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ORIGINAL ARTICLE

Preclinical studies of treosulfan demonstrate potent activity in Ewing's sarcoma

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Abstract
Objectives High-dose chemotherapy with the alkylating agent busulfan has been widely used in the treatment of patients with high-risk Ewing's sarcoma. Because of risks for toxicity, busulfan and radiotherapy can not be applied together, leading to the omission of one effective therapy component. Treosulfan is a derivative of busulfan which has a lower side effect profile than busulfan and which can be used together with radiotherapy. We investigated the effect of treosulfan in a panel of Ewing's sarcoma cell lines on cell survival, cell cycle and apoptosis in vitro and compared it to busulfan. Furthermore, the anti-tumor effect of treosulfan was studied in an orthotopic Ewing's sarcoma mouse xenograft model.
Methods Cell survival was measured by MTT assay and cell cycle analysis by flow cytometry. Apoptosis was analyzed via detection of DNA fragmentation, Hoechst 33258 staining, Annexin V, and cleavage of caspases-3 and 9. The effect of treosulfan and busulfan on primary tumor growth was assessed in Ewing's sarcoma xenografts in NOD/SCID mice (10 mice per group), pharmacokinetics of treosulfan were analyzed in nude mice.
Results Treosulfan inhibited cell growth to at least 70% in all cell lines at concentrations achievable in vivo. Treosulfan had a greater effect on the inhibition of cell growth at equivalent concentrations compared to busulfan. The growth inhibitory effect of treosulfan at low concentrations was mainly due to a G2 cell cycle arrest, whereas at higher concentrations it was due to apoptosis. Apoptosis was induced at lower concentrations compared to busulfan. In contrast to busulfan, treosulfan induced cell death in an apoptosis-deficient cell line at concentrations achievable in vivo. In mice, treosulfan suppressed tumor growth at dosages of 2,500 and 3,000 mg/kg. Pharmacokinetic exposures of treosulfan in mice were similar to previous reports in human patients. At maximal tolerated dosages treosulfan had a higher anti-tumor activity than busulfan.
Conclusions Our results suggest that treosulfan has efficacy against Ewing's sarcoma cells in vitro and in mice. Therefore, controlled trials examining the role of treosulfan in patients with Ewing's sarcoma are warranted.

Keywords Ewing's sarcoma · Treosulfan · Busulfan · Chemosensitivity · Apoptosis · Mouse · Xenograft · Pharmacokinetics

Abbreviations

ES	Ewing's sarcoma
MTT	3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide
AUC	Area under the curve

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Introduction

Ewing's sarcoma (ES) is the second most common bone tumor in children and adolescents [1]. The tumors are of neuroectodermal origin and are characterized by a translocation between the *EWS* gene and a member of the *ETS* family of transcription factors [2]. Cure rates for patients with localized tumors are approximately 70% using a combination of chemotherapy, surgery and/or radiotherapy [3]. Unfortunately, the prognosis for patients with metastatic or relapsed ES is poor [4, 5]. High dose chemotherapy followed by autologous stem cell rescue has been demonstrated to be associated with higher cure rates than conventional chemotherapy in relapsed patients [6]. Further analysis has shown that patients who received busulfan as part of their conditioning regimen during their autologous transplant had a better outcome than patients treated on other conditioning regimens [7]. The use of busulfan, however, may be associated with severe side effects such as CNS toxicity and acute lung injury leading to respiratory failure and death [8]. Since busulfan toxicity is increased by radiation therapy to the trunk, high dose chemotherapy with busulfan and radiotherapy are mutually exclusive in these patients. A large proportion of patients with metastatic ES, however, present with pulmonary or bone metastases involving the trunk. For these patients, one efficacious therapy component, either high-dose chemotherapy with busulfan or radiotherapy must be withheld [9].

Treosulfan (L-threitol-1,4-bis-methanesulfonate) is a water-soluble bifunctional alkylating agent, initially introduced for the treatment of ovarian cancer [10, 11]. In contrast to its parent component busulfan (Fig. 1), major pulmonary or CNS toxicity has not been described, even in the combination with radiotherapy [12]. Treosulfan has been used in various studies as part of the conditioning regimen for allogeneic stem cell transplantation [13–16]. It has been shown to be myeloablative and has been well tolerated in children [17–19]. Thus far, the efficacy of treosulfan in ES is not well defined [20–22]. If treosulfan would be at least as efficacious against ES tumors as busulfan, it would be preferable due to its favourable side effect profile and potential combination with radiation in patients with metastatic ES.

In this report, we compare the effects of treosulfan and busulfan on a panel of ES cell lines with respect to cell survival, cell cycle, and apoptosis in vitro and on tumor growth in a mouse xenograft model. Furthermore, we present pharmacokinetics for treosulfan in mice.

Materials and methods

Cell lines

ES cell lines A4573, CHP-100, JR (NCI-EWS 94), RD-ES, SK-N-MC, TC32, and TC71 were kindly provided by Jeff Toretsky (Georgetown University, Washington, DC), ES cell line SB (NCI-EWS 95) by Crystal Mackall (NCI, Bethesda, MD). All cell lines have been characterized previously and bear the t(11;22) translocation [23–28]. Except of TC32, none of the cell lines has a functional p53 status (data not shown). Cell lines were maintained in RPMI supplemented with 10% FCS and 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine.

Reagents

Busulfan was obtained from Sigma (Muenchen, Germany), treosulfan (Ovastat) from Medac GmbH (Hamburg, Germany). Stock solutions for busulfan were prepared in DMSO at 40 mg/ml and for treosulfan at 50 mg/ml in sterile water. For in vitro studies, further dilutions were made in cell culture medium, for in vivo studies, busulfan was diluted in PBS to a final concentration of 20% DMSO.

MTT assay

To assess cell survival, cells were plated in quintuplicates in flat bottom wells at 10,000 cells/well in 96-well microtiter plates and allowed to attach to the plate. The following day treosulfan or busulfan was added at the indicated concentrations. To minimize the effect of airborne toxicity of the treosulfan metabolite diepoxybutane, untreated controls were placed next to wells with the two lowest concentrations of treosulfan, 1 and 5 µmol/l. Also, exposure of cells to treosulfan or busulfan was always on separate 96-well plates. Forty-eight hours after the addition of drugs, cell viability was assessed by the MTT dye reduction assay as described previously [29]. IC50 values were determined by plotting compound concentration versus cell viability.

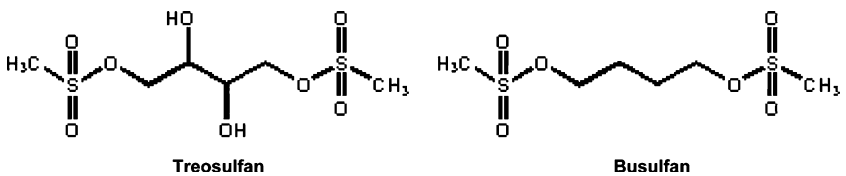


Fig. 1 Structural formula of treosulfan (L-threitol-1,4-bis-methanesulfonate) and busulfan (1,4-butanediol dimethansulfonate)

Cell cycle analysis and measurement of apoptosis by sub-G1 content

Cell cycle distribution and apoptotic cells were determined by the propidium iodide method [30]. In brief, ES cells were plated overnight in a 24-well plate (Falcon Microtest, Becton Dickinson) at a concentration of 5×10^4 cells/well. The following day treosulfan or busulfan was added at the indicated concentrations. Forty-eight hours later, cells in suspension and adherent cells were collected in 12 × 75 mm Falcon Polystyrene tubes and centrifuged at 200 × *g*. The cell pellet was resuspended in 400 µl of a hypotonic buffer (propidium iodide, 50 µg/ml, 0.1% sodium citrate plus 0.1% Triton X-100) and placed at 4°C in the dark overnight. Flowcytometric analysis was performed using a FACScan analyzer (Becton Dickinson). The propidium iodide fluorescence of individual nuclei was measured in the red fluorescence and the data registered in a logarithmic scale. At least 10^4 cells of each sample were analyzed.

Chromatin staining with Hoechst 33258

Cells were incubated at the indicated concentrations of treosulfan for 48 h in cell culture plates. Cells were then trypsinized and washed twice with PBS followed by centrifugation at 800 U/min × 10 min for cytospin preparation. Slides were incubated with Hoechst 33258 for 15 min. The cell preparations were examined under UV illumination with the Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany). Apoptosis was defined when apoptotic bodies, chromatin condensation, or fragmented nuclei were observed.

Detection of apoptosis via Annexin V

Apoptotic cells were determined by staining cell suspensions with 2 µg/ml propidium iodide (PI) plus FITC-coupled Annexin V followed by analysis in a FACScan (Becton Dickinson) as described previously [31].

Measurement of active Caspase-3

For the detection of active caspase-3, cells were washed twice in PBS, then fixed and permeabilized in 4% paraformaldehyd/ 0,5% saponine for 20 min on ice. FITC-stained anti-active caspase-3 antibody (Becton Dickinson) was used for intracellular staining in an 1:5 dilution in PBS/saponine and detected by FACScan.

Immunoblot

Protein was extracted from cells by detergent lysis with a buffer containing 10% SDS. The lysates were boiled for 10 min, and cellular debris was then removed by centrifugation (10 min at 14,000 rpm). The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Forty microgram of protein were boiled 10 min before loading on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and then blocked for 30 min in PBS containing 5% dry milk and 0.1% Tween (Sigma). Membranes were incubated with a goat polyclonal anti-caspase-9 antibody (R&D, Wiesbaden, Germany), at a 1:1000 dilution overnight in blocking buffer, washed three times in PBS/Triton, incubated for an additional 30 min with a secondary antibody conjugated to horseradish peroxidase at a 1:2000 dilution, washed three times with PBS/Triton, and then developed using enhanced chemoluminescense (Amersham, Braunschweig, Germany).

Xenograft tumor study

Subconfluent CHP-100 or TC71 cells were harvested by trypsinization and resuspended in Hank's balanced salt solution at 2×10^7 cells/mL. Two million Ewing's sarcoma cells were injected into the gastrocnemius of 4- to 8-week-old female NOD/SCID mice (Taconic, Germantown, NY). Each mouse had a single palpable tumor evident at 14–21 days after inoculation. At a tumor volume of 100–500 mm^3, mice were randomly assigned to receive different treatments (10 mice/group). In the dose-finding experiment mice received intraperitoneally either treosulfan at 3,000 mg/kg, treosulfan at 2,500 mg/kg or vehicle (PBS/20% DMSO). When treosulfan was compared to busulfan, mice received intraperitoneally either treosulfan at 3,000 mg/kg, busulfan at 60 mg/kg or vehicle (PBS/20% DMSO). Tumor dimensions were measured twice a week with digital calipers to obtain two diameters of the tumor sphere. The lower extremity volume at the site of the tumor was determined by the formula $(D \times d^2/6) \times p$, where D is the longer diameter and d is the shorter diameter. Lower extremity volumes without tumor were approximately 50 mm^3. Xenograft studies were approved by the National Cancer Institute's Animal Care and Use Committee, and all animal care was in accordance with institutional guidelines.

Pharmacokinetic studies

For the pharmacokinetic analysis, tumor-free nude mice, aged 7–8 weeks, received a single dose of treosulfan at 3,000 mg/kg intraperitoneally. Three animals each were sacrificed by carbon dioxide inhalation before and after the administration close to the following time points: 10, 20, 30, 45, 60, 90 min, 2, 3, 4, 6, 9, and 24 h. A total blood sample (1.26 ml) was immediately collected by cardiac puncture and adjusted to a final pH of 5.5 by citrate to avoid

artificial ex vivo degradation of treosulfan. Plasma was separated by centrifugation at 4°C and 1,000 × *g* for 10 min. At each time point, plasma samples from the three animals were separated and frozen at −80°C until analysis. Samples were microfiltrated and treosulfan was separated by a validated RP-HPLC method and quantified by refractometrical detection as described previously [32]. The limit of quantification for treosulfan was given to 1 µg/ml in plasma. Reproducibility was 99 ± 3%, recovery was 96 ± 4%, and linearity was from 1 µg/ml to 50 mg/ml treosulfan (correlation coefficient, 0.99). Pharmacokinetic parameters were evaluated by two-compartment disposition modelling using the data analysis system TOPFIT 2.0 [33].

Results

Treosulfan decreases cell viability in ES cell lines in vitro at lower concentrations than busulfan

In order to investigate the effect of treosulfan on cell survival, cells of eight different ES cell lines were incubated with increasing dosages of treosulfan for 48 h; viability was then tested by MTT assay. Since in humans maximal plasma concentrations of approximately 1,000 µmol/l have been achieved in dose escalation trials, this dosage was used as the upper concentration limit [13, 34]. Figure 2a shows that the number of viable cells is decreased at concentrations as low as 1 µmol/l compared to untreated controls. The IC_{50} for the cell lines ranged between 5 and 250 µmol/l (Table 1). Even in the least sensitive cell line, JR, survival decreased by 90% at 1,000 µmol/l. Incubation of cells with busulfan also resulted in a dose-dependent decrease in cell survival (Fig. 2b). However, busulfan dosages required to achieve an equal effect on survival as treosulfan were higher, with IC_{50}s starting at 200 µmol/l.

Table 1 IC_{50}s of treosulfan and busulfan in Ewing's sarcoma cell lines

Cell line	IC_{50}	
	Treosulfan (µmol/l)	Busulfan (µmol/l)
A4573	35	250
CHP-100	70	500
RD-ES	100	750
JR	250	>1,000
SB	75	625
SK-N-MC	5	ND
TC32	50	250
TC71	10	200
C_{max} in vivo	1,120 [13]	18.5 [35]

For the cell line JR, cell survival was only decreased by 22% at the highest concentration of 1,000 µmol/l. This shows that in vitro, treosulfan decreases cell viability to a greater extent than busulfan at comparable concentrations. Since, in humans, maximal plasma concentrations for busulfan have been described to be approximately 20 µmol/l [35], the results suggest that in vivo treosulfan might be more effective against ES cells than busulfan.

Inhibition of cell proliferation predominates at low concentrations of treosulfan whereas cell death prevails at higher concentrations

Since the MTT assay does not allow differentiating between cell cycle effects and cell death, cells of different ES cell lines were incubated with increasing dosages of treosulfan for 48 h and examined by phase contrast microscopy for cell number and morphology. As demonstrated in Fig. 3, confluency of cells of ES cell line TC32 was lower at 5 µmol/l of treosulfan when compared to the untreated control. No

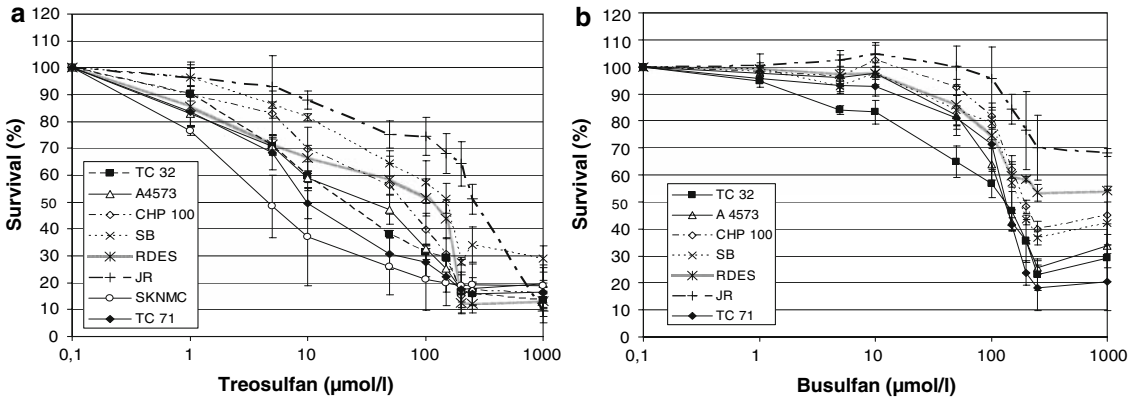


Fig. 2 Cell survival of ES cell lines after incubation with treosulfan (**a**) and busulfan (**b**). Cells were incubated for 48 h at indicated concentrations of drugs. Surviving cells were then analyzed by MTT assay. Treosulfan decreases cell viability in ES cell lines in vitro at equal concentrations to a higher extent than busulfan. *Points* indicate mean survival for at least three separate experiments conducted in quintuplicates. *Bars* indicate SD

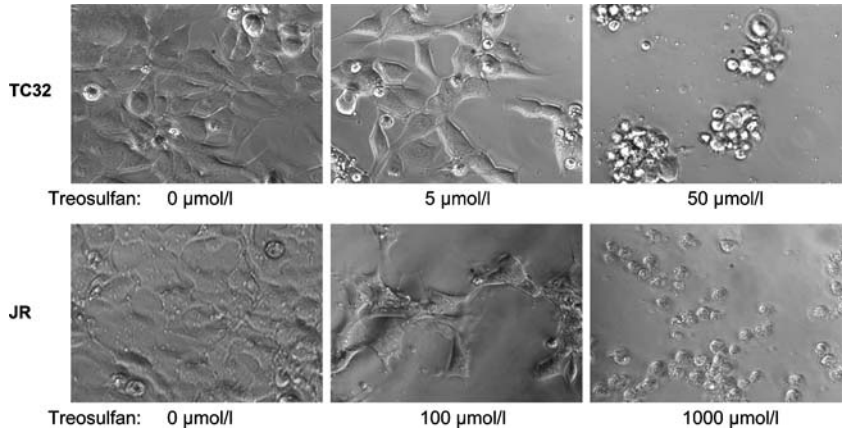


Fig. 3 Treosulfan predominately inhibits cell proliferation at low concentration and cell death prevails at high concentrations. Photographs were taken using a phase contrast microscope (×32) after 48 h incubation with indicated concentrations of treosulfan. At a concentration of 5 µmol/l in cell line TC32 and 100 µmol/l in JR cells, the cell number was decreased and some cells showed morphological features of cell death such as rounding and detachment from the plate. At a concentration of 50 µmol/l for TC32 and 1,000 µmol/l for JR cells, almost all cells showed features of cell death

changes in the morphology of the majority of cells were observed, suggesting inhibition of cell cycle at this concentration as the predominant cause for decreased MTT uptake. At 50 µmol/l almost all cells demonstrated signs of cell death including rounding and detachment from the plate surface. In the cell line JR, reduction in cell number without morphological changes was seen at concentrations up to 100 µmol/l, whereas cell death was only observed at higher concentrations, which was also confirmed by trypan blue dye exclusion (data not shown). This suggests that the effect of treosulfan on cell number at low concentrations is mainly due to its inhibition of cell cycle, whereas at higher concentrations it is predominately caused by cell death.

Treosulfan induces G2 cell cycle arrest at low concentrations

In order to further investigate the antiproliferative effect of treosulfan on ES cells, cell lines were incubated with increasing dosages of treosulfan and cell cycle was analyzed by flow cytometry of propidium iodide stained nuclei. As demonstrated in Fig. 4a, b, treosulfan induced a profound G2 arrest both in cells of the wt p53 cell line TC32 as well as in cells of the mut p53 cell line JR. The highest percentage of cells in G2 phase was achieved in both cell lines at a concentration of 5 µmol/l. Whereas interpretation of cell cycle changes in the TC32 cells at concentrations above 5 µmol/l is hampered due to increasing apoptosis, the cell cycle pattern of JR cells which only show minor signs of apoptosis approaches that of untreated cells suggesting that cells cannot proceed further in the cell cycle. Similar effects on cell cycle were observed when cells were incubated with busulfan reaching the maximum percentage of cells in G2 at 100 µmol/l (Fig. 4b).

Treosulfan induces apoptosis starting at concentrations as low as 1 µmol/l

Cells of ES cell lines A4573, JR, TC32 and TC71 were incubated with increasing dosages of treosulfan for 24 and 48 h. Apoptosis was measured as percentage of cells with subdiploid DNA content (sub-G1). In ES cell lines A4573, TC32 and TC71 apoptosis was already observed at concentrations as low as 1 µmol/l at 48 h (Fig. 5a, b). The percentage of apoptotic cells increased with higher concentrations of treosulfan peaking at 100 µmol/l at 48 h. At higher concentrations, the percentage of cells with nuclear fragmentation decreased suggesting that other mechanisms leading to cell death are involved at these concentrations. In cell line JR, only a small percentage of cells showed nuclear fragmentation of DNA starting at a concentration of 250 µmol/l and peaking at about 22% of cells at 1,000 µmol/l. Therefore, it appears that cell death at this high concentration is not predominately mediated by the classical apoptotic pathway involving nuclear fragmentation. In contrast to treosulfan, busulfan only induced apoptosis in cell lines A4573, TC32 and TC71 starting at concentrations of 50 µmol/l, which are higher than maximal concentrations achieved in vivo (Fig. 5a). Significant nuclear fragmentation was not induced in cell line JR. Induction of apoptosis was also demonstrated by staining of nuclei with Hoechst 33258 showing nuclear condensation and apoptotic bodies at concentrations of 10 and 100 µmol/l of treosulfan in ES cell lines A4573 and TC32 (Fig. 5c). In cell line JR, nuclear condensation was observed in a minority of cells at 1,000 µmol/l suggesting that at this concentration cell death is only partly mediated by apoptosis. A similar observation was made when cells were labeled with

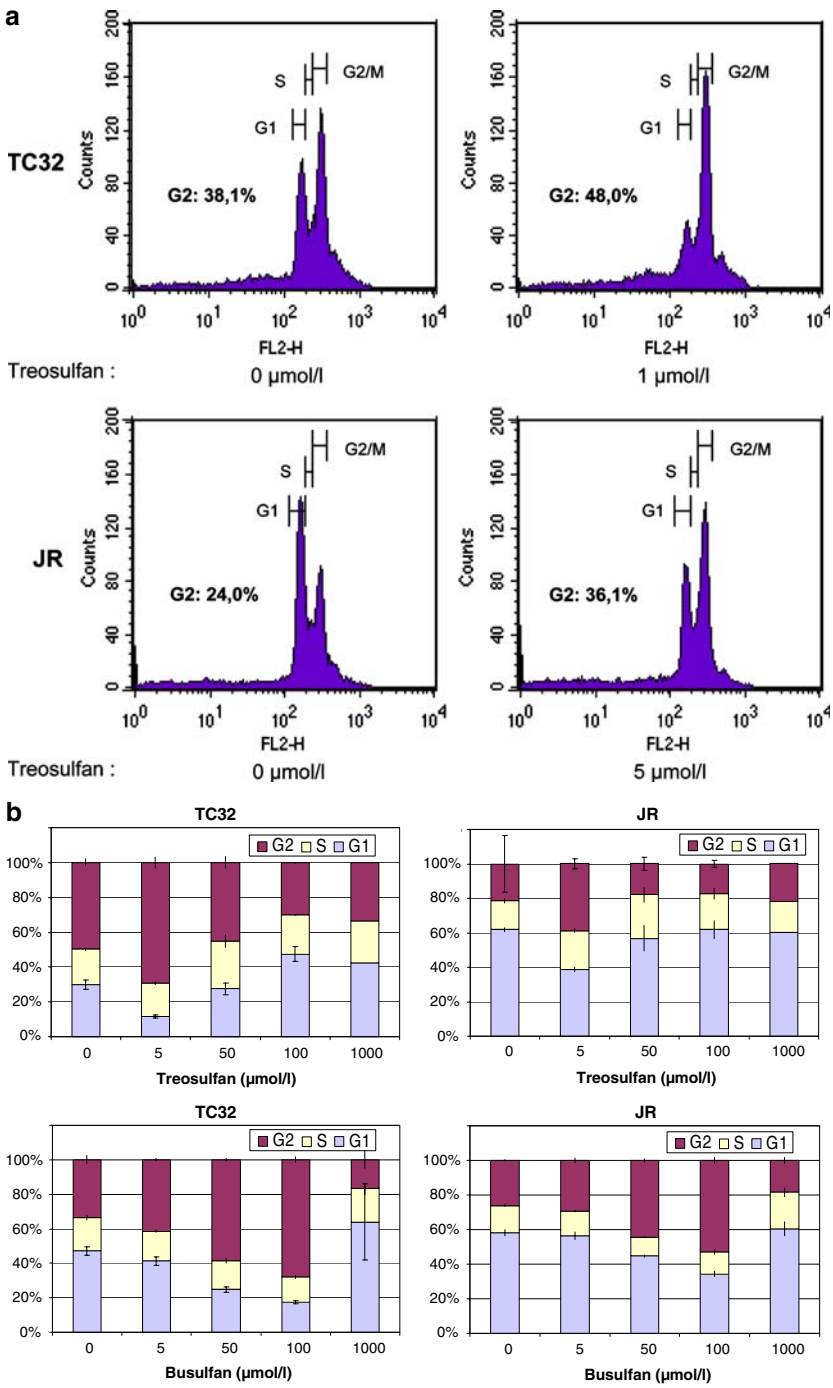


Fig. 4 Treosulfan induces G2 cell cycle arrest at low concentrations. ES cell lines A4573, JR, TC32 and TC71 were incubated with indicated concentrations of treosulfan or busulfan for 48 h and cell cycle distribution was measured by flow cytometry after propidium iodide staining of nuclei. **a** Cell cycle profiles of cells of cell lines TC32 and JR after treatment with 1 and 5 µmol/l treosulfan, respectively. **b** Cell populations in G1, S and G2 phases. *Columns* represent a mean of two experiments and *bars* specify SD

annexin V and propidium iodide (Fig. 5d). Whereas treatment with treosulfan resulted in both an annexin V-positive/PI-negative and an annexin V-positive/PI-positive cell population in cell line TC32, cells of cell line JR only showed the double positive phenotype. However, only cells positive for annexin V and negative for PI are uniquely apoptotic, since this reflects the flip-over of phophatidylserines from the inner cell membrane to the outside in early apoptosis. In contrast, cells positive for both annexin V and PI enclose late apoptotic cells as well as necrotic cells [36]. In the latter population annexin V-positivity reflects staining of inner membrane phophatidylserine after loss of membrane integrity [33].

Treosulfan induces apoptosis via activation of caspases-9 and 3

To investigate whether apoptosis induced by treosulfan was caspase-dependent, activation of effector caspase-3 was analyzed by flowcytometry. As shown in Fig. 6a, treatment of

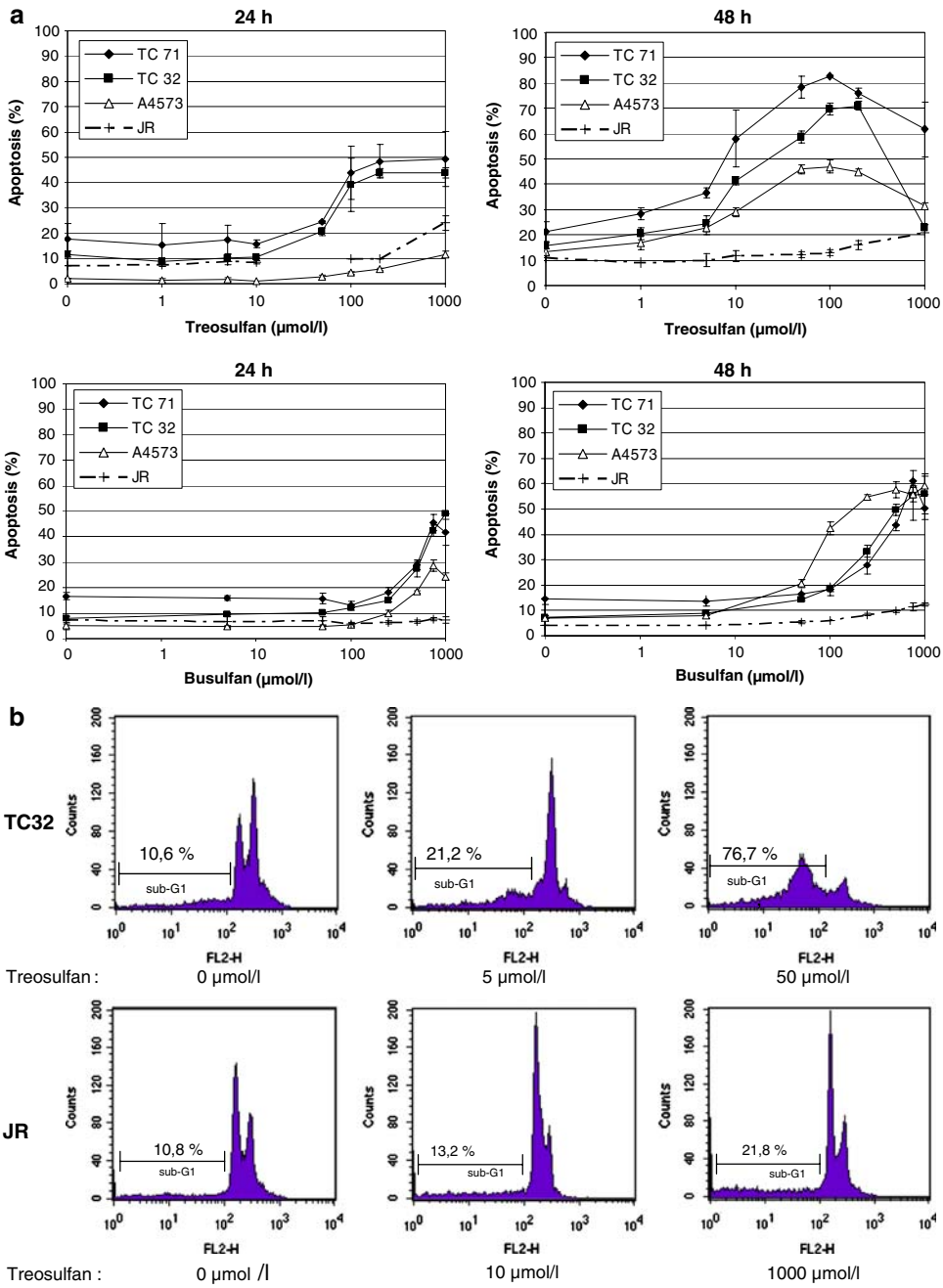


Fig. 5 Treosulfan induces apoptosis in ES cell lines. Cells of cell lines A4573, JR, TC32 and TC71 were incubated with treosulfan or busulfan for 24 or 48 h. **a** Treosulfan induces apoptosis at concentrations as low as 1 µmol/l in TC71 cells after 48 h. Higher dosages of busulfan are needed for equal effect on apoptosis. Apoptosis was determined by measuring sub-G1 content of nuclei using the method by Nicoletti et al. [30]. *Columns* indicate a mean of two experiments; *bars* indicate SD. **b** FACS profiles of TC32 and JR cells. Marked increase in sub-G1 fraction is observed in cell line TC32 at 5 µmol/l, only moderate increase in apoptotic cells is seen in JR cells at 1,000 µmol/l. **c** Hoechst 33258 staining of A4573, TC32 and JR cells after a 48 h incubation with treosulfan. Nuclear fragmentation and condensation (*arrows*), signs of apoptosis, are seen with increasing concentrations of treosulfan. Few cells of cell line JR show morphological changes consistent with apoptosis at 1,000 µmol/l. **d** Annexin-V staining of TC32 and JR cells after 36 h incubation with treosulfan. Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cell populations in TC32 cells at 100 µmol/l of treosulfan; in JR cells no Annexin V-positive/PI-negative cells which are specific for apoptosis are seen, neither at 100 nor 500 µmol/l of treosulfan

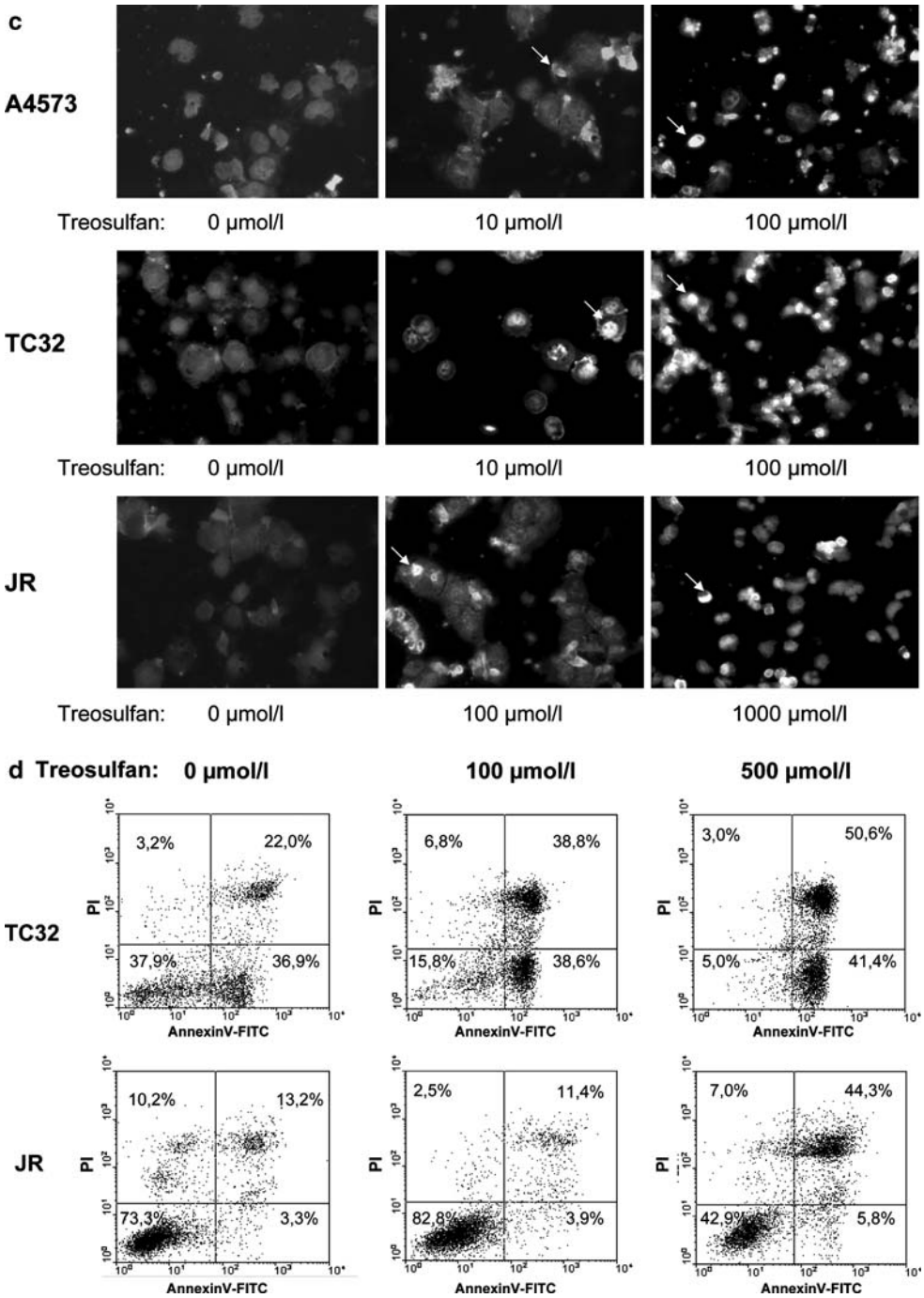
cells of cell line TC32 lead to the appearance of active caspase-3. Cells of cell line JR which undergo cell death after incubation with high concentrations of treosulfan did not show activation of caspase-3, underlining the observation of the experiments above that these cells do not die via apoptosis.

Since chemotherapeutics induce apoptosis mainly via the intrinsic pathway with caspase-9 as initiator caspase [37], we examined the expression of caspase-9 in cell lines A4573 and TC32 at increasing concentrations of treosulfan by immunoblot. Figure 6b demonstrates that expression of caspase-9 was reduced in cell line TC32 starting at 50 µmol/l, and in cell line A4573 at 200 µmol/l suggesting that caspase-9 is activated and cleaved in treosulfan-induced apoptosis in both cell lines.

Treosulfan suppresses tumor growth of Ewing's sarcoma cells in an orthotopic mouse xenograft model

The in vivo activity of treosulfan was tested by administering treosulfan intraperitoneally to mice with Ewing's sarcoma xenografts. Since the maximal tolerable dose of treosulfan in mice was reported to be 3,000 mg/kg [38], we

Fig. 5 continued



chose this dose and the lower dose of 2,500 mg/kg for these initial experiments. NOD/SCID mice with a palpable Ewing's sarcoma xenograft tumor were given treosulfan at 2,500 and 3,000 mg/kg or PBS/20% DMSO as a control twice 7 days apart. Tumor dimensions were measured every 3 or 4 days. Both dosages of treosulfan suppressed tumor growth when compared to sham treated mice. Whereas tumors in sham-treated mice grew exponentially, tumors in the group receiving treosulfan doses of 3,000 mg/kg grew slowly throughout the course of treatment (Fig. 7). Treosulfan was tolerated well at both dose levels, and no animal died during the observation period.

Pharmacokinetics of treosulfan in mice

To investigate whether the anti-tumor effect of treosulfan in mice could be obtained with plasma levels achievable in humans, pharmacokinetics of treosulfan in mice were analyzed. Treosulfan was administered to tumor free nude mice at a concentration of 3,000 mg/kg once intraperitoneally.

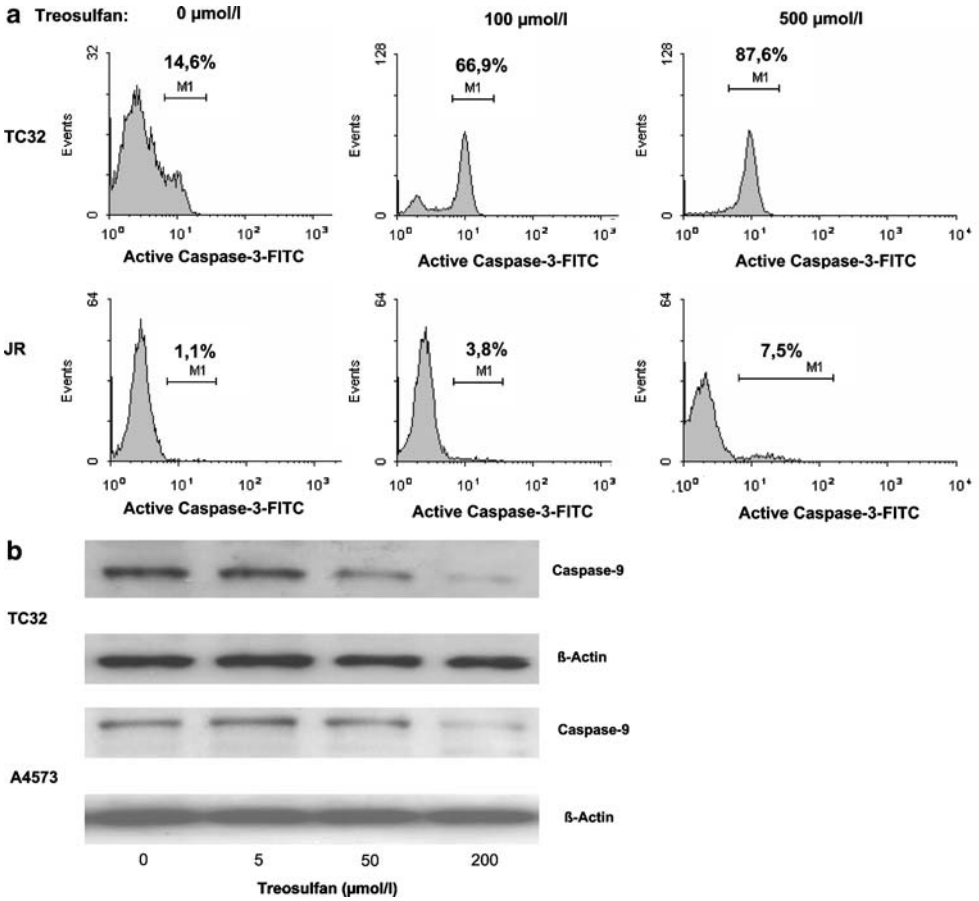


Fig. 6 Activation of caspases during treosulfan-induced apoptosis. **a** Detection of active, cleaved caspase-3 after a 36 h incubation with treosulfan in TC32 cells at a concentration of 100 and 500 µmol/l; only weak expression of active caspase-3 in JR cells treated with 500 µmol/l of treosulfan. **b** Decreased expression of caspase-9 in A4573 and TC32 cells after a 48 h incubation in increasing concentrations of treosulfan. ß-actin was used as a loading control

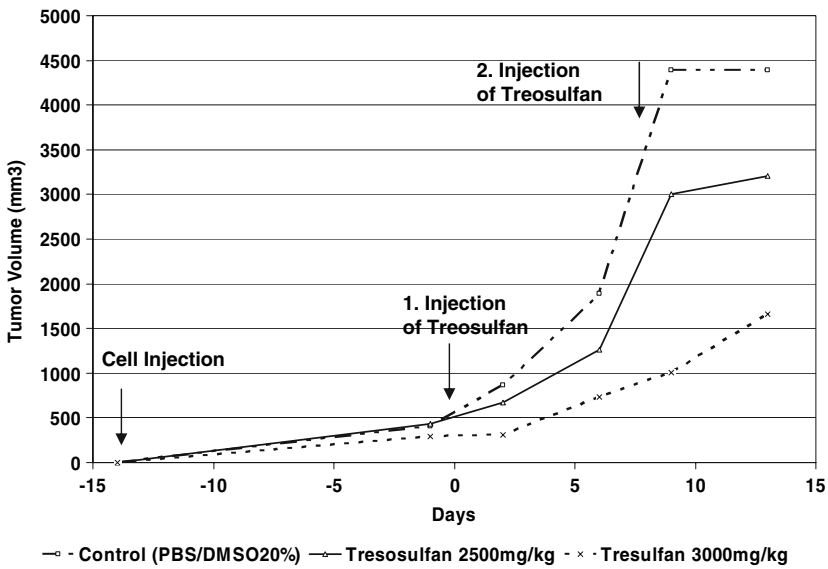


Fig. 7 Tresoulfan inhibits growth of ES cells in a mouse xenograft model. NOD/SCID mice (10 per group) were injected with 2 Mio cells of ES cell line TC71. When tumors were palpable, mice were injected i.p. with either treosulfan at 3,000 or 2,500 mg/kg or vehicle (day 0). Injection was repeated on day 7. Treosulfan inhibited tumor growth at both concentrations, with a more pronounced effect at the higher concentration

Treosulfan plasma levels were determined before administration and at various time points afterwards. As in humans, plasma concentrations of treosulfan declined exponentially and are described by a first-order elimination process best fitted by a two-compartmental model (Fig. 8). Peak plasma level of treosulfan was reached at 0.21 h after administration, and terminal half-life of treosulfan in plasma was calculated to 1.8 h. The AUC was comparable to the AUC in humans after i.v. administration of 30 g/m^2 [32–34]. Therefore, treosulfan is active against Ewing's

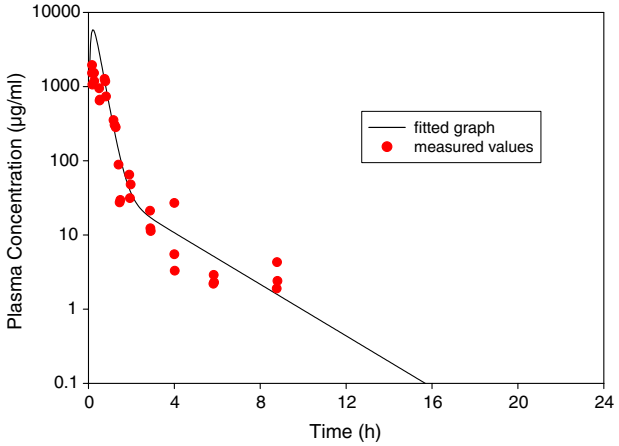


Fig. 8 Concentration-time curve of treosulfan plasma in mice. Nude mice (3 group) were injected with treosulfan (3,000 mg/kg i.p.). Plasma levels of treosulfan were measured before and at indicated time points after administration of the drug. Plasma concentration declines exponentially and is best fitted by a two-compartmental model

sarcoma tumors in mice at concentrations achievable in humans.

Treosulfan is more active than busulfan in suppressing growth of Ewing's sarcoma cells in mice

In order to compare the anti-tumor effect of busulfan and treosulfan in mice, Ewing's sarcoma bearing NOD/SCID-mice were randomized to one single intraperitoneal treatment with either treosulfan (3,000 mg/kg), busulfan (60 mg/kg) or vehicle (PBS/20% DMSO). Both concentrations have been described in the literature before as maximal tolerable dosages in mice [38]. The experiments were done in mice either bearing tumors of Ewing's sarcoma cell line TC71 or CHP-100. As demonstrated in Fig. 9a treosulfan suppressed tumor growth of both Ewing's sarcoma cell lines in the mouse-xenograft model. Only a modest effect on tumor growth was seen when mice were treated with busulfan. Whereas busulfan only slowed down initial tumor growth in mice bearing TC71 cells, a moderate suppression of tumor growth in mice harboring CHP-100 tumors was seen starting at day 5 of treatment (Fig. 9b).

Discussion

The alkylating agent treosulfan is of interest for the treatment of ES, because of a more favourable side effect profile in comparison with the structural related compound busulfan and its potential for combination with radiotherapy. Data on the efficacy of treosulfan in ES, however, have been limited [20–22]. Our results demonstrate that treosulfan is able to suppress growth of Ewing's sarcoma cells in vitro and in vivo in an orthotopic mouse xenograft model. In vitro, treosulfan inhibits growth of ES cell lines up to at least 70% at concentrations achievable in vivo. The

