

Arsenic trioxide enhances the cytotoxic effect of thalidomide in a KG-1a human acute myelogenous leukemia cell line

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Abstract. Studies have shown that thalidomide exerts modest activity as a single agent in the therapy of acute myeloid leukemia (AML). The present investigation was conducted to test the hypothesis that the cytotoxic effect of thalidomide is enhanced when properly combined with other chemotherapeutic agents. The human AML cell line KG-1a was used in this study. Cells were cultured for 48 h in the presence or absence of thalidomide, arsenic trioxide and a combination of the two substances. Results obtained indicate that thalidomide at concentrations of 1, 2 and 5 mg/l produced a dose-dependent cytotoxic effect and at 5 mg/ml resulted in late apoptosis in 49.39% of the total cell population (as compared to 5.35% in the control cells). When the cells were incubated with arsenic trioxide alone (4 μ M), late apoptosis was detected in 16.97% of the total cell population. However, when cells were incubated with a combination of thalidomide (5 mg/l) and arsenic trioxide (4 μ M), late apoptosis was noted to be 80.6% in the total cell population. This percentage of late apoptosis was statistically significant from that observed when cells were incubated with thalidomide alone. These findings clearly indicate that arsenic trioxide enhances the cytotoxic effects of thalidomide.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignant disease with diverse biological features. AML occurs in approximately 60% of patients, most of whom are older than 60 years (1). These elderly patients normally do not respond as well to the current conventional chemotherapy as their younger counterparts. This is due to the intrinsic resistant nature of their leukemic cells and/or poor tolerance to conventional chemotherapy regimens (2). Progress has been ongoing in the treatment outcome of AML, especially in elderly patients.

However, outcome has been poor with only 25-30% of adult patients being cured (3-5). Although prognosis varies among AML subtypes, the majority of patients relapse following an initial complete response (CR) and ultimately succumb due to resistant disease. Patients with AML who experience a particularly short first CR and those who fail to achieve CR after two induction attempts are unlikely to respond to any currently available chemotherapeutic agents. Similarly, patients with high-risk myelodysplastic syndromes likely to progress to AML [refractory anemia with excess blasts (RAEB) or refractory anemia with excess blasts in transformation (RAEBT)] have an estimated survival of less than one year (6). Supportive therapy remains the standard care for this population since intensive chemotherapy regimens, such as those used in AML, were reported to produce high rates of treatment-related mortality with rare durable remissions (7,8).

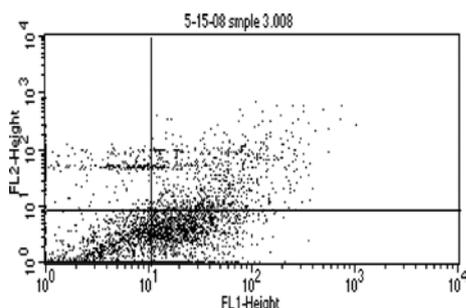
Therefore, novel approaches and alternative therapeutic strategies need to be explored. The role of angiogenesis in hematologic malignancies has been elucidated by several investigators; thus, inhibitors of angiogenesis are currently under investigation in these disorders. Thalidomide has antiangiogenic and immunomodulatory properties. However, thalidomide has shown only modest activity as a single agent in the therapy of AML. In addition, no study in the literature identifies a dose or schedule that should be followed when using thalidomide. Therefore, more studies are required to design a strategy to enhance the antileukemic effect of thalidomide. Such an approach is beneficial if the proper combination with other chemotherapeutic agents, such as interleukin-2 (IL-2) and arsenic trioxide (As_2O_3), is used. The current study examined the effect of the combination of thalidomide and the suggested chemotherapeutic agents on the KG-1a (human AML with early phenotype) cell line. The present study aimed to test the hypothesis that combining thalidomide with the correct chemotherapeutic agent/agents may be more efficient in the therapy of AML.

Materials and methods

Human KG-1a acute myeloid leukemia cells. The KG-1a cells, which are an early phenotype of human AML (American Type Culture Collection, Manassas, VA, USA), were grown in complete growth medium (Iscove's Modified Dulbecco's Medium; American Type Culture Collection) supplemented

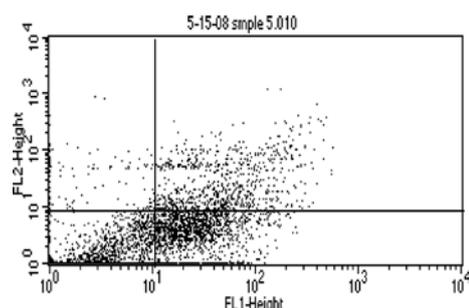
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2.68%	6.88%
75.94%	14.50%

Figure 1. Flow cytometry analysis of KG-1a cells treated with arsenic trioxide (2 μ M). The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.



