The Effects of Arsenic Trioxide in Combination with Retinoic Acids on Cutaneous T-Cell Lymphoma Cell Lines

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Key Words

Arsenic trioxide · Combination therapy · Sézary syndrome · Retinoids · All-*trans*-retinoic acid · Bexarotene · Cutaneous T-cell lymphoma · Mycosis fungoides

Abstract

Cutaneous T-cell lymphomas (CTCL) are characterized by an infiltration of the skin with malignant T cells. Curative treatments for aggressive entities such as Sézary syndrome have not been identified yet. Arsenic trioxide (AsO₃) is used for the treatment of acute promyelocytic leukemia in combination with retinoids. As the latter are established treatment options in CTCL, we sought to evaluate the efficacy of AsO₃ for mono- and combination therapy in vitro. Analyses for apoptosis, cell cycle inhibition, cytotoxicity and cell viability were made after incubation of CTCL cells with AsO₃ alone or in combination with the retinoids all-*trans*-retinoic acid or bexarotene. While AsO₃ induced apoptosis, retinoids did not at the time point of analysis. However, retinoids strongly reduced cell viability. Due to the efficient apoptosis induction, AsO₃ might be a potentially suitable agent for CTCL treatment, although this effect was not increased by retinoids.

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Introduction

Sézary syndrome belongs to the group of primary cutaneous T-cell lymphoma (CTCL), characterized by a cutaneous infiltration as well as leukemic distribution of malignant T cells and a 5-year survival of only 24% [1, 2]. Even considering the overall scarcity of CTCL, it is a rare condition with a reported incidence of 0.1/1 million person-years [3]. Despite many of the mechanisms leading to malignancy being still unclear, resistance to apoptosis seems to be one of the major features of disease propagation in CTCL [4]. In this context, overexpression of NF-κB, bcl-2, and c-FLIP among others is known to contribute to apoptosis resistance [5]. Furthermore, it has been shown that agents inducing apoptosis in CTCL cells often act via downregulation of these overexpressed anti-apoptotic factors [6, 7]. This is of interest, because the initial treatment of CTCL like mycosis fungoides and Sézary syndrome is preferably done with immunomodu-

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latory agents such as interferon- α , photochemotherapy (PUVA), extracorporeal photopheresis or retinoids like bexarotene, the latter even being able to be delivered topically [8, 9]. Although these therapies have proven efficacious, remissions in general are not long lasting. Cytoreductive therapies, despite also being effective, achieve neither long-lived remissions nor do they alter the course of disease when used early after diagnosis [10, 11]. Furthermore, they impair the immune system that is already afflicted by the lymphoma itself. The severity of Sézary syndrome and a limited existing arsenal of treatment options underline the necessity for conducting research on novel therapeutical modalities. In this respect, arsenic trioxide (AsO_3) is a substance with known efficacy for the treatment of hematologic malignant diseases. Having been used since antiquity for a variety of ailments [12], AsO_3 has been identified as the effective component of a traditional Chinese medicine in the treatment of acute promyelocytic leukemia (APL) and is nowadays approved for this disease [13]. Furthermore, antineoplastic effects were observed in a variety of tumor cell lines, mostly due to cell cycle arrest or direct induction of apoptosis [14–16]. Due to this, in particular two factors make this substance a promising agent for CTCL treatment: the first is the fact mentioned above, namely that the mode of action seems to rely on induction of apoptosis and among others downregulates bcl-2 that as stated mediates apoptotic resistance in CTCL [17]. Secondly AsO_3 proved to be particularly effective in APL when being combined with the retinoid all-*trans*-retinoic acid (ATRA), achieving complete remissions in 11 of 12 patients in a pilot study [18]. Concerning CTCL, in vitro experiments have shown that AsO_3 can induce apoptosis in CTCL cell lines. Furthermore, intratumoral injection of AsO_3 led to complete remission in a mouse model of CTCL, and treatment of 2 CTCL patients with AsO_3 led to stable disease in the one and partial remission in the other patient [19, 20]. The finding of a favorable combination of AsO_3 and retinoids in APL is especially interesting, as the latter represent a mainstay in CTCL treatment. They exert a multitude of effects in a variety of cells, affecting proliferation, differentiation, immune response and apoptosis and act by binding to specific nuclear receptors [21]. Two receptor classes are known, retinoid A receptor (RAR, comprised of three different known types), targeted among others by ATRA, and retinoid X receptor (RXR), the target of bexarotene. Studies indicate that in CTCL treatment, but of other malignancies such as melanoma cell lines as well, cell cycle inhibition seems to be the most important antineo-

