

# Arsenic trioxide down-regulates antiapoptotic genes and induces cell death in mycosis fungoides tumors in a mouse model

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**Background:** Mycosis fungoides (MF) is the most frequent cutaneous T-cell lymphoma (CTCL). Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has recently been shown to be effective against leukemias, so we studied whether As<sub>2</sub>O<sub>3</sub> induces apoptosis of CTCL cells *in vitro*. We further investigated if As<sub>2</sub>O<sub>3</sub> is effective in a MF mouse model.

**Material and methods:** Annexin V/7-amino-actinomycin-D stainings were carried out to investigate if As<sub>2</sub>O<sub>3</sub> induced apoptosis of CTCL cell lines. To study the underlying mechanisms, the effects of As<sub>2</sub>O<sub>3</sub> on various transcription factors and apoptosis regulating proteins were analyzed by western blots, electrophoretic mobility shift assays and transcription factor enzyme-linked immunosorbent assays. The ability of As<sub>2</sub>O<sub>3</sub> to induce tumor regression was investigated in a MF mouse model.

**Results:** As<sub>2</sub>O<sub>3</sub>-induced apoptosis was paralleled by a reduction of the DNA-binding activities of transcription factors of the NFκB and signal transducer and activator of transcription gene families and reduced expression of the antiapoptotic proteins bcl-1, bcl-xL and mcl-1. Local injections of 200 μM As<sub>2</sub>O<sub>3</sub> into tumors caused complete remissions in five of six mice and one partial remission.

**Conclusions:** As<sub>2</sub>O<sub>3</sub> induced apoptosis of CTCL cells by the down-regulation of transcription factors that stimulate the expression of antiapoptotic genes. Local injection of As<sub>2</sub>O<sub>3</sub> into MF tumor-bearing mice resulted in tumor regression.

**Key words:** cancer therapy, cell death/survival genes, cutaneous T-cell lymphoma, gene regulation, oncogenes, transcription factors

## introduction

Cutaneous T-cell lymphomas (CTCL) are a class of non-Hodgkin's lymphoma and are the second most common extranodal lymphomas, characterized by a clonal proliferation of neoplastic T lymphocytes [1, 2]. The two main forms are mycosis fungoides (MF), which is the most common type of CTCL, and its leukemic form, the Sézary syndrome (SS). MF remains confined to the skin and often presents with patches and plaques or in more advanced forms with tumors and a generalized erythema (erythroderma). Most patients will only experience skin symptoms without serious complications. In early clinical stages, malignant T cells are present in the epidermis, particularly along the basal layer and adjacent to Langerhans cells. However, as disease progresses, neoplastic cells are detected within dermal infiltrates in increasing

numbers, at times entailed with loss of epidermotropism. In contrast, SS patients show a generalized erythroderma, leukemic T cells in the blood and a reduced life expectancy compared with MF patients with only ~30% of patients surviving beyond 5 years after diagnosis. This is probably due to the circulating malignant T cells which produce various immunosuppressing molecules such as interleukin (IL)-10, which might lead to a down-regulation of the immunological tumor surveillance.

There are various therapeutic modalities that show activity in CTCL [3, 4]. MF patients in early stages are generally treated with glucocorticoids, retinoids, extracorporeal photopheresis or more effectively with psoralen and UVA (PUVA). PUVA therapy shows complete remission for 6–30 months in >80% of the patients, but the patients relapse. Advanced MF stages and SS are treated with PUVA in combination with interferon-α, locally applied cytostatics such as 1,3-bis(2-chloroethyl)-1-nitrosourea or radiation therapy [4, 5]. Currently, no cure for either form of CTCL has been found.

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As mentioned above, CTCL tumors remain restricted to the skin for most of the time as the disease progresses. The low number of mitotic cells led to the idea that the increase of the tumor mass is due to the overexpression of cell survival genes such as *bcl-2* which protect tumor cells from programmed cell death (apoptosis) [6] rather than oncogenes that promote cell growth. Indeed, it has been found that the antiapoptotic genes *bcl-2*, *bcl-xL* and *mcl-1* are expressed in CTCL cells and that the expression levels of the latter increase with the progression of the disease [7].

IL-7 and IL-15 are growth factors for CTCL cells that are produced by skin keratinocytes and CTCL cells themselves [8–11]. Both ILs stimulate the expression of *bcl-2* [12]. CTCL cells possess also constitutive NFκB, c-myc and signal transducer and activator of transcription (STAT)5 [12–17] activities that may stimulate the transcription of the apoptosis inhibitors cIAP2 [17] and *bcl-2*. NFκB activities may therefore also increase the survival of CTCL cells. Agents that suppress NFκB activity and/or *bcl-2* expression may be good candidates for the treatment of CTCL, as they should be able to induce apoptosis in CTCL cells.