1.16%	7.12%
76.03%	15.69%

Figure 2. Flow cytometry analysis of KG-1a cells treated with arsenic trioxide (2 μ M) + ascorbic acid (100 μ M). The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.

with 20% fetal bovine serum (Sigma-Aldrich, UK) and 1% penicillin-streptomycin (Gibco Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in a humidified 5% CO₂ incubator.

Treatment of human KG-1a acute myeloid leukemia cells.

The KG-1a cells were cultured for 48 h in 12-well tissue culture plates, each containing complete growth medium at a concentration of 2x10⁶ cells/ml. Each well contained a total volume of 2 ml. Thalidomide (Tocris Bioscience, Ellisville, MO, USA) was added at concentrations of 5 mg/l, either alone or in combination with other chemotherapeutic agents. IL-2 (Proleukin®, Aldesleuken for injection) (Chiron Therapeutics, Emeryville, CA, USA) was added at a concentration of 200 IU/ml, either alone or in combination with thalidomide and As₂O₃. As₂O₃ (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added at two concentrations of 2 and 4 μ M with and without 100 μ M of ascorbic acid (AA) in the first flow cytometry study, and at 4 μ M in the remaining studies; either alone or combined with thalidomide and IL-2. A control culture containing neither thalidomide nor IL-2 nor As₂O₃ was established in conditions that were otherwise identical. Duplicate control and treated cultures were established and incubated for 48 h at 37°C in a humidified 5% CO₂ incubator. The incubation time was selected to allow adequate time for apoptosis and necrosis to occur in the KG-1a human myeloid leukemia cells [modified from Lu and Hassan (9)].

Detection of apoptosis and necrosis using flow cytometry.

The detection of apoptosis and necrosis by Annexin V-FITC assay using flow cytometry was performed as described in the Annexin V-FITC apoptosis detection kit manual (BioVision, Inc., Mountain View, CA, USA). Staurosporine (Sigma-Aldrich, Inc.) was used as a positive control at a concentration of 10 μ M and was incubated for only 24 h with the leukemia cells. Briefly, following incubation for 48 h, the KG-1a cells were harvested by centrifugation at 1,300 rpm for 5 min. Cells were resuspended in 500 μ l of 1X binding buffer. Annexin

V-FITC (5 μ l) and propidium iodide (PI) (5 μ l) were added to the tubes (except the negative control which contained no staining nor treatment where only Annexin V-FITC was added for the Annexin V-FITC controls and only PI was added for the PI control). Tubes were incubated at room temperature for 5 min in the dark. Samples were then checked by the FACSCalibur Flow Cytometer (BD-Biosciences, San Jose, CA, USA) and apoptotic and necrotic cells were counted according to their staining with Annexin V or PI, respectively [modified from Yang *et al* (10)].

Statistical analysis. Results were subjected to one-way ANOVA. Statistical significant differences between means was set at p<0.05.

Results

First flow cytometry study. This study was mainly designed to determine whether AA enhances As₂O₃-induced cytotoxicity in the KG-1a human AML cell line. When the KG-1a cells were incubated with the chemotherapeutic agents for 48 h, results obtained indicated that 2 μ M of As₂O₃ alone resulted in early and late apoptosis, and necrosis (14.5%, 6.88% and 2.68%, respectively) of the total cell population (Fig. 1). Moreover, 2 μ M of As₂O₃ in the presence of 100 μ M of AA resulted in 15.69% early apoptosis, 7.12% late apoptosis and 1.16% necrosis of the total cell population (Fig. 2).

When the cells were incubated with 4 μ M of As₂O₃ alone, early and late apoptosis, as well as necrosis (20.87%, 8.31% and 1.26%, respectively) of the total cell population were observed (Fig. 3). Conversely, when 4 μ M of As₂O₃ was used in the presence of 100 μ M of AA, 21.84% early apoptosis, 8.14% late apoptosis and 0.77% necrosis of the total cell population were noted (Fig. 4). These findings indicate that AA did not significantly enhance the cytotoxicity of As₂O₃, and as a result our study utilized the higher dose of As₂O₃ (4 μ M) without the addition of AA.

