Emodin and DHA potently increase arsenic trioxide interferon-α–induced cell death of HTLV-I–transformed cells by generation of reactive oxygen species and inhibition of Akt and AP-1

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Abstract

Introduction

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disease of poor clinical prognosis associated with infection by the human T-cell leukemia virus type I (HTLV-I). The use of arsenic trioxide (As2O3) has been shown to effectively treat acute promyelocytic leukemia (APL) with greater than 80% of patients achieving complete remission. The combination of arsenic and interferon has also shown promising results in the treatment of ATL.

The requirement for slow dosage increases of arsenic and the time required to achieve a pharmacologic active dose in patients is a major obstacle because median survival of patients with ATL is about 6 months. In this study we report a potent synergistic effect of the combination of arsenic trioxide and interferon α (As/IFN-α) with emodin and DHA on cell-cycle arrest and cell death of HTLV-I–infected cells. Importantly, we found that clinically achievable doses of DHA and emodin allowed for reduced arsenic concentrations by 100-fold while still remaining highly toxic to tumor cells. Our data provide a rationale for combined use of As/IFN-α with emodin and DHA in patients with ATL refractory to conventional therapy. (Blood. 2007;109:1653-1659)

Keywords: arsenic trioxide, interferon, adult T-cell leukemia, apoptosis, reactive oxygen species, proteasome inhibitors

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Materials and methods

Drugs

IFN-α 2 (Cell Sciences, Canton, MA) and arsenic trioxide (Sigma, St Louis, MO) were used at 100 U/mL and 1 μM, respectively, as previously described. DHA (Sigma) was dissolved in ethanol and used at 10 to 50 μM. Emodin (Sigma) was dissolved in DMSO and used at 10 to 50 μM. Ascorbic acid (Sigma) was dissolved in sterile dH2O and used at 5 mM.

Cell lines

C8166 and MT-2 are HTLV-I–transformed T-cell lines. Jurkat and CEM HTLV-I–negative T-cell lines were grown in RPMI-1640 (Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum, gentamycin, and penicillin-streptomycin. 1185 and LAF, IL-2–dependent T-cell lines immortalized by HTLV-I, and peripheral blood mononuclear cells (PBMCs) were cultured in 20% serum and 40 U/mL IL-2.

Cell-proliferation assay

Cell proliferation was measured using the Cell Proliferation Kit II (XTT; Roche, Mannheim, Germany) according to manufacturer’s instructions. Cells (10^5/mL) were treated 48 to 72 hours in a 96-well plate and then incubated with tetrazolium salt XTT for 4 hours. Proliferation was quantified by measuring cleavage of XTT to an orange formazan dye using an enzyme-linked immunoabsorbent assay (ELISA) reader at 450 nm.

Cell-cycle analysis

Cells (2 × 10^6) were treated 24 to 72 hours then washed with PBS. After resuspension, cells were fixed on ice with 80% EtOH for 30 minutes. Cells were washed with PBS and treated with RNase for 15 minutes at 37°C, followed by staining with 50 μg/mL propidium iodide for 15 minutes at room temperature. Cells were then washed and resuspended in PBS and analyzed by flow cytometry.

Measurement of intracellular ROS production

Production of ROS in cells was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA; Sigma), an oxygen-sensitive fluorescent probe. Cells (5 × 10^5) were incubated for 12 hours, as indicated, with arsenic and IFN-α with or without ascorbic acid, then DHA and emodin were added for another 12 hours. DCFH-DA was added at 5-nM final concentration to each sample and incubated at 37°C for 15 minutes. Cells were then collected and washed with cold PBS. Cells were analyzed by flow cytometry after resuspension in 500 μL PBS.