Arsenic-containing compounds like As<sub>2</sub>O<sub>3</sub> and melarsoprol have been shown to induce *bcl-2* down-regulation and apoptosis in acute promyelocytic leukemia (APL) [18, 19] and chronic B-cell leukemia [20] at nontoxic concentrations. The inhibition of *bcl-2* leads to the activation of the proteolytic caspases, which regulate the further steps of cell death [21]. As<sub>2</sub>O<sub>3</sub> has been used for APL treatment and led to complete remission in 90% of the patients and prolonged survival [19, 22]. SS patients have been treated systematically with As<sub>2</sub>O<sub>3</sub> with little success, as the As<sub>2</sub>O<sub>3</sub> levels in the skin have not been high enough [23].

In this study, we investigated the effects of As<sub>2</sub>O<sub>3</sub> on the cell survival/death genes *bcl-2*, *bcl-xL*, *mcl-1*, *bax*, *bad* and the transcription factors NFκB and STAT5 by western blot and electrophoretic mobility shift assays (EMSA) as well as transcription factor enzyme-linked immunosorbent assays (ELISAs), using nuclear and cytoplasmic extracts from CTCL cell lines Hut78 and MyLa 2059. We also investigated whether As<sub>2</sub>O<sub>3</sub> leads to cell death in CTCL cell lines *in vitro* and whether As<sub>2</sub>O<sub>3</sub> causes remission of human CTCL tumors in nude mice *in vivo*.

## material and methods

### cells and cell culture

The cell line Hut78 (SS) was obtained from ECACC. The cell line MyLa 2059 (MF) was a kind gift of Dr Keld Kaltoft, University of Aarhus, Denmark. Hut78 and MyLa cells were grown in Hepes-buffered RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, 0.25 mg/ml amphotericin B, 100 U penicillin G, 100 U streptomycin and 1 mM pyruvate (all from Invitrogen, Basel, Switzerland).

### induction of apoptosis

Apoptosis was assessed *in vitro* after growing  $2 \times 10^5$  Hut78 or MyLa 2059 cells in the presence of 0.5–2 μM As<sub>2</sub>O<sub>3</sub> for 10–14 days, depending on the sensitivity of the tested cell line to the corresponding agent. Cell death was monitored by observation of cell morphology, cell counting and trypan blue staining as well as MTT assays and flow cytometric analysis of Annexin

V/7-amino-actinomycin-D (AAD)-stained cells as further described below. Fresh MF and SS cells from patients were not analyzed, as they survive only a short time in culture and do not propagate.

### MTT assay

Cells were cultured in the presence of various concentrations of As<sub>2</sub>O<sub>3</sub> for 3 days. Cells were thereafter washed and resuspended in DMEM containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Buchs, Switzerland) and incubated for 3 h at 37°C. Cells were then washed and the converted dye solubilized with 1 ml acidic isopropanol (0.04 M HCl in absolute isopropanol). The absorbance of the converted dye was subsequently measured at a wavelength of 570 nm.

### flow cytometry

As<sub>2</sub>O<sub>3</sub>-treated cell suspensions were stained with Annexin V-PE and 7-AAD following the manufacturer's guidelines and analyzed on a FACScalibur machine (both BD Biosciences, Switzerland). Annexin V-PE positive, 7-AAD negative cells were considered early apoptotic and cells that were in late apoptosis or already dead were both Annexin V-PE and 7-AAD positive.

### western blotting

The antibodies against *bcl-2* (sc-1041), *bcl-x* (sc-1041), *mcl-1* (sc-819) and beta actin (sc-1616) were from Santa Cruz Biotechnology Inc. For western blotting, 30 μg of protein of cytoplasmic extracts were run on a 9% sodium dodecyl sulfate polyacrylamide gel and separated by polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose filter using a Mini Trans Blot Cell (BioRad, Reinach, Switzerland) following the manufacturer's guidelines. Unspecific antibody-binding sites were blocked by incubating the membrane overnight at 4°C in 2% milk in tris-buffered saline (TBS), pH 8.0 containing 0.3% Tween 20 (TBS-T). The membrane was then incubated with the corresponding first antibody (1:1000 dilution) for 4 h at room temperature in 1% milk in TBS-T. The incubation with the secondary antibody (anti-rabbit, Santa Cruz Biotechnology Inc., 1:1000 dilution) was done in TBS-T for 4 h at room temperature. The signal was detected by incubating the membrane with BM purple AP substrate (Roche Biochemicals, Rotkreuz, Switzerland) following the manufacturer's instructions.

