected to western blot as described above. Expression of PML–RARα was detected by polyclonal anti-PML (H-238; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). In order to ascertain whether the anti-leukemia activity of GMZ27 depended on the degradation of the PML–RARα fusion protein, NB4 and U937 cells (a monocytic leukemia cell line that does not express PML–RARα) were treated with GMZ27 and the antiproliferative effects of GMZ27 on these cell lines were evaluated by a 72-hour MTS assay.

Measurement of GSH content

NB4 cells (20×10⁶) at a density of 0.4 million/ml were harvested by centrifuging at 1500 rpm for 3 minutes. Cells were then washed with PBS once and stored at −80°C until further use. Three freeze-thaw cycles were performed in order to lyse the cells completely. Cell lysates were mixed with protein assay solution (Bio-Rad, Hercules, CA, USA) and the O.D. value was measured at 595 nM to determine protein concentration. Subsequently, 250 μl 10% metaphosphase (MPA) were added to 250 μl of cell lysate, mixed, and centrifuged at 3000 g at 4°C for 10 minutes. Next, 200 μl of supernatant were mixed with 700 μl of potassium phosphate buffer containing diethylenetriamine pentaacetic acid and lubrol, vortexed, and added to 50 μl R1 solution (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate). After vortexing, 50 μl of R2 solution (30% NaOH) were added and the mixture incubated at room temperature for 10 minutes in the dark. The OD values of samples were measured at 400 nM.

Western blotting analysis

Following incubation with different concentrations of GMZ27 or ATO for different time periods western blot analysis was performed using the following primary antibodies: anti-β-actin (Sigma Chemical Co.), anti-BCL2 (Upstate Biotechnology, Waltham, MA, USA), anti-
Results

**GMZ27 induces dose-dependent inhibition of cell proliferation in leukemia cell lines**

The cytotoxic properties of GMZ27 were explored in two leukemia cell lines, NB4 and HL60. Cell proliferation was assessed by the MTS assay 72 hours after incubation with GMZ27 or ATO. As shown in Figure 1A and 1B, both ATO and GMZ27 produced dose–dependent inhibition of proliferation in both cell lines. However, GMZ27 exhibited stronger inhibition compared with that of ATO. The IC_{50} of GMZ27 ranged from 0.53 μM to 0.82 μM when tested in various leukemia cell lines, which is significantly lower than those of ATO (Table I). To confirm and extend these findings, Trypan blue dye exclusion assay was also performed. In each cell line tested, a 72-hour treatment with GMZ27 produced significant dose-dependent growth inhibition (Figure 1C).

**GMZ27 inhibits cell growth independently of PML–RARα expression**

The aberrant PML–RARα fusion protein results from the t(15;17) translocation between the zinc-finger protein PML and the retinoic acid receptor α. To assess whether the effects of GMZ27 were mediated through inhibition/degradation of PML–RARα, NB4 cells, which consistently express the PML-RARα fusion protein, were treated with different doses of GMZ27 for 24 hours. Dose-dependent PML–RARα protein degradation upon GMZ27 exposure was detected by western blot analysis (Figure 2A). In order to establish whether the PML-RARα fusion protein in leukemia cells contributed to their sensitivity to GMZ27, U937 cells, which do not carry PML–RARα and are known to be resistant to ATO, were treated with different concentrations of GMZ27 and ATO and cell proliferation was evaluated by the 72-hour MTS assay.

As expected, U937 cells showed significant resistance to ATO treatment. However GMZ27 was very effective at inhibiting the proliferation of U937 cells (Figure 2B). These results indicated that although GMZ27 did induce degradation of PML-RARα, the antileukemia effect of GMZ27 was not completely dependent on the latter. We then determined whether the decrease in cell proliferation was associated with changes in cell maturation. Expression of CD11b, a maturation marker for myeloid cells, on ATO- and GMZ27-treated NB4 cells was analyzed by flow cytometry. We detected only a marginal increase in the percentage of CD11b-positive NB4 cells after a 72-hour treatment with 0.1-0.6 μM of ATO compared to that of GMZ27-treated cells, except when cells were treated at the highest concentration (0.6 μM) of ATO, which induced significant induction of CD11b expression (Figure 2C).