plastic effect of retinoids and to offer the possibility of increased efficacy when combined with AsO_3 [22, 23]. These data represented the rationale for in vitro assessing the efficacy and mode of action of AsO_3 alone and in combination with several established retinoids for the treatment of cutaneous lymphoma cell lines.

Materials and Methods

Cell Lines and Culture Conditions

The two CTCL cell lines SeAx [24] and Hut-78 [25], both established from patients with Sézary syndrome, were cultured at 37°C, 5% CO_2 in RPMI 1640 medium (Invitrogen/Life Technologies) supplemented with L-glutamine, fetal bovine serum, and antibiotics (100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, all PAA, Austria). AsO_3 (Sigma-Aldrich, Germany) stock solution was dissolved in 1 mM NaOH at 30 μM and diluted in cell culture medium for the final dose. ATRA (Sigma-Aldrich) and bexarotene (Calbiochem, USA) were dissolved in dimethylsulfoxide, ascorbic acid and N-acetylcysteine in sterile water. For all experiments with single substances, cells were cultured in multiwell plates with the respective substance for 24–72 h. For combined treatment of cells with ascorbic acid and N-acetylcysteine, cells were preincubated with the respective substance for 1 h before addition of AsO_3 . For combined treatment with retinoids, cells were preincubated with ATRA or bexarotene for 48 h and then treated with AsO_3 for 24 h. The respective solvents were used as controls.

Cell Cycle Analysis and Detection of Apoptosis

Cells were harvested by centrifugation, washed once with cold phosphate-buffered saline, fixed with prechilled 70% ethanol at –20°C for 24 h, and stained for 30 min with phosphate-buffered saline containing 0.1% sodium citrate, 20 $\mu\text{g}/\text{ml}$ propidium iodide, 0.2 mg/ml RNase A and 0.1% Triton X-100. Cells were then analyzed on a flow FACSCalibur flow cytometer (BD Bioscience) using CellQuest Pro (BD Bioscience), WinMDI 2.9 (Scripps Research Institute, La Jolla, Calif., USA).

Cytotoxicity Assay

Cytotoxicity was determined by measuring the amount of lactate dehydrogenase (LDH) in the cell culture supernatant using the Cytotoxicity Detection Kit from Roche Diagnostics according to the recommendations of the manufacturer.

Apoptosis Detection by DNA Fragmentation ELISA

Apoptosis was measured by detection of DNA fragmentation using the Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's recommendations. Optical density values were normalized by division through the optical density of LDH activity (Cytotoxicity Detection Kit, Roche; see above) of the cell lysate to compensate for different amounts of lysed cells.

Cell Viability Assay

Cell viability was analyzed with the cell proliferation reagent WST-1 (water-soluble tetrazolium; Roche), as recommended by the manufacturer. The results correlate with the number of metabolically active cells.