### nuclear and cytoplasmic cell extracts

Extracts were prepared by the following method. Cells ( $1-2 \times 10^6$ ) were swollen in 400 μl hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1 mM benzamidine, 1 mM EGTA, pH 8, 1 mM EDTA, pH 8) for 20 min at 4°C and thereafter passed 10 times through a 25-gauge needle. The suspensions were then centrifuged at 16 000g for 30 s and the supernatants were used as cytoplasmic extracts. The nuclear pellets were extracted with 60 μl hypertonic buffer C (20 mM Hepes pH 7.9, 25% glycerol 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA pH 8, 1 mM benzamidine). The supernatants were centrifuged for 5 min and then used as nuclear extracts for EMSAs.

### electrophoretic mobility shift assay

We carried out EMSAs with nuclear extracts of Hut78 and MyLa 2059 cell lines as described earlier [24]. The nuclear extracts were incubated with a radioactively labeled oligonucleotide containing the binding sites for NF $\kappa$ B. We compared nuclear extracts from untreated Hut78 and MyLa 2059 cells and Hut78 and MyLa 2059 cells which had been treated with 1  $\mu$ M (Hut 78 cells) or 2  $\mu$ M As<sub>2</sub>O<sub>3</sub> (MyLa 2059 cells) for 1, 2 and 4 days. The DNA–protein complexes were analyzed on a non-denaturing 4% polyacrylamide gel. The oligonucleotide for the DNA-binding site was synthesized by Microsynth, Balgach, Switzerland.

### transcription factor ELISA

We used the NF $\kappa$ B family transcription factor ELISA from Active Motif, Rixenart, Belgium. A DNA oligonucleotide containing the DNA-binding sequence of NF $\kappa$ B was attached to the surface of a 96-well plate. After rehydration, the nuclear extracts of the MF and SS cells were added and incubated for 1 h at room temperature to let the NF $\kappa$ B protein bind to their DNA motif. The plates were then washed to remove unbound proteins, and antibodies against the different proteins of the NF $\kappa$ B family were added. After 1 h incubation at room temperature, the first antibodies were washed away and a second antibody coupled to horseradish peroxidase (HRP) was added for 1 h at room temperature. The addition of the HRP substrate to the well started a color reaction, which allowed the measurement of the DNA-bound transcription factor. The specificity of the DNA-binding reaction was monitored by control experiments where oligonucleotides containing the wild-type or mutated DNA-binding motif of interest were added as competitors.

### mice, tumor induction and As<sub>2</sub>O<sub>3</sub> treatment

Athymic nude mice from Harlan, Oxon, UK were kept under sterile conditions in the central animal laboratory of the University Hospital Zurich;  $3 \times 10^6$  MyLa 2059T cells were injected s.c. into the right flank of the mice. Tumors were visible after 2–3 weeks in 70%–80% of the mice. The tumor sizes were measured by calipers and the volumes were calculated by the formula  $V = (\text{length} \times \text{width} \times \text{height})/2$ . The MyLa 2059T cells were derived from a tumor induced in athymic nude mice by standard MyLa 2059 cells. The cells were

examined for the presence of murine pathogens before injection. The cells were injected s.c., since the skin of nude mice is extremely thin (3–6 cell layers) and does not allow intradermal injections. The tumors were injected with As<sub>2</sub>O<sub>3</sub> directly, as the tail veins of mice are fragile and break down after daily repeated injections.

As<sub>2</sub>O<sub>3</sub> treatment (50 or 200  $\mu$ M in sterile PBS) was started when the tumors had reached a minimal volume of 100 mm<sup>3</sup>. Using an insulin syringe, 50  $\mu$ l of As<sub>2</sub>O<sub>3</sub> were injected when the tumor volume was <200 mm<sup>3</sup> and 100  $\mu$ l As<sub>2</sub>O<sub>3</sub> were injected when the tumor volume was >200 mm<sup>3</sup>.

The experiments were carried out following the ethic guidelines for animal experiments of the Swiss National Fund and were approved by the Veterinary Authorities of the Kanton of Zurich, Switzerland (license no. 163/2003).