**Impact of GMZ27 on cell cycle and apoptosis in leukemia cell lines**

In order to determine whether the mechanism of action of GMZ27 might involve cell cycle perturbations, the effects of GMZ27 on cell cycle progression were examined. To that end, we treated HL60 cells with different doses of GMZ27 and we analyzed the percentage of cells in the G_2/M phase of the cell cycle as assessed by flow cytometry using PI staining.
The drug dose range employed was selected based on the *in vitro* sensitivities of the cell lines to GMZ27 used in the experiment. After 24-hour and 48-hour treatment with GMZ27, no significant induction of cell cycle arrest was observed in HL60 cells (Figure 3A). Similarly, no significant accumulation of cells in the sub-G1 phase of the cell cycle was observed at different concentrations of GMZ27 (Figure 3B).

Given the lack of significant effects on the cell cycle, we next investigated the effect of GMZ27 on apoptosis of HL60 cells. Cell apoptosis was assessed by flow cytometry using three markers directed at different targets involved in the apoptotic process: compromised cell membrane integrity (Figure 4A), activation of caspase 3 (Figure 4B) and dissipation of mitochondrial transmembrane potential (Figure 4C). By all three measures, both ATO and GMZ27 induced apoptosis in a concentration- and time-dependent fashion. However, GMZ27 induced apoptosis at significant lower concentrations compared to ATO, indicating a more potent proapoptotic effect associated with GMZ27.

**GMZ27 induces apoptosis through activation of the intrinsic pathway and caspase 9**

To determine whether induction of apoptosis in HL60 cells by GMZ27 involves activation of specific apoptotic pathways, HL60 cells were treated for 24 hours with vehicle or with GMZ27. As shown in Figure 5A, treatment with GMZ27 induced activation of caspase-9 (demonstrated by cleavage of caspase-9), but failed to activate caspase-8. These observations suggest that GMZ27-mediated apoptosis may occur preferentially through the intrinsic apoptotic pathway, which involves activation of caspase-9 and dissipation of the mitochondrial transmembrane potential (Figure 4C). Furthermore, immunoblotting demonstrated cleavage of PARP (manifested as an 89kDa band in the western blot; Figure 5A), thus confirming induction of apoptosis by GMZ27. To further investigate the mechanism of apoptosis induction, degradation of Bcl-2 family proteins (BCL-2, BCL-xl and BAX) was analyzed in NB4 cells treated with different concentrations of GMZ27 for 24 hours. No significant alteration of the expression of BCL-2 proteins was detected (Figure 5B).

**The antileukemia activity of GMZ27 is dependent on modulation of intracellular oxidative status**

Next, we evaluated the redox status of leukemia cell lines upon exposure to GMZ27. We modulated the cell redox status by either NAC (which enhances GSH synthesis), or by BSO (which blocks GSH synthesis). HL60 cells were treated with no drug, 100 μM BSO, or 10 mM NAC for 14 hours and different concentration of GMZ27 were then added to the cell culture. MTS assays were performed at specific time points to evaluate cell proliferation. Our experiments show unequivocally that depleting intracellular GSH with BSO enhances the antiproliferative activity of GMZ27 (Figure 6A). This is confirmed by the fact that increasing cellular reduction by pre-incubation with NAC protects cells from the antiproliferative activity of GMZ27 (Figure 6B). The opposing effects of BSO and NAC on the activity of GMZ27 indicate that ROS generation is likely involved in the mechanism of action of GMZ27.
To determine whether treatment with GMZ27 induced the production of ROS, superoxide levels were assessed in HL60 cells treated with vehicle alone, or with 1 μM GMZ27 for 0 to 8 hours. Superoxide production increased in a dose- and time-dependent manner after treatment with GMZ27 that peaked at 4 hours post exposure to the drug (Figure 6C and D). Furthermore, intracellular GSH was measured in NB4 cells after exposure to increasing concentrations of ATO or GMZ27. The intracellular concentration of GSH decreased markedly 8 hours after treatment with GMZ27 (Figure 7), which was even more remarkable after 14 hours (data not shown). This effect was not observed in cells treated with ATO. These results indicate that in addition to ROS generation, GMZ27 also further reduces intracellular GSH which seriously weakens the cellular antioxidant capacity, thus enhancing the detrimental intracellular effects of ROS. Again, similar effects on intracellular GSH were not observed in ATO-treated cells (data not shown).