Apoptosis assay

Cells (5 × 10^6) were treated as described in “Measurement of intracellular ROS production” for the measurement of ROS production, then collected and washed with cold PBS. Cells were then stained with annexin V/propidium iodide using the Vybrant Apoptosis Assay Kit no. 2 (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

Mitochondrial membrane potential

Cells (1 × 10^6) were treated for 24 hours, then changes in the mitochondrial membrane potential (ΔΨm) were measured using the ApoAlert Mitochondrial Membrane Sensor Kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions.

Western blot analysis

Cells were washed with PBS containing phosphatase inhibitors and lysed in RIPA buffer with phosphatase and protease inhibitors (Complete Cocktail; Roche) after 24 to 48 hours of treatment. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon PVDF membranes (Millipore, Billerica, MA), visualization was done with the Supersignal West Dura enhanced chemiluminescence system (Pierce, Rockford, IL). Spot densitometry quantification of expression was performed as previously reported and was expressed as a percentage of the appropriate control sample referred as 100%. The study protocol was approved by the institutional review board at the University of Kansas Medical Center.

Results

Effects of As/IFN and their combination with DHA and emodin on cell proliferation of HTLV-I–immortalized and –transformed T cells

Cellular proliferation was assessed using the Cell Proliferation Kit II (XTT; according to the manufacturer’s instructions. Proliferation was quantified by measuring cleavage of XTT to an orange formazan dye using an enzyme-linked immunoabsorbent assay (ELISA) reader at 450 nm. Results are expressed as the percentage of control-treated cells and represent the mean values of at least 2 independent experiments performed in triplicate. Our results indicated a very strong synergy between As/IFN-α and DHA/emodin at all time points and doses used in the HTLV-I–infected cells C8166 and MT-2, as well as the IL-2–dependent 1185 and LAF cell lines. This was in contrast to those effects not seen in the control PBMCs (Figure 1A-D) or with buffer controls (data not shown). Consistent with previous reports, MT-2 cells were more resistant to As/IFN-α–induced cell death when compared with C8166, LAF, or 1185 cells. However, all HTLV-I cell lines appeared to be equally and highly sensitive to the combination of As2O3/IFN-α with emodin and DHA (AIDE) (Figure 1A).

The effect of emodin and DHA was also tested in the absence of arsenic trioxide and IFNα. As reported in Figure 1B, no effect on cell growth was detected for C8166 cells when cultured in the presence of emodin and DHA at a concentration of 10 μmol/L, which confirms the synergistic action of emodin and DHA with arsenic trioxide and IFN-α (Figure 1A). Because of arsenic trioxide toxicity, a major limitation to its use as a therapeutic agent for patients with ATL is the time required to achieve the clinically active dose. Because emodin and DHA showed a strong synergy with As/IFN-α, we tested whether we could lower the dose of arsenic while still preventing proliferation of tumor cells. The combination of emodin and DHA allowed up to a 100-fold decrease in arsenic concentrations with little loss in efficacy against tumor-cell proliferation (Figure 1E), whereas control cell lines Jurkat and CEM were not significantly affected (Figure 1F).

We next analyzed cell-cycle progression following treatment of HTLV-I cells with As/IFN-α (AI) or AIDE.

Results revealed a marked G0/G1 arrest of tumor cells cultured in the presence of emodin and DHA along with As/IFN-α (Figure 2A). In HTLV-I–infected cells the retinoblastoma protein (Rb) is constitutively phosphorylated, thereby allowing tumor cells to progress to S phase. In agreement with cell-cycle results presented in Figure 2A, we found that Rb accumulated in its hypophosphorylated form in 1185 and C8166 cells (Figure 2B). This effect was more pronounced in cells treated with As/IFN-α with emodin and DHA than those treated with As/IFN alone.
Emodin and DHA reroute As/IFN-treated cells to caspase-independent cell death

Changes in mitochondrial membrane potential, ΔΨm, are a critical step in cells undergoing apoptosis, regardless of the death signal. Therefore, ΔΨm was measured in C8166 cells (Figure 3A). As previously reported As2O3/IFN-α–treated cells that entered apoptosis showed an altered mitochondrial membrane permeability that led to ΔΨm collapse. Consistent with the increased ROS generation, mitochondrial membrane permeability collapse was much more pronounced when DHA and emodin were added to As2O3/IFN-α (Figure 3A). By contrast, control C8166 cells treated with buffer did not show any change in ΔΨm.