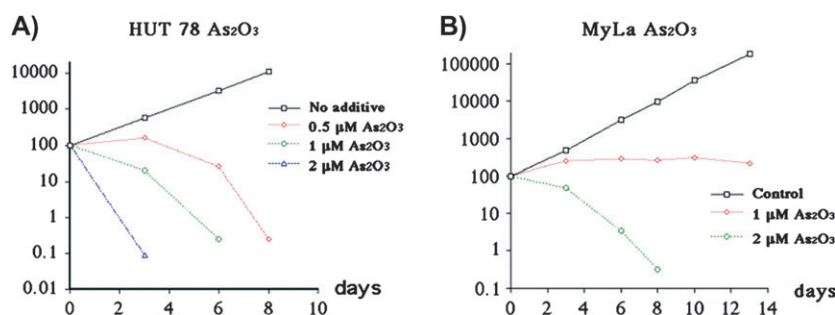
## results

### As<sub>2</sub>O<sub>3</sub> causes cell death of cutaneous T cell lines *in vitro*

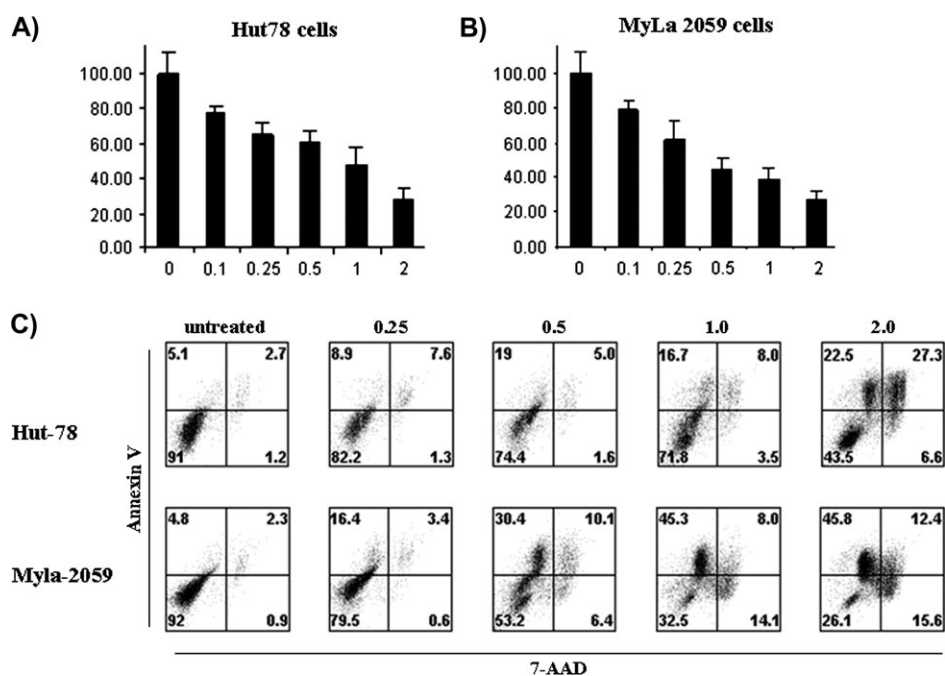
It has been shown that As<sub>2</sub>O<sub>3</sub> concentrations of  $\sim 1$   $\mu$ M cause cell death of leukemia cells [20]. To investigate the effects of As<sub>2</sub>O<sub>3</sub> on the proliferation and viability of Hut78 and MyLa 2059 cells, cells were cultured in the presence of various As<sub>2</sub>O<sub>3</sub> concentrations.

Microscopic inspection of cell cultures showed the presence of both Hut78 and MyLa 2059 cells displaying the typical features of apoptosis such as cell shrinkage, membrane blebbing, nuclear fragmentation and cellular debris in a dose-dependent manner (data not shown). By determining the number of viable cells using the trypan blue exclusion test, we found that culturing the cells in the presence of 0.5–2  $\mu$ M As<sub>2</sub>O<sub>3</sub> significantly decreased the number of viable Hut78 and MyLa 2059 cells. Figure 1A shows that As<sub>2</sub>O<sub>3</sub> induced cell death of most of the Hut78 cells at 0.5  $\mu$ M within 8 days; 1 and 2  $\mu$ M As<sub>2</sub>O<sub>3</sub> had a comparable effect within 6 and 3 days, respectively. MyLa 2059 cells were more resistant against As<sub>2</sub>O<sub>3</sub> (Figure 1B). In the presence of 1  $\mu$ M As<sub>2</sub>O<sub>3</sub>, the number of MyLa 2059 cells remained nearly constant.

We confirmed these results by performing MTT assays which showed a dose-dependent decrease of viable Hut78 and MyLa 2059 cells after treatment with various concentrations of As<sub>2</sub>O<sub>3</sub> for 3 days (Figure 2A).



**Figure 1.** Arsenic trioxide causes cell death of (A) Hut78 and (B) MyLa 2059 cells. The cells were treated with different As<sub>2</sub>O<sub>3</sub> concentrations as indicated. The number of cells (*y*-axis) is given in percent (100% =  $2 \times 10^5$  cells). The time of treatment is indicated on the *x*-axis.

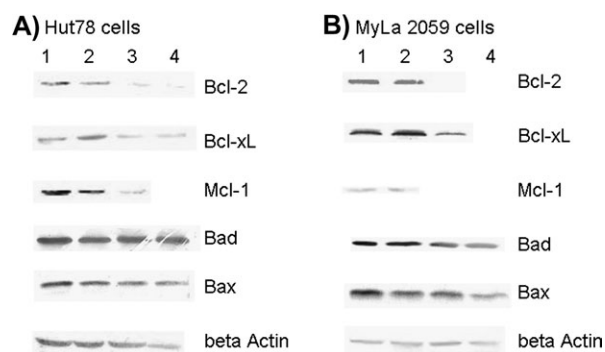


**Figure 2.** As<sub>2</sub>O<sub>3</sub> induces apoptosis in Hut78 and MyLa 2059 cells. To quantify the effect of As<sub>2</sub>O<sub>3</sub> on cell viability and growth, (A) Hut78 and (B) MyLa 2059 cells were grown for 3 days in the presence of various concentrations of As<sub>2</sub>O<sub>3</sub>. Cell viability was thereafter assessed by MTT assays. To determine whether As<sub>2</sub>O<sub>3</sub>-induced cell death was due to apoptosis, the two cell lines were cultured in the presence of various As<sub>2</sub>O<sub>3</sub> concentrations for 3 days and thereafter stained with Annexin V-PE and 7-amino-actinomycin-D (7-AAD).

To study whether the observed decrease in growth was due to necrosis or apoptosis, Hut78 and Myla 2059 cells were cultured in the presence of various concentrations of As<sub>2</sub>O<sub>3</sub> for 3 days and thereafter stained with Annexin V/7-AAD and subsequently analyzed by flow cytometry. Results demonstrated that both Hut78 and MyLa 2059 cells underwent apoptosis in a dose-dependent manner. Flow cytometry showed that the cells moved from viable (Annexin V-PE and 7-AAD negative) to early (Annexin V-PE positive and 7-AAD negative) and finally late apoptosis (Annexin V-PE and 7-AAD positive) as the As<sub>2</sub>O<sub>3</sub> concentration was increased (Figure 2B). These data demonstrate that apoptosis is the main reason for As<sub>2</sub>O<sub>3</sub>-induced cell death.

### the influence of As<sub>2</sub>O<sub>3</sub> on apoptosis-regulating proteins in CTCL cell lines

MF and SS cells express the antiapoptotic proteins *bcl-2*, *bcl-xL*, *mcl-1* and the proapoptotic genes *bad* and *bax* [7]. To test whether As<sub>2</sub>O<sub>3</sub> influences the abundance of these proteins during the induction of apoptosis, we analyzed their expression levels while culturing Hut78 and MyLa 2059 cells in the presence of 1 μM As<sub>2</sub>O<sub>3</sub>. Figure 3 shows that *bcl-2* and *mcl-1* were faster down-regulated in the presence of As<sub>2</sub>O<sub>3</sub> than the beta actin control in both Hut78 and MyLa 2059 cells. The *bcl-xL* protein shows another kinetic since its levels increased on day 2, but subsequently dropped down to levels that are lower than those in untreated cells, indicating that there may be a defence mechanism that protects cells from low concentration and short time exposure to As<sub>2</sub>O<sub>3</sub>. The proapoptotic gene *bax* behaved similar to the beta actin control



**Figure 3.** The influence of prolonged arsenic trioxide treatment on the expression of the apoptosis-regulating *bcl-2*, *bcl-xL*, *mcl-1*, *bad* and *bax* genes and the beta actin control gene in (A) Hut78 and (B) MyLa 2059 cells. The tested gene is indicated on the right. Lane 1: untreated cells, lane 2: cell treated for 2 days, lane 3: cell treated for 3 days, lane 4: cell treated for 4 days with 1 μM arsenic trioxide.

and showed only a weak decrease after prolonged As<sub>2</sub>O<sub>3</sub> exposure, whereas *bad* was more strongly down-regulated. The data shown are representative of three independent experiments.

### the influence of As<sub>2</sub>O<sub>3</sub> on constitutive transcription factor activities in MF and SS cells

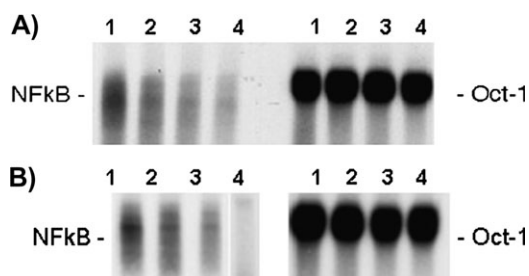
MF and SS cells contain constitutive NFκB activities [13, 15] that may protect them from apoptosis. When measuring the binding of NFκB to its DNA-binding sequence using EMSAs, we found that binding was strongly reduced after prolonged

As<sub>2</sub>O<sub>3</sub> treatment at 1 μM. However, the DNA-binding activity of the ubiquitous Oct-1 transcription factor remained nearly unchanged (Figure 4).

To determine the effects of As<sub>2</sub>O<sub>3</sub> on the DNA-binding activities of the different members of the NFκB transcription factor family, we carried out transcription factor ELISAs using the corresponding antibodies. Since MF and SS cells have also been found to contain constitutive STAT5a and STAT5b activities that may also protect MF and SS cells from apoptosis, we also included these two proteins in our analysis as well. Figure 5 shows that As<sub>2</sub>O<sub>3</sub> strongly reduced the DNA-binding activities of all the seven tested transcription factors p50, p65 (RelA), p52, RelB, c-Rel, STAT5a and STAT5b.

### the influence of As<sub>2</sub>O<sub>3</sub> on tumor growth in a mouse model for MF

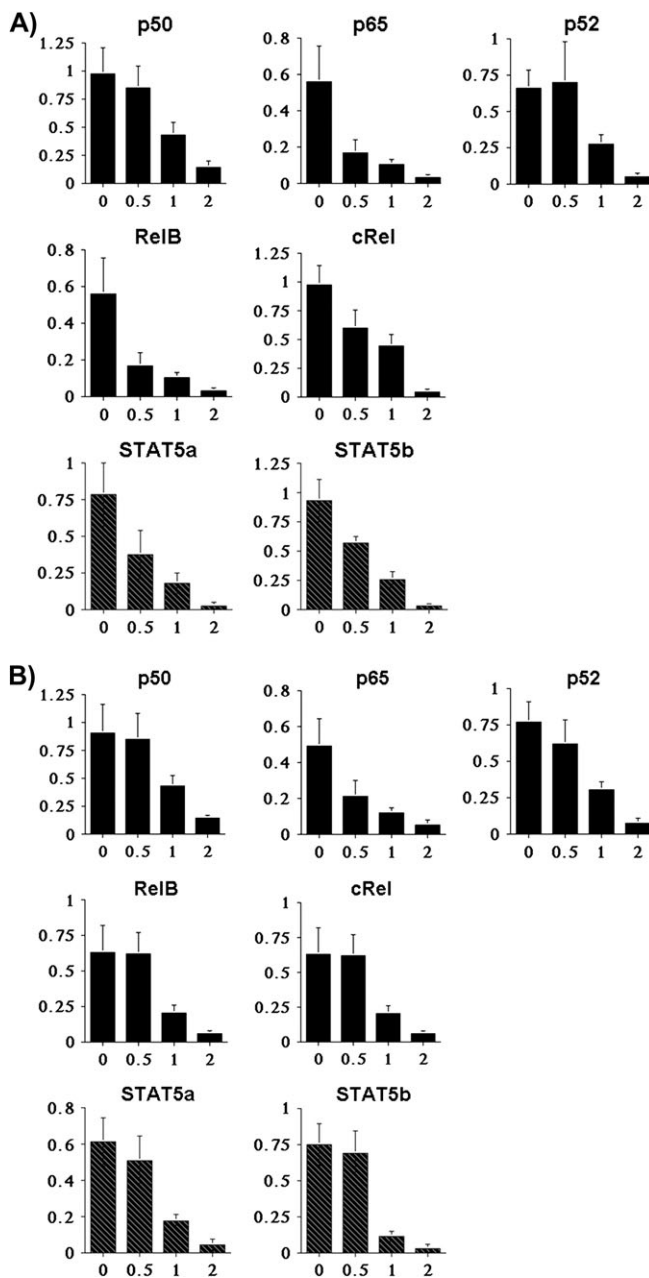
A mouse model for MF has recently been established using the MyLa 2059 cell line and athymic nude mice [25]. To study whether As<sub>2</sub>O<sub>3</sub> can also slow down tumor growth *in vivo*, 3 × 10<sup>6</sup> cells were injected into the flanks of athymic nude mice. Once the tumors reached a size of 100 mm<sup>3</sup> As<sub>2</sub>O<sub>3</sub> treatment was started. Since cells in a solid tumor are much more resistant against antitumor agents, we started with the injection of 50 μM As<sub>2</sub>O<sub>3</sub> into the center of the lesion. The dose was increased to 100 μl once the tumors reached a size >200 mm<sup>3</sup>. Tumors <800 mm<sup>3</sup> were treated with a volume of 150 μl. In control mice that were injected with PBS alone, the tumors reached a maximal volume of 1000 mm<sup>3</sup> within 17–20 days after which they were killed (Figure 6A). The treatment with 50 μM As<sub>2</sub>O<sub>3</sub> delayed the growth of the tumors in three mice and caused a complete remission in one animal (Figure 6B). To investigate if a higher As<sub>2</sub>O<sub>3</sub> concentration would result in a higher percentage of remissions, we treated a second group of mice with 200 μM As<sub>2</sub>O<sub>3</sub>. This higher concentration turned out to be much more effective and caused four complete remissions within 22–40 days, one complete remission after 90 days and delayed tumor growth in another animal (Figure 6C), demonstrating a dose-dependent effect of As<sub>2</sub>O<sub>3</sub> on *in vivo* tumor growth. At both concentrations, we could often observe the formation of an ulcer-like lesion in the skin adjacent to the



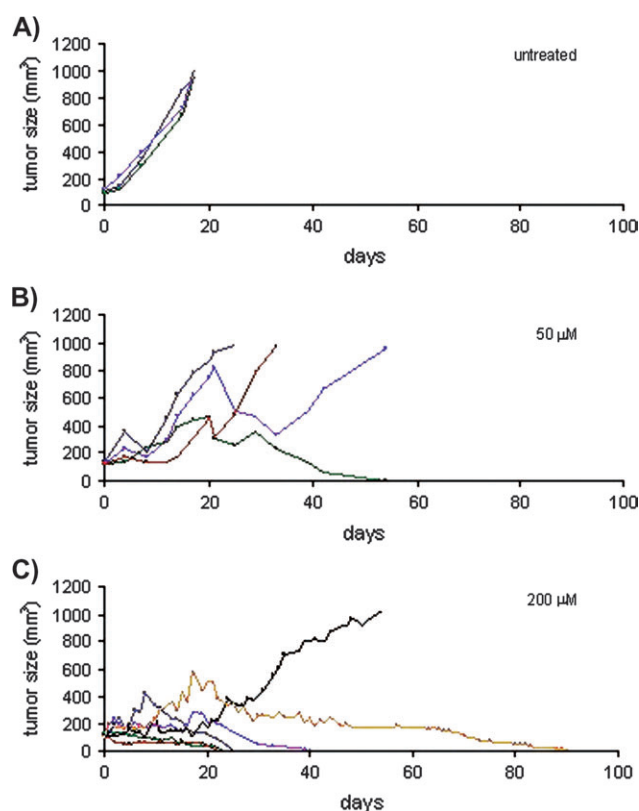
**Figure 4.** Prolonged arsenic trioxide treatment reduces the binding of the NFκB transcription factor to its recognition sequence in (A) Hut78 and (B) MyLa 2059 cells. The DNA binding of the Oct-1 transcription factor remains unchanged. Lane 1: untreated cells, lane 2: cell treated for 2 days, lane 3: cell treated for 3 days, lane 4: cell treated for 4 days with 1 μM arsenic trioxide.

tumor which got smaller and finally disappeared as the tumor volume decreased. There was no lasting visible scar formation in the mouse skin after treatment (Figure 7B and C). No lasting side effects of As<sub>2</sub>O<sub>3</sub> on the surrounding skin or other tissues and organs could be observed.

Tumors that showed partial remission of both series of experiments were excised and underwent histological investigation (Figure 7D). The greatest cell destruction was observed in the center of the lesions where tissue was



**Figure 5.** Transcription factor enzyme-linked immunosorbent assays of NFκB and signal transducer and activator of transcription proteins of As<sub>2</sub>O<sub>3</sub>-treated and -untreated (A) Hut78 and (B) MyLa 2059 cells. The As<sub>2</sub>O<sub>3</sub> concentration is indicated by the x-axis and the relative amount of specific DNA-binding protein (extinction at OD595) is indicated by the y-axis.



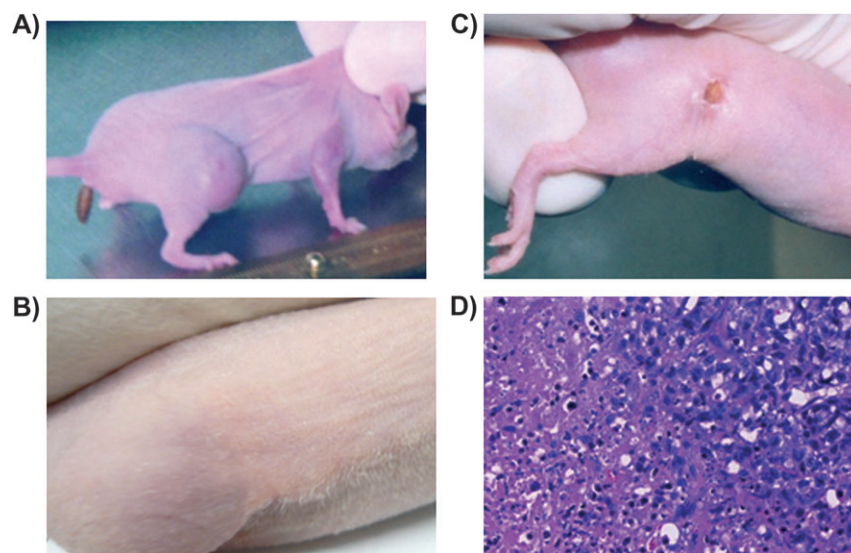
**Figure 6.** Growth of MyLa 2059 cells derived tumors on immune-deficient athymic nude mice;  $3 \times 10^6$  MyLa 2059T cells were injected s.c. into nude mice and treatment was initiated once the tumors had reached a volume of  $100 \text{ mm}^3$ . Mice received daily intralesional injections of (A) PBS, (B)  $50 \mu\text{M}$  or (C)  $200 \mu\text{M}$   $\text{As}_2\text{O}_3$ . Day 0 represents the time point when the tumors had reached a volume of  $\sim 100 \mu\text{l}$  and when the treatment was started. Mice were euthanized once the tumors reached a volume of  $1 \text{ ml}$  ( $1000 \text{ mm}^3$ ), according to the directions of the Swiss Animal Protection Law.

fragmented to acellular debris. This area was surrounded by dead cells with fragmented nuclei followed by cells in several stages of apoptosis. Living cells could be detected in the periphery of the tumors. Unfortunately, the unspecific reactions of the antibodies with the cellular debris of the  $\text{As}_2\text{O}_3$ -treated tumor lesions prevented the analysis of these lesions for the expression of apoptosis-regulating genes by specific antibodies. The tissue surrounding the tumor showed no damage caused by  $\text{As}_2\text{O}_3$  and no significant scarring could be detected.

## discussion

Here, we demonstrate that the treatment of MF skin tumors with  $\text{As}_2\text{O}_3$  may be feasible. Recent data of Michel et al. [23] have shown that intravenous concentrations of  $1\text{--}2 \mu\text{M}$   $\text{As}_2\text{O}_3$  are sufficient to kill malignant SS T cells in the blood but had no effect on skin lesions, suggesting that the resulting nanomolar  $\text{As}_2\text{O}_3$  concentrations in the skin were too low. Our findings that up to  $200 \mu\text{M}$  were needed to achieve a complete remission confirm this assumption. Fortunately, such high concentrations were well tolerated by the surrounding tissues when the  $\text{As}_2\text{O}_3$  was injected into the tumor or its close vicinity. There were no signs of significant scarring in the mouse skin although we often observed a temporary formation of an ulcer-like lesion in the skin surrounding the tumor.

Michel et al. [23] observed that  $\text{As}_2\text{O}_3$ -triggered apoptosis induced the translocation of cytochrome c from the mitochondria to the cytoplasm. Here, we demonstrate that  $\text{As}_2\text{O}_3$  down-regulates the antiapoptotic genes *bcl-2*, *bcl-xL* and *mcl-1* which counteract the release of cytochrome c from mitochondria. The down-regulation of these genes is therefore a good explanation for the observed translocation of cytochrome c from the mitochondria. This mechanism would be comparable to the one observed in anoxia-induced apoptosis [26].



**Figure 7.** Tumor regression during (A–C) arsenic trioxide treatment and (D) biopsy of a treated tumor. (A) Untreated tumor, (B) regression and formation of a central necrotic area in an  $\text{As}_2\text{O}_3$ -treated tumor, (C) site after complete tumor regression, (D) biopsy of a treated tumor. Dead tumor tissue can be seen on the left with fragmented nuclei and on the right living tumor tissue showing first signs of apoptosis (chromatin condensation).

The proapoptotic *bax* gene was also somewhat down-regulated by  $As_2O_3$ , but the concentration of the bad protein remained nearly unaffected. Thus, in a sum the equilibrium between antiapoptotic and proapoptotic proteins that is crucial for the survival of the cell [27, 28] was clearly skipped to the side of the proapoptotic proteins. The data of the annexin V/7-AAD assay confirm that  $As_2O_3$  induces cell death mainly by apoptosis.

$As_2O_3$  also down-regulates the constitutive DNA-binding activities of the transcription factors of the NF $\kappa$ B and STAT gene families. Especially the STATs may be important for the regulation of the *bcl-2*, *bcl-xL* and *mcl-1* genes, since a STAT5-binding site has been identified in the regulatory gene of the *bcl-2* gene [29]. Thus, the down-regulation of STAT5 and other STATs should result in a reduced expression of the antiapoptotic genes.

The relationship between NF $\kappa$ B and these antiapoptotic genes in MF and SS is less clear, since the NF $\kappa$ B-regulated antiapoptotic cIAP genes are not expressed in these cells (J.-Z. Qin, C.-L. Zhang and U. Döbbling, unpublished data), although they possess constitutive NF $\kappa$ B activities. The literature on the effect of NF $\kappa$ B on *bcl-2* and related proteins is contradictory. An NF $\kappa$ B-regulated gene that could be important for cell survival of CTCL cells is FLIP which is an important mediator of antiapoptotic signals and has been shown to play a role in the pathogenesis of anaplastic large-cell lymphoma by protecting cells from apoptosis [30].

Taken together, our results show that the death of MF and SS cells by  $As_2O_3$  is caused by reduced expression levels of antiapoptotic proteins, which are paralleled by the down-regulation of STAT and NF $\kappa$ B transcription factors. The *in vivo* tumor regression upon local  $As_2O_3$  treatment demonstrates that  $As_2O_3$  could be a novel and effective approach in the treatment of CTCL tumors.

## funding

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