In vivo toxicity study

In order to characterize the toxicity of GMZ27 *in vivo*, a single injection of this compound was given to wild-type Swiss-Webster mice via tail vein in groups of 6, on a 10 mg-dose escalating schedule with doses ranging from 0 mg/kg to 150 mg/kg. *In vivo* toxicity was measured on the basis of mortality and was expressed as the lethal dose at which half the mice in a group died (LD$_{50}$). The LD$_{50}$ was reached at 100 mg/kg, a dose significantly higher than that of ATO, which has been reported to be 10 mg/kg.

Discussion

The striking activity of ATO, an inorganic arsenical compound, against APL cells (7) resulted in the establishment of ATO as standard treatment for patients with APL. The role of ATO in other types of hematological cancer is, however, limited (9-12). In spite of its impressive activity in APL, the potential for systemic toxicity with ATO therapy is of concern (9-12). Inorganic arsenic compounds such as ATO affect many organ systems, including the cardiovascular system, gastrointestinal tract, kidneys, skin, nervous system and hematopoietic organs. Inorganic compounds can be particularly toxic to the liver, causing infiltration, central necrosis and cirrhosis (13-16). There is now sufficient evidence that inorganic arsenic compounds are skin and lung carcinogens in humans (16). The toxicity of a given arsenic compound is related to the rate of its clearance from the body and to the extent of its tissue accumulation (15). Unlike inorganic arsenic compounds, no deaths or serious toxic effects associated with OAD have been reported. In mammals, the methylation of inorganic arsenic has been considered as a detoxification reaction because of the lower toxicity of methylated OAD, and the faster excretion and reduced retention (16, 17). A good example is that of dimethylarsinic acid, an organic compound, which is the predominant urinary metabolite excreted by most mammals after exposure to inorganic arsenic, including ATO. The LD$_{50}$ of ATO in mice is reported to be 10 mg/kg (18), while that for dimethylarsinic acid is 50-fold higher at 500 mg/kg (19). The antileukemia potential of melarsoprol, the only OAD currently manufactured for human use, has been evaluated. Studies unexpectedly showed that melarsoprol has activity at least equivalent to that of ATO against both APL and non-APL cell lines (20, 21). A limited clinical study of melarsoprol
was initiated in the U.S. in patients with advanced leukemia (22). A total of eight patients were treated on the schedule used for the treatment of central nervous system trypanosomiasis (three days per week for three consecutive weeks). Under this schedule, only one patient (with chronic lymphocytic leukemia) displayed antitumor effects and most patients experienced neurological side-effects. These early results suggest that while this or similar agents might have clinical benefit, the dosing schedule developed for treatment of trypanosomiasis is excessively toxic in patients with leukemia and that further preclinical experiments to develop novel OADs with equivalent activity and less toxicity would be necessary.

GMZ27 was picked as a leading compound among various OADs due to its potent activity against various kinds of tumor cell lines included in the NCI 60 cell line panel (data not shown). In the initial experiments presented here, cell proliferation and apoptosis were assayed after treatment of cells with either ATO or GMZ27. GMZ27 exhibited potent activity against acute leukemia cell lines, with an IC50 significantly lower than that of ATO. Most importantly, in in vivo experiments, GMZ27 exhibited less toxicity in wild-type mice than what was reported for ATO, which suggests its suitability for future clinical testing in leukemia. Based on these results, GMZ27 was chosen as the primary leading compound for further investigation regarding its antileukemic mechanism of action.