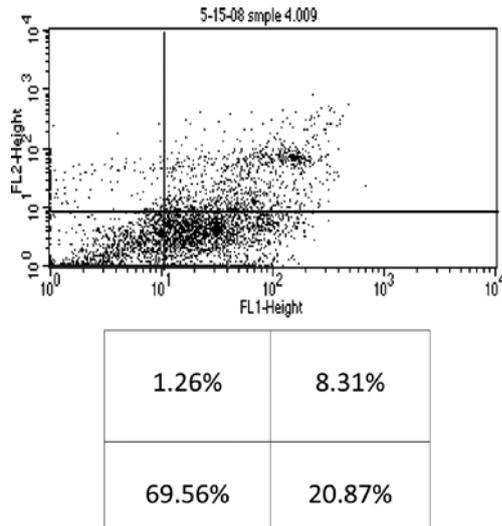


Figure 3. Flow cytometry of KG-1a cells treated with arsenic trioxide (4 μ M). The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.

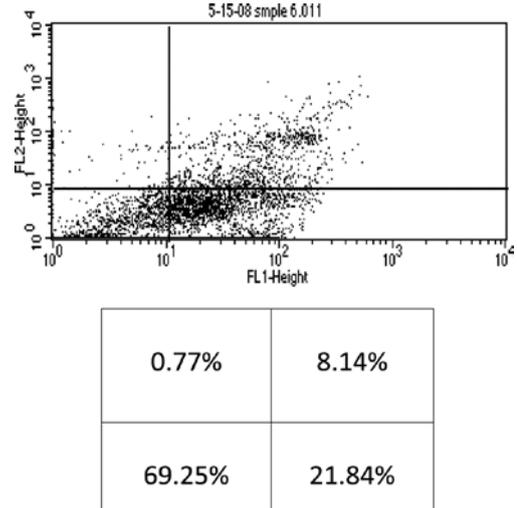


Figure 4. Flow cytometry of KG-1a cells treated with arsenic trioxide (4 μ M) + ascorbic acid (100 μ M). The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.

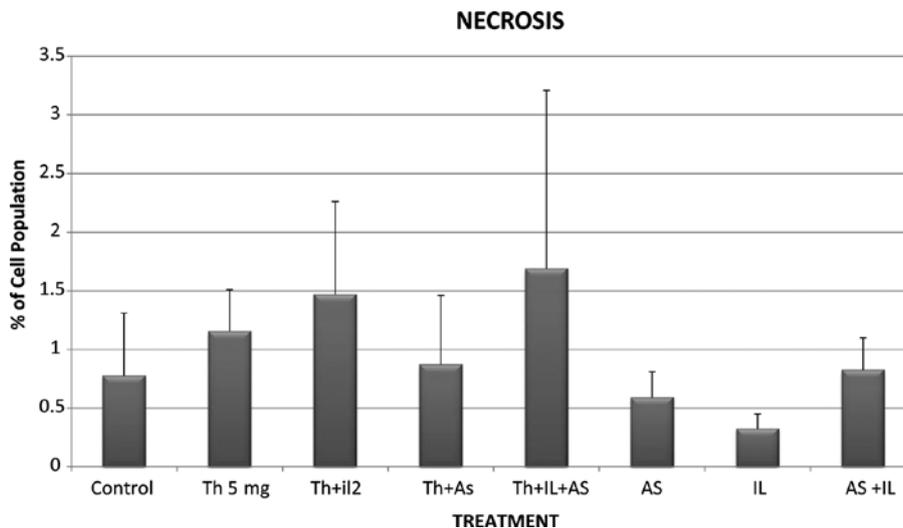


Figure 5. Necrosis of KG-1a cells incubated with various chemotherapeutic agents. Bars represent mean \pm SE. Cells were incubated for 48 h with thalidomide (Th) (5 mg/ml), arsenic trioxide (As) (4 μ M) and interleukin-2 (IL-2) (200 IU/ml) alone or in combination.