ΔΨm collapse is usually associated with the activation of caspases. Caspase-3 is a critical downstream protease in the caspase cascade, and poly-ADP-ribose polymerase (PARP) is a substrate of caspase-3 protease activity that is associated with apoptosis. Several studies have reported that As2O3/IFN-α–mediated apoptosis of HTLV-I–infected cells is partially a caspase-dependent process and partially caspase independent. To gain insight into the mechanism used by emodin and DHA, cells were analyzed by flow cytometry using double staining (annexin V/PI). Apoptotic cells were scored as annexin V−/PI− versus necrotic dead cells, which were PI−. Results indicated that DHA and emodin did not significantly increase the apoptotic activity of As2O3/IFN-α but, instead, triggered a potent necrotic-cell death signal, resulting in nearly 90% cell death after 48 hours of treatment (Figure 3B). Similar results were observed with lower doses of DHA and emodin and longer incubation times (data not shown).

These results were further confirmed by Western blot analysis of procaspase 3 and PARP (Figure 3C). Our results indicate that DHA and emodin are not associated with cleavage of the PARP, because the 85-kDa fragment representing the cleaved form was not detected after treatment of HTLV-I cells by As2O3/IFN-α with DHA and emodin.

Together, with the flow cytometry data, this suggests that the combination of As2O3/IFN-α with emodin and DHA is a potent inducer
of necrotic-cell death in HTLV-I–infected cells (Figure 3B-C). Spot densitometry quantification analyses of the expression of several proteins implicated in survival pathways revealed a decrease in Bcl-2 expression of 37% and 68% following treatment with Al and AIDE, respectively (data not shown). Concomitantly, we found an increase in Bax expression, 200% and 150%, following treatment with Al and AIDE, respectively (data not shown). In contrast to a previous report, the proapoptotic cleaved form of Bcl-2 was not detected in our assay (data not shown). This is possibly due to differences in experimental procedures because we did not use purified mitochondria in our study. Other markers, such as Bcl-xL, were not significantly affected in our experimental conditions (data not shown).

**Emodin and docosahexaenoic acid dramatically increase generation of ROSs and loss of mitochondrial membrane potential in As/IFN-treated cells**

Because previous studies have shown that lower levels of ROSs promote apoptosis and higher intracellular levels of ROSs are associated with necrosis, we tested whether emodin and DHA treatment may increase the concentration of intracellular ROSs in HTLV-I cells following treatment with As/IFN-α and DHA/ emodin. ROS production was not changed in As/IFN-α-treated C8166 cells, but ROS production significantly increased when DHA and emodin were added with As/IFN-α. PBMCs used as control were not affected under the same experimental conditions (Figure 4A).

To demonstrate that ROSs were involved in HTLV-I–cell death, we used ascorbic acid, a well-known ROS scavenger. Treatment with ascorbic acid effectively reduced the generation of ROSs in C8166 cells treated with As/IFN-α and emodin and DHA (data not shown). In parallel experiments the presence of ascorbic acid efficiently prevented the death of HTLV-I cells treated with As/IFN-α and emodin and DHA as demonstrated by propidium iodide and FACS analysis (Figure 4B; compare left and right panels). Generation of ROSs has been linked to increased Bax expression, and ROS scavengers can prevent cell death by reducing Bax protein expression. In fact, when we performed Western blot analysis in HTLV-I cells treated with As/IFN-α and emodin and DHA in the absence or in the presence of ascorbic acid, we found that the presence of ascorbic acid reduced Bax expression by 30% to 35% (data not shown).