Arsenic produces profound cellular alterations, including induction of apoptosis, inhibition of proliferation, stimulation of differentiation, and inhibition of angiogenesis. APL is characterized by the t(15;17) translocation, which results in the fusion of the PML gene to the RARα gene (23, 24). The resulting PML–RARα fusion protein blocks the expression of genes required for normal myeloid differentiation. The block in myeloid differentiation by PML–RARα can be released by treatment with pharmacological-levels of retinoic acid, providing the basis for (ATRA) therapy in APL. Arsenic trioxide has been proposed as an alternative to treatment with ATRA because it can induce complete remission in patients with both RA-sensitive and RA-resistant APL. In our studies, GMZ27 and ATO (data not shown) can both induce degradation of the PML–RARα fusion protein. However, GMZ27 is effective against both NB4 cells, which express PML–RARα, as well as against U937 cells, which is not an APL cell line and therefore does not express PML–RARα. These results indicate that the main mechanism of action of GMZ27 is independent of PML–RARα degradation and induction of myeloid maturation, with the latter effect being similar with both ATO and GMZ27.

Arsenic exposure has been shown to activate caspases both in vitro and in vivo. ATO-induced apoptosis is associated with a loss of inner mitochondrial transmembrane potential and release of cytochrome c into the cytosol (25-27). Other mechanisms involved in ATO-induced apoptosis, such as a altered level of the BCL-2 family of proteins and decrease in telomerase transcription, remain controversial (28, 29). GMZ27 induced apoptosis in both APL and non-APL cell lines as evidenced by activation of caspase-3 and caspase-9, collapse of mitochondrial membrane potential, and PARP cleavage. However, expression of BCL-2 family proteins, was not altered by GMZ27 treatment. It has been reported that arsenic treatment can induce cell cycle arrest at the G2/M checkpoint in various leukemia cell lines (25-27). However, cell cycle arrest at the G1 or the G2/M checkpoints was not observed upon
treatment of NB4 and HL60 cell lines with GMZ27. The latter indicates that although both inorganic and OAD contain arsenic, their mechanisms of action are different. It is well known that various heavy metal molecules induce oxidative damage including arsenic, iron, copper, nickel, chromium, cadmium, lead, and mercury (16). It is also accepted that induction of ROS is one of many anticancer mechanisms induced by ATO (18-20). Intracellular GSH constitutes the most important reductive buffer against oxidative stress (21, 22). Through experimental manipulations we were able to alter GSH levels that changed the apoptotic response of leukemia cells to GMZ27. Reduction of intracellular GSH with BSO in leukemia cells resulted in enhanced sensitivity to GMZ27, whereas administration of free radical scavengers (i.e. N-acetylcysteine) increased GSH and abrogated the cytotoxic effects of GMZ27 (19). In contrast with ATO, GMZ27 directly reduced the intracellular GSH level which made cells more sensitive to ROS-induced stress. Furthermore, GMZ27 induced production of intracellular superoxide in a dose and time dependent manner. Overall, our results indicate that ROS generation is the single most important inducer of leukemic cell apoptosis after treatment with GMZ27.

In summary, GMZ27 inhibits proliferation and induces apoptosis of the AML cell lines studied here. This activity is independent of PML–RARα expression and degradation, and is not mediated by induction of myeloid maturation or cell cycle arrest. Instead, the activity of GMZ27 appears to be mediated through ROS generation and modulation of the intracellular oxidative status, leading to apoptosis. This novel OAD with enhanced antileukemia activity in vitro and a more favorable toxicity profile in vivo compared with ATO holds promise as an antileukemia agent.