Cytotoxic effects of thalidomide, arsenic trioxide and interleukin-2 alone or in combination in KG-1a leukemia cells. Subsequent flow cytometry studies were conducted to test the cytotoxicity of chemotherapeutic agents both individually and in combination in KG-1a human leukemia cells in order to evaluate whether thalidomide cytotoxicity was enhanced by either IL-2 and/or As₂O₃.

Control KG-1a cells that were incubated for 48 h showed 0.775% necrosis, 9.89% early apoptosis and 5.345% late apoptosis in the total cell population (Figs. 5-7). When the cells were incubated with thalidomide only in a concentration of 5 mg/l, the percentages noted were: necrosis, 1.155%; early apoptosis, 7.92% and late apoptosis, 49.385% in the total cell population (Figs. 5-7). These results indicate that thalidomide exerted significant cytotoxicity in the KG-1a cells, and this cytotoxicity was mainly due to late apoptosis.

When the cells were incubated with 4 μ M of As₂O₃ the percentages of total cell population noted were: necrosis, 0.59%; early apoptosis, 10.095% and late apoptosis, 16.965% (Figs. 5-7). These results indicate that As₂O₃ alone had a modest cytotoxic effect since the percentages of cells in early and late apoptosis as well as necrosis were not statistically significantly different from values noted in the control cells. A similar conclusion was drawn with regard to cytotoxicity resulting from incubation of the KG-1a cells with 200 IU/ml of IL-2 alone. IL-2 alone resulted in 0.325% necrosis, 8.54% early apoptosis and 4.585% late apoptosis (Figs. 5-7). These values are not significantly different from those observed in the control KG-1a cells incubated under the same conditions.

However, when thalidomide and As₂O₃ were combined, the percentages observed were: necrosis, 0.87%; early apoptosis, 3.455% and late apoptosis, 80.6% (Figs. 5-7). These findings

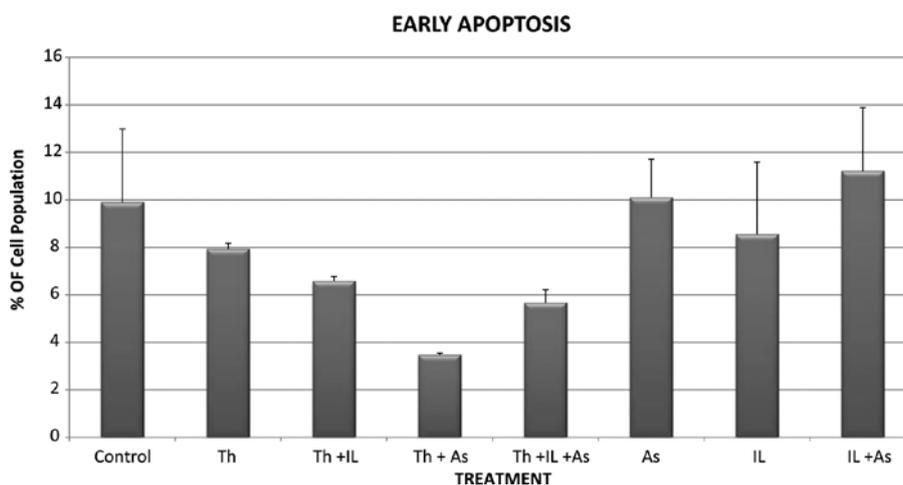


Figure 6. Early apoptosis of KG-1a cells incubated with various chemotherapeutic agents. Bars represent mean \pm SE. Cells were incubated for 48 h with thalidomide (Th) (5 mg/ml), arsenic trioxide (As) (4 μ M) and interleukin-2 (IL-2) (200 IU/ml) alone or in combination.