Fig. 1. Effects of AsO₃ on CTCL cells. Values represent means $\pm$ SD. **a** Cell lines Hut-78 (squares) and SeAx (circles) were treated for 24 h with AsO₃ or solvent control (indicated at 0 μ M) and analyzed subsequently by flow cytometry for the percentage of cells in the subG₁ fraction to determine induction of apoptosis (n = 3, * p < 0.05, ** p < 0.001). **b** Hut-78 cell culture supernatants were analyzed for LDH content by ELISA to determine cytotoxicity (n = 3, * p < 0.001). **c, d** The effect of vitamin C (vit. C) and N-acetylcysteine (NAC) on apoptosis induction by 2.5 and 5 μ M AsO₃, respectively, in Hut-78 cells was analyzed by flow cytometry as described (n = 3, * p < 0.01).

Statistics

Statistics were done using Graph Pad Prism 5 (GraphPad Software Inc., La Jolla, Calif., USA); statistical analyses were done by t test or one-way ANOVA where appropriate. Significance levels are given in the text or the figure legends.

Results

Effects of AsO₃ on CTCL Cells

First we tested the effect of AsO₃ on apoptosis induction in CTCL cells. To this end, the CTCL cell lines Hut-78 and SeAx were incubated with different concentrations of AsO₃ for 24 h, and the rate of apoptotic cells was determined by flow-cytometric analysis (FACS) of the subG₁ population. In both cell lines, the percentage of apoptotic cells increased concentration dependently, becoming significantly different from the control at 2.5 μ M for Hut-78 and at 5 μ M for SeAx cells (fig. 1a). To test for cytotoxic effects of AsO₃ on CTCL cells, we conducted an assay analyzing the release of LDH as a parameter for

damaged cells. Hut-78 cells were incubated as described above, and LDH content in the cell culture supernatant was measured at 24 h. The amount of LDH was significantly increased at a concentration of 5 μ M (fig. 1b).

The efficacy of AsO₃ for inducing apoptosis depends on the antioxidative potential of cells. Prior studies have shown that apoptosis induction by AsO₃ occurs via modulation of the antioxidant glutathione [26]. Furthermore, it has been demonstrated that induction of apoptosis is increased by depletion of intracellular glutathione with ascorbic acid (vitamin C) in cancer cells including lymphoma cell lines [19, 27] but inhibited by N-acetylcysteine, which increases intracellular glutathione levels in carcinoma and myeloma cells [28, 29].

To test if these mechanisms apply to Hut-78 and SeAx cells, both cell lines were incubated with either 1 mM N-acetylcysteine or 100 μ M vitamin C for 1 h before addition of either 2.5 μ M AsO₃ to vitamin C or 5 μ M AsO₃ to N-acetylcysteine for 24 h. Apoptosis induction was measured by determination of the subG₁ population by flow cytometry as well as DNA fragmentation by ELISA. Incu-

Fig. 2. Effects of retinoids on CTCL cells. Values represent means ($n = 9$) $\pm$ SD. **a** Hut-78 cells were treated for 72 h with ATRA, bexarotene or solvent control (indicated at 0 μM) and analyzed subsequently by flow cytometry for the percentage of cells in the subG₁ fraction to determine induction of apoptosis (* $p < 0.05$, ** $p < 0.01$).

White bars represent ATRA, gray bars represent bexarotene. **b** Cell viability after retinoid treatment was analyzed with the WST reagent and photometry (** $p < 0.01$); triangles represent ATRA, squares represent bexarotene.

bation of cells with vitamin C or N-acetylcysteine as a control did not induce apoptosis at the respective concentrations. While vitamin C led to a 3-fold increase, N-acetylcysteine led to a 2.2-fold decrease in AsO₃-induced apoptosis in Hut-78 cells (fig. 1c, d). These effects were also seen in the DNA fragmentation ELISA and observed after treatment of SeAx cells (data not shown). Due to the demonstration of induction of apoptosis without concomitant cytotoxic effects, we used 2.5 μM AsO₃ for further experiments. The induction of apoptosis at this dose was also confirmed by demonstration of a significant increase in DNA fragmentation in Hut-78 cells after treatment with 2.5 μM AsO₃ for 24 h. It is noteworthy that the dose of 2.5 μM AsO₃ has been shown to be readily achievable in patients during treatment [30].

Effects of the Retinoids ATRA and Bexarotene on CTCL Cells Alone and in Combination with AsO₃

The main scope of our study was to test whether retinoids are able to augment AsO₃-induced effects in CTCL cells as it is seen in APL. For these experiments ATRA, which ligates the RAR and is used as combination partner in APL, and bexarotene, an RXR-specific retinoid approved for the treatment of primary CTCL, were used. Hut-78 cells were incubated for 72 h, as this time span was used in previous studies, with 1, 3, 6, 10, and 25 μM of ATRA or bexarotene [31]. It has to be noted in this re-

spect that at the dosages approved for clinical use ATRA and bexarotene readily reach a plasma concentration of about 1 and 3 μM , respectively, as could be shown in pharmacokinetic studies [32, 33].

To determine the induction of apoptosis by retinoids, the percentage of cells in the subG₁ populations was measured by flow cytometry. Surprisingly, a significant reduction was seen after treatment with 1 μM ATRA and all concentrations tested for bexarotene (fig. 2a). For ATRA a dose-dependent increase in apoptosis induction with a significant difference between 1 and 25 μM was seen.

To assess cell viability, we conducted a WST assay, which correlates with the number of metabolically active cells. Despite low levels of apoptosis, a significant reduction of cell viability was seen at the concentration of 1 μM ATRA or bexarotene, which was not increased dose dependently up to 25 μM (fig. 2b). To exclude a cytotoxic effect of the retinoids on CTCL cells, an LDH assay was made and did not demonstrate significant differences in comparison to control treatment (data not shown).

As clear dose-dependent effects were not demonstrated for both retinoids, we chose to evaluate the effects in combination with AsO₃ at the minimal and maximal dose of 1 and 25 μM .

Cells were first incubated for 48 h with the retinoid alone and then for another 24 h with the retinoid in combination with 2.5 μM AsO₃. Analysis of the subG₁ fraction

Fig. 3. Effects of combination with AsO₃ and retinoids on CTCL cells. Hut-78 cells were treated for 72 h with ATRA, bexarotene or solvent control. AsO₃ was added in indicated (+) samples at 2.5 μM for the last 24 h of the incubation period. Cells were subsequently analyzed by flow cytometry for the percentage of cells in the subG₁ fraction to determine induction of apoptosis (**a**) or with the WST reagent and photometry to determine cell viability (**b**). Significant differences are described in the Results section. Bars show means (n = 3) ± SD. **c** Distribution of cells in the G_{0/1} (■), S (▨) and G₂ phases (□) was analyzed by FACS. Columns represent mean values from triplicates.

demonstrated that the percentage of apoptotic cells after combination treatment was significantly higher compared to ATRA but significantly lower in comparison to AsO₃ alone. However, the overall effects observed were rather small. Comparable results were seen with the combination of bexarotene and AsO₃, though no significant reduction of AsO₃-induced apoptosis was seen at the dose of 25 μM (fig. 3a).

The WST assay showed that in all settings, treatment led to a decreased cell viability compared to the control. While addition of retinoids to AsO₃ resulted in a significant decrease ($p < 0.01$) compared to the AsO₃ alone, the combination did not further increase retinoid-induced reductions of cell viability (fig. 3b). The LDH activity in the cell culture supernatant was measured, and none of the combinations exhibited significant cytotoxic effects (data not shown). Regarding the distribution of cells in the different phases of the cell cycle, we observed only a slight reduction in cells in the S phase after ATRA but not

bexarotene treatment while an arrest in the G₁ phase was observed for AsO₃, which was not influenced by preceding retinoid treatment (fig. 3c).

Discussion

Induction of apoptosis and cell cycle arrest are thought to be major mechanisms of treatment modalities used for the treatment of CTCL like retinoids, interferons and photochemotherapy [34–36]. Previous data on the successful use of AsO₃ in conjunction with the retinoid ATRA in APL led us to the question whether this combination might also prove beneficial in the therapy of CTCL. In APL a distinct mutation resulting in a fusion gene of the RAR-α and tumor-propagating genes are thought to represent the target for ATRA. Although such a driving mutation has not been demonstrated in CTCL, both ATRA and bexarotene have been reported to be efficient

in CTCL treatment and to affect the microenvironment of the tumor cells, e.g. protein expression in keratinocytes [37–41]. Our results demonstrate that incubation of CTCL cell lines with AsO₃ leads to a dose-dependent increase in apoptosis without cytotoxic side effects at concentrations that can be reached under therapeutical conditions. Only at higher concentrations like 5 μM was LDH significantly increased arguing for a direct cytotoxic effect of AsO₃ at this concentration. Additionally, we confirmed the augmenting effects of vitamin C on apoptosis induction and introduce data that N-acetylcysteine also in CTCL cell lines almost completely countermands the effects of AsO₃ underlining the importance of the cell's redox system for both the apoptotic effect of AsO₃ and carcinogenesis in CTCL or in general [42, 43]. Furthermore, the concentration of vitamin C used is nearly reached in the plasma under normal nutritional conditions, leading to the question whether a dietarily induced higher uptake could influence the efficacy of treatment [44].

When investigating the effects of retinoids alone, we found a significant increase in apoptosis neither for ATRA nor for bexarotene after 72 h of treatment. This time span has been sufficient in some but not in other reports on apoptosis induction of retinoids in CTCL cells. As different working groups have employed varying analytic settings regarding dose range, incubation periods and cell type, the results obtained are not easily comparable. In our experiments neither RAR- nor RXR-specific retinoids induced significant apoptosis but reduced cell viability in CTCL cells after treatment for 72 h. These observations argue for an antiproliferative effect preceding apoptosis induction as the reduction in cell number was not explained by direct cytotoxic effects. This is in line with a paper pointing towards a proliferative arrest at different cell cycle stages by bexarotene [20, 22]. Furthermore, induction of apoptosis in vivo could not be demonstrated. Finally, other modes of action such as reduction of T-cell migration, e.g. via expression of homing receptors expressed by the tumor cells, may also play a role in bexarotene-induced responses seen in CTCL patients [45, 46]. In conclusion, in our experiments retinoids did not induce significant alterations of the cell cycle as it has been described before for bexarotene. In contrast to a strong G₁ arrest in adult T-cell leukemia cells by AsO₃ treatment, incubation of CTCL cells with AsO₃ led to only a slight increase in the G_{1/0} fraction [47, 48].

In combination with AsO₃ both retinoids reduced apoptosis induction by AsO₃ significantly, whereas reduction in cell viability was unaltered in all combinations

applied. Therefore, our experiments did not demonstrate an additive or synergistic effect with regard to induction of apoptosis or reduction of cell viability. Furthermore, we did not observe cytotoxic effects of the combination treatment in vitro at pharmacologically tolerated doses, which correlates with the tolerability of combined ATRA and AsO₃ treatment of APL patients. In the literature, AsO₃ has so far only been used in 2 patients with Sézary syndrome or erythrodermic mycosis fungoides, resulting in stable disease and partial remission, respectively. These remissions, though, were reported to last for only a week after discontinuation of therapy because of adverse events [19]. These results suggest the necessity of a combination partner other than retinoids, should AsO₃ play a future role in CTCL treatment. In adult T-cell leukemia, several studies report on the successful combination of AsO₃ with interferon-α [49, 50]. As the latter represents another important substance for CTCL therapy, this might be an interesting combination for future studies.

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Disclosure Statement

Chalid Assaf received honoraria for advisory board meetings and presentations from TEVA and Cephalon oncology. Marc Beyer received research grants and honoraria for a presentation from Cephalon oncology and for an advisory board meeting from TEVA. Ioana Cosagarea received a travel grant from TEVA.

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