References


52. American Conference of Governmental Industrial Hygienists, Inc. (ACGIH). Documentation of the Threshold Limit Values and Biological Exposure Indices. sixth edition. 1991. Arsenic and soluble compounds, including arsine.
Figure 1.
Activity of GMZ27 towards different leukemia cell lines. Summary of GMZ27-induced cell growth inhibition in 72-h MTS assays performed with HL60 (A) and NB4 (B) cells. Growth inhibition by GMZ27 of the HL60, NB4, U937, and KBM-5 leukemia cell lines in 72-h MTS assays (Table I). Viability of the HL60, NB4, U937, and KBM-5 leukemia cell lines after GMZ27 treatment in 72-h Trypan blue assays (C). Data represent results obtained in three independent experiments.
The antileukemia effect of GMZ27 is PML–RARα independent. NB4 cells were treated with GMZ27 for 24 hours and dose-dependent PML–RARα protein degradation was assayed by western blot using a rabbit anti-PML-RARα antibody (A). NB4 and U937 cells were incubated with GMZ27 for 72 hours and subjected to MTS assay to evaluate cell proliferation. U937 was sensitive to GMZ27 but not to arsenic trioxide (ATO) (B). After 72 hours of incubation with GMZ27 or ATO, NB4 cells were incubated with phycoerythrin-conjugated anti-CD11b monoclonal antibody (dilution 1:10) and then subjected to flow cytometry analysis using CellQuest software. GMZ27 treatment induces marginal myeloid maturation compared to ATO (C).
Figure 3.
GMZ27 does not induce cell cycle arrest. HL60 cells were treated with GMZ27 for 24 and 48 hours. Cell cycle analysis showed no arrest. The percentage of Sub G\textsubscript{1} phase of cells after treatment with of GMZ27 is also presented (B).
Figure 4.
GMZ27 induces apoptosis of HL60 cells in a dose- and time-dependent manner. HL60 cells were treated with ATO or GMZ27 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 are shown from experiments performed in triplicate.
Figure 5.
GMZ27 induces apoptosis in HL60 cells through activation of caspase-9. After incubation with GMZ27 for 24 hours, whole-cell lysate was subjected to western blot to detect pro-caspase-8, pro-caspase-9 and their active, cleaved fragments. PARP cleavage was also detected by western blotting. GMZ27-mediated apoptosis occurs preferentially through the intrinsic apoptosis pathway (A). GMZ27 does not alter the expression levels of Bcl-2, BAX and BCL-xl. Expressions of BCL family proteins were detected by anti-BCL-2, anti-BCL-xl and anti-BAX antibodies. β-Actin expression was detected by the anti-β-Actin antibody and was used as loading control (B).
Figure 6.
The antileukemia activity of GMZ27 is mediated through modulation of intracellular oxidative status and production of reactive oxygen species (ROS). HL60 cells were pre-incubated with or without 100 μM L-buthionine-[S,R]-sulfoximine (L-BSO) (A) or 10 mM N-acetyl-L-cysteine (NAC) (B) for 24 hours, and were then treated with GMZ27 at different concentrations. After 72 hours MTS assay was performed in order to evaluate cell proliferation. GMZ27 treatment results in production of superoxide. HL60 cells were treated with GMZ27 for 2, 4, 6, 8 hours. Superoxide production was assessed using a dihydroethidium probe. Each experimental point represents the mean±SD of three independent experiments (C, D).
Figure 7.
GMZ27 treatment depletes intracellular GSH content in NB4 cells. After 2, 8, 14, 24 hours of GMZ27 treatment, 20×10⁶ NB4 cells were harvested and the intracellular GSH was measured with 4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate as per the manufacturer’s instructions. The GSH content is expressed as μmol/μg protein.
Table I

IC$_{50}$ of GMZ27 tested in various leukemia cell lines.

<table>
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<tr>
<th>Cell line</th>
<th>IC$_{50}$ (μM)</th>
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<tr>
<td>HL60</td>
<td>0.79±0.22</td>
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<tr>
<td>NB4</td>
<td>0.5±0.18</td>
</tr>
<tr>
<td>U937</td>
<td>0.82±0.31</td>
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<tr>
<td>KBM-5</td>
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