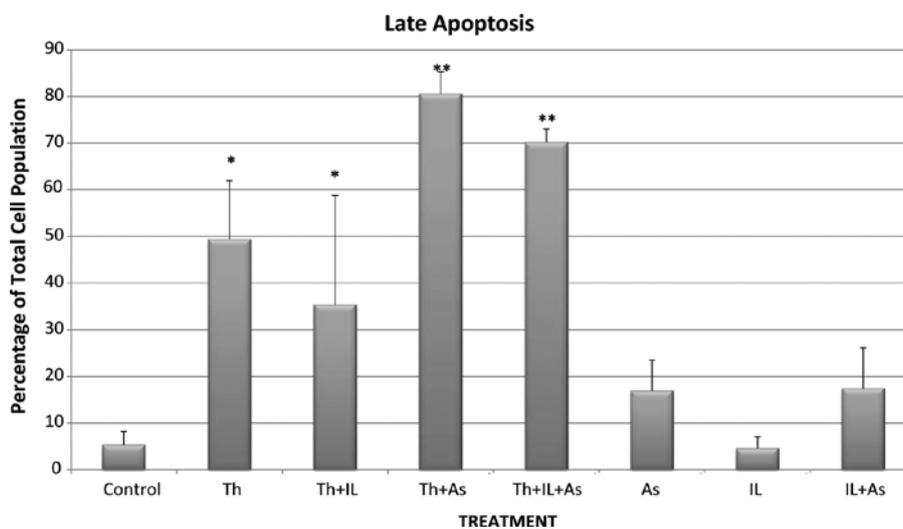


Figure 7. Late apoptosis of KG-1a cells incubated with various chemotherapeutic agents. Bars represent mean \pm SE. Cells were incubated for 48 h with thalidomide (Th) (5 mg/ml), arsenic trioxide (As) (4 μ M) and interleukin-2 (IL-2) (200 IU/ml) alone or in combination. *Significantly different from control ($p < 0.05$); **significantly different from the control and thalidomide-treated cells ($p < 0.05$).

indicate that As_2O_3 enhanced the cytotoxicity induced by thalidomide since the percentage of cells in late apoptosis was 49.385% when the cells were incubated with thalidomide alone, but increased to 80.6% when thalidomide was combined with As_2O_3 .

When IL-2 was combined with thalidomide no enhancement of cytotoxicity was noted as the percentages of total cell population showing necrosis, early apoptosis and late apoptosis were: 1.155, 7.92 and 49.385%, respectively, with thalidomide alone and 1.47, 6.58 and 35.385%, respectively, when a combination of thalidomide and IL-2 were used (Figs. 5-7).

IL-2 was also unable to enhance the cytotoxicity of the combination of thalidomide and As_2O_3 when the chemotherapeutic agents were used together since the percentages of total cell population showing necrosis, early and late apoptosis were 1.69, 5.655 and 70.135%, respectively. These values were not statistically significantly different from those observed with thalidomide and As_2O_3 .

Discussion

This study was conducted to evaluate the efficacy of thalidomide in the management of AML, as well as to examine the possibility of increasing its cytotoxicity by combining it with other chemotherapeutic agents, such as IL-2 and As_2O_3 . The variant subline KG-1a of the human acute myelogenous leukemia cell line KG-1 (11) was used as a test model.

The present study investigated the effect of AA on As_2O_3 cytotoxic activity. Glutathione (GSH) was shown to be an inhibitor of As_2O_3 -induced cell death either by conjugating As_2O_3 or by sequestering reactive oxygen induced by As_2O_3 . Consistent with this possibility, increasing GSH levels with N-acetylcysteine attenuated As_2O_3 cytotoxicity (12). Decreases in GSH levels were associated with AA metabolism. Clinically relevant doses of AA decreased GSH levels and potentiated the As_2O_3 -mediated cell death of the four multiple myeloma cell lines. Similar results were obtained in freshly isolated

human multiple myeloma cells (12). AA is widely heralded as an antioxidant (13). However, it was shown that AA acts as an oxidizing agent, particularly in the presence of compounds that increase the production of reactive oxygen species (14). The pro-oxidant effects of AA and potentiation of cell death induced by free radicals appear to involve the production of hydrogen peroxide (H_2O_2) (14,15). However, AA alone had no effect on cell viability suggesting that AA does not produce a sufficient level of H_2O_2 to initiate oxidative damage. Instead, AA treatment increases basal levels of cellular H_2O_2 (12). Grad *et al* showed that clinically-relevant doses of AA decreased GSH levels and potentiated the As_2O_3 -mediated cell death of four types of multiple myeloma cell lines (12).

Accordingly, the likelihood that AA increases the cytotoxicity of As_2O_3 in the KG-1a human leukemia cell line was investigated. Two doses of As_2O_3 , 2 and 4 μM , were tested in the presence or absence of AA in a concentration of 100 μM . The results obtained indicate that 2 μM of As_2O_3 alone resulted in 6.88% late apoptosis compared to 7.12% of late apoptosis in the presence of AA. In addition, late apoptosis induced by 4 μM of As_2O_3 alone was 8.31 compared to 8.14% in the presence of AA. These findings indicate that in our protocol, AA did not enhance the cytotoxicity of As_2O_3 . Therefore, subsequent studies were conducted without adding AA to As_2O_3 .

Thalidomide (Thalomid[®]), α -(N-phthalimido) glutarimide, is an immunomodulatory agent. Thalidomide has numerous characteristics that may contribute to its role as a potential agent in the treatment of malignant and immunological diseases. The drug inhibits angiogenesis by blocking basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (16), modulates various cytokines (17), enhances cell-mediated immunity by directly co-stimulating T-cells (18) and alters adhesion molecule expression (19).

Thalidomide at 5 mg/l (19 μM) resulted in 49.385% late apoptosis compared to 5.345% of the control. These results were consistent with Du *et al* (20), who stated that thalidomide inhibited tumor growth in a concentration-dependent manner in MCF-7 and HL-60 cell lines. Moreover, IC50S (inhibitory concentration of 50%) for the cell lines were 18.36 \pm 2.34 and 22.14 \pm 2.15 μM , respectively (20).

Efforts were made in our study to increase the cytotoxicity of thalidomide by combining it with other chemotherapeutic agents. The first agent used was IL-2. IL-2 (Proleukin, Aldesleuken for injection), currently being used to treat adults with metastatic melanoma and renal cell carcinoma, was also examined in this study to see whether it was able to enhance thalidomide cytotoxicity. IL-2 alone resulted in 4.59% late apoptosis compared to 5.35% late apoptosis in the control. The combination of thalidomide and IL-2 resulted in 35.39% late apoptosis compared to 49.39% late apoptosis for thalidomide alone. Thus, it was shown that IL-2 neither enhanced thalidomide toxicity nor was cytotoxic itself when used as a single therapeutic modality.

The reason that IL-2 was not cytotoxic to the leukemic cell line in this setting may be due to an unfavorable microenvironment since this was an *in vitro* study. Other studies investigated the development of an approach aimed at preventing relapse after autologous hemopoietic cell transplantation (AHCT). Post-transplant relapse is due to minimal residual disease in

the body of the patient that persists after the conditioning regimen and/or presence of leukemic cells in the auto graft (21). Following an allogeneic transplant, the rate of leukemic relapse is lower for two reasons: the graft is free of leukemic cells and, more importantly, the graft vs. leukemia (GVL) effect associated with the transfer of donor T- and natural killer-cells is able to immunologically eradicate host leukemic cells (22). Therefore, efforts have been made to lower leukemic relapse after AHCT by either purging the leukemic cells in the auto graft (23) or inducing an autologous GVL effect by immunotherapy (24). Since the results of the two approaches were not always satisfactory, other approaches which actively eradicated the residual disease from the patient's body, as opposed to those that only eliminated the contaminating leukemic cells in the graft, were adopted to ensure long-term disease-free survival (25). Reported immunotherapeutic approaches are diverse but the majority include the use of *in vivo* IL-2 with or without adoptively transferred lymphokine-activated killer (LAK) cells. However, this approach is limited by the fact that the doses of IL-2 required to maintain LAK activity *in vivo* cause undesirable side effects (26). Alternatively, several other cytokines including IL-1 (27), IL-4 (28), IL-7 (29), IL-12 (29) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (30) have shown to be able to induce LAK activity themselves or in combination with IL-2. Rojas *et al* (31) demonstrated that immunotherapy with IL-2 + GM-CSF after total body irradiation results in a net improvement in survival in BALB/C mice injected with LSTRA leukemic cells (31). Other investigators reported the *in vitro* and *in vivo* generation of LAK activity by the synergistic effects of IL-2 and GM-CSF (30,32). However, the ability of this approach to cure leukemia or reduce the post-transplant relapse rate has yet to be conclusively demonstrated. Thus, IL-2 would be more effective if combined with a cytokine such as GM-CSF.

As_2O_3 is a chemotherapeutic agent used in the current study to enhance the cytotoxicity of thalidomide. As_2O_3 (Trisenox[®]) is currently used in the treatment of the first relapse of acute promyelocytic leukemia. The detailed mechanisms of As_2O_3 cytotoxicity are not completely known, but various preclinical studies have provided insight into the processes involved. The mechanisms include cellular differentiation, induction of apoptosis, and degradation of specific acute promyelocytic leukemia (APL) transcripts, antiproliferation, and inhibition of angiogenesis (33). Numerous studies examining the activity of arsenic utilized the prototype APL cell line NB4, which carries the t(15;17) translocation involving the RAR- α and PML genes (34). This activity generates a PML/RAR- α fusion protein between RAR- α , a nuclear receptor for retinoic acid and PML, a growth suppressor localized on nuclear-matrix-associated bodies. In APL studies, As_2O_3 induced a differential effect that was shown to be dose-dependent in that it induced partial differentiation at low concentrations (0.1-0.5 mmol/l) and induced apoptosis at relatively high concentrations (0.5-2.0 mmol/l) (35). Apoptosis in APL patients was shown to be, in part, secondary to the down-regulation of bcl-2 gene expression at the protein and mRNA levels as well as through modulation of PML-RAR- α and PML (36,37). The down-regulation of bcl-2 protein is independent of PML and PML/RAR- α expression (38). Davison *et al* showed that the apoptotic effect of As_2O_3 in APL is partially dependent on JNK

activation (39). The antileukemic effects of all-trans retinoic acid and As₂O₃ target RAR- α and PML, respectively, while both induce the degradation of PML/RAR- α fusion proteins in NB4 cells (35,40). As₂O₃ induces the degradation of PML/RAR- α (as well as the wild-type PML) over a wide range of concentrations (0.5-2.0 mmol/l) (35). Moreover, As₂O₃ was shown to induce the degradation of the PML/RAR- α fusion protein in retinoic acid-resistant cells (35,41).

In related hematologic malignancies, such as multiple myeloma and lymphoma, preclinical studies of As₂O₃ have demonstrated similar apoptotic effects (15,36,42,43). Park *et al* demonstrated that As₂O₃-induced G1 and/or G2M phase arrest in myeloma cells (36). Simultaneous induction of cyclin-dependent kinase inhibitor, p21 was also noted. Liu *et al* showed that As₂O₃ induced apoptosis in resistant cell lines and fresh myeloma cells through p53-dependent cell cycle arrest and activation of extrinsic and intrinsic caspase pathways (43). Evidence exists of an immune mechanism with As₂O₃ in myeloma cells with elevated LAK and other immune cells (44). A similar immune mechanism has yet to be demonstrated in As₂O₃-treated APL.

The anti-leukemic effect of As₂O₃ may partially be related to the inhibition of angiogenesis due to interruption of the reciprocal stimulant loop between endothelial cells releasing cytokines which stimulate leukemic cells to release growth factors such as VEGF (through apoptosis of the two cell types) (45). Another mechanism of As₂O₃-induced apoptosis is through the activation of caspases. As₂O₃ activates these proteases, which play an important role in the degradation phase of apoptosis, in NB4 cell lines (43,46,47). As₂O₃ is able to lead to potential changes in the membrane and increased membrane permeability with the resultant degradation phase of apoptosis. Furthermore, the ability of As₂O₃ to induce apoptosis is dependent on the generation of reactive oxygen species (46,48). This suggests that the effect of As₂O₃ is potentiated through modulation of the glutathione redox system.

As₂O₃ was used in the current study to test its efficacy in the enhancement of the cytotoxicity of thalidomide in an acute myelogenous human leukemia cell line. It was noted that As₂O₃ alone resulted in 16.97% late apoptosis compared to 5.35% late apoptosis in the control. When As₂O₃ was used concurrently with thalidomide it resulted in 80.6% late apoptosis compared to 49.39% late apoptosis of thalidomide alone. The combination of thalidomide, As₂O₃ and IL-2 resulted in 70.14% of late apoptosis. Thus, in our study As₂O₃ enhanced the cytotoxic effect of thalidomide in contrast to IL-2 which did not manifest any noticeable cytotoxicity. Further studies are warranted to prove or disprove the potential role of IL-2 to enhance the cytotoxicity of thalidomide.

In conclusion, this study showed that the cytotoxic effect of thalidomide is significantly enhanced when combined with As₂O₃ in the KG-1a human AML cell line. These findings point to the potential use of As₂O₃ in combination with thalidomide for a more efficient management of AML by increasing the rate of complete remission for AML patients and the least chance of relapse. Further studies are needed to confirm the current findings. However, the results obtained are promising since thalidomide effectiveness in the therapy of AML may be significantly enhanced by the concurrent use of As₂O₃. This may aid patients who suffer from this disease.

Acknowledgements

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