![Figure 3. AIDE induces the collapse of mitochondrial membrane potential and increased Bax and decreased Bcl-2 expression and tumor-cell death.](image)

As/IFN with emodin and docosahexaenoic acid inhibit Akt pathway and reduce levels of JunD and JAB1 expression in HTLV-I cells

Recent studies suggest that DHA inhibits activator protein 1 (AP-1) activity. AP-1 plays a critical role in HTLV-I oncogenesis, is expressed at high levels in ATL patient samples, and may be required for maintenance of the malignant phenotype. Among AP-1’s components, c-Jun is a potent transcriptional activator, and its activity is regulated through phosphorylation by Jun N-terminal kinase (JNK), a member of the MAP kinase family. AP-1 becomes activated through phosphorylation of Jun family transcription factors by members of the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) family. Previously, constitutive JNK activation has been reported in HTLV-I–infected T cells, suggesting that HTLV-I–induced JNK activation may contribute to the activation of AP-1 in T cells. However, in our experiments only a small decrease in phosphorylation of JNK2 or JNK3 was detected in HTLV-I cells treated with As/IFN-α with emodin and DHA (data not shown). Previous studies found that JunD is the major component of the AP-1–DNA complex in HTLV-I–infected T-cell lines. Others have reported that the
Jun-activating binding protein (JAB1) selectively potentiates trans-activation by JunD. Interestingly, the expression of both JunD and JAB1, in cells treated with As2O3 with DHA and emodin, was reduced (Figure 5A). Because recent studies suggest that Akt, an essential survival pathway, is activated by HTLV-I, we also tested expression of Akt in HTLV-I cells treated with As2O3 and with emodin and DHA. Although As2O3 had no significant effect on Akt expression, the addition of emodin and DHA strongly down-regulated Akt expression by 25% to 50% (Figure 5B).

Spot densitometry quantification indicated that a longer incubation time or a higher dose was required to achieve inhibition levels comparable to those observed on Akt, and that JunD was more affected than JAB1 (Figure 5C). However, proliferation assays demonstrated that HTLV-I-transformed cells are more susceptible to As2O3 with emodin and DHA than normal PBMCs (Figure 5D). Antiproliferative effects were more pronounced when emodin and DHA were used at 25 and 50 μmol/L, suggesting that the inhibition of both AP-1 and Akt may have an additive effect on preventing growth of HTLV-I-transformed cells.

Discussion

Disruption of cellular death pathways can contribute to a number of diseases, including cancer. It is now well established that anticancer agents induce apoptosis or necrosis and that disruption of cellular death pathways can reduce treatment sensitivity. In vitro and in vivo HTLV-I-transformed cells are highly resistant to most death-inducing agents. Although the basis for this resistance is not fully understood, it has been reported that HTLV-I-infected cells overexpress antiapoptotic factors such as Bcl-2, Bcl-xL, survivin, and I-309.

Studies suggest a broad spectrum of antileukemic activity for As2O3 because of its ability to increase intracellular ROS production. In As2O3-sensitive cells, such as the APL-derived cell line NB-4, increased intracellular levels of ROSs lead to collapse of the mitochondrial membrane potential, activation of the caspases, and apoptosis. However, cells with intracellular antioxidant defense mechanisms, such as the acute myeloid leukemia (AML)-derived cell line HL-60, are resistant to As2O3. To render As2O3-resistant tumor cells more susceptible to As2O3-mediated cell death, ways to enhance the effects of As2O3 have been investigated. Importantly, agents modulating the GSH redox system as well as agents increasing intracellular ROS concentrations have been shown to increase As2O3 efficacy in cells otherwise resistant to As2O3. It is well known that clinically achievable concentrations of As2O3 (that is, 1–2 μM) induce insufficient amounts of ROSs to induce apoptosis of HL-60 cells in vitro. However, recent studies have shown an enhancing effect of DHA on As2O3-mediated cytotoxicity in some As2O3-resistant cell lines, indicating that the combined effect of As2O3 and DHA is not restricted to AML-derived HL-60 cells but may also be observed in other As2O3-resistant hematologic malignancies, including Burkitt lymphoma and hairy-cell leukemia. Recent clinical studies have shown some antileukemic effect of As2O3, suggesting that ATL cells may respond by apoptosis or cell-cycle arrest in vivo. Differences in sensitivity to arsenic, as well as the toxicity associated with its use, make it worthwhile to investigate drug combinations that could enhance the actions of arsenic and reduce the level necessary for treatment.

In this study, we found a synergistic effect of the combination of As2O3 with emodin and DHA in HTLV-I-infected cells. IL-2–dependent or IL-2–independent HTLV-I–infected cells, as well as freshly isolated patient samples, were all highly sensitive to this drug combination. The rationale for this drug combination was because emodin has been shown to reduce invasiveness of tumor cells and to enhance As-mediated ROS production. In addition, DHA was added to this combination because DHA sensitizes tumor cells to ROS-inducing agents (As and emodin in our case).

Our results indicate a potent antiproliferative effect on tumor cells, which was linked to the accumulation of hypophosphorylated Rb and G1/G0 arrest. Recent reports have shown that Tax can induce JNK activity and e-Jun phosphorylation, leading to the formation of Smad3/c-Jun complexes and inhibition of TGF-β signaling. In fact, in ATL and in normal T cells transduced by Tax, c-Jun is constitutively phosphorylated and acts as a repressor of TGF-β signaling. Thus, our results suggest that As2O3 in the presence of emodin and DHA may lead to reactivation of TGF-β, thereby preventing the growth of tumor cells. Further investigations in this area are warranted. We also found a significant reduction in Akt expression which may in part be responsible for increased cell death in treated cells.

Previous studies have shown that HL-60 cells are resistant to clinically relevant doses of arsenic but have 90% reduction in viability, increased intracellular ROSs, and up-regulation of Bax.
when treated with a combination of arsenic and DHA. Similarly, we readily detected increased ROS production in HTLV-I–infected cells, but not in healthy PBMCs, treated with As/IFN-α and emodin and DHA. To further demonstrate that ROS production played a role in tumor growth suppression and death of HTLV-I–expressing cells we measured cell death by FACS in the absence or presence of ascorbic acid, a ROS scavenger. Results clearly demonstrate the importance of ROSs, because significant cell death was prevented by ascorbic acid with concomitant down-regulation of Bax to normal endogenous levels. In human hematopoietic malignancies, continuous AP-1 DNA binding activity involving JunD has been found in cell lines of cutaneous T-cell lymphoma and patients with Sézary syndrome. Previously, it has been shown that HTLV-I–transformed cells and samples from patients with ATL have constitutive AP-1 and JNK activity and that these may play a role in the maintenance of the tumor growth potential. In fact, our data are in agreement with these observations, because we found an inhibition of the AP-1 pathway characterized by a dose-dependent decrease of JunD and JAB1. Inhibition of NF-kB pathway or survivin expression by As/IFN-α have also been reported by other groups and are likely to play a role in the suppression of tumor-cell proliferation by arsenic. However, they do not appear to be involved in the synergy described here between As/IFN-α, emodin, and DHA and, therefore, were not further investigated.

In conclusion, we have shown high synergy of As/IFN-α with emodin and DHA treatment with regard to the inhibition of proliferation and induction of cell death of HTLV-I–transformed T cells. In addition, the synergistic effectiveness of the combination treatment at pharmacologic doses was specific for HTLV-I–infected cells and had marginal effect on PBMCs isolated from healthy donors. The drug combination described here allows the As concentration to be decreased by 100-fold while preserving its antitumor activity, suggesting a broad application in the treatment of leukemias and lymphomas and warrants consideration for a phase 2 clinical study.

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Authorship

Contribution: M.B. performed experiments described in Figures 1 to 5; M.B. performed some experiments described in Figure 3; C.N. is responsible for the design of the project and interpretation of the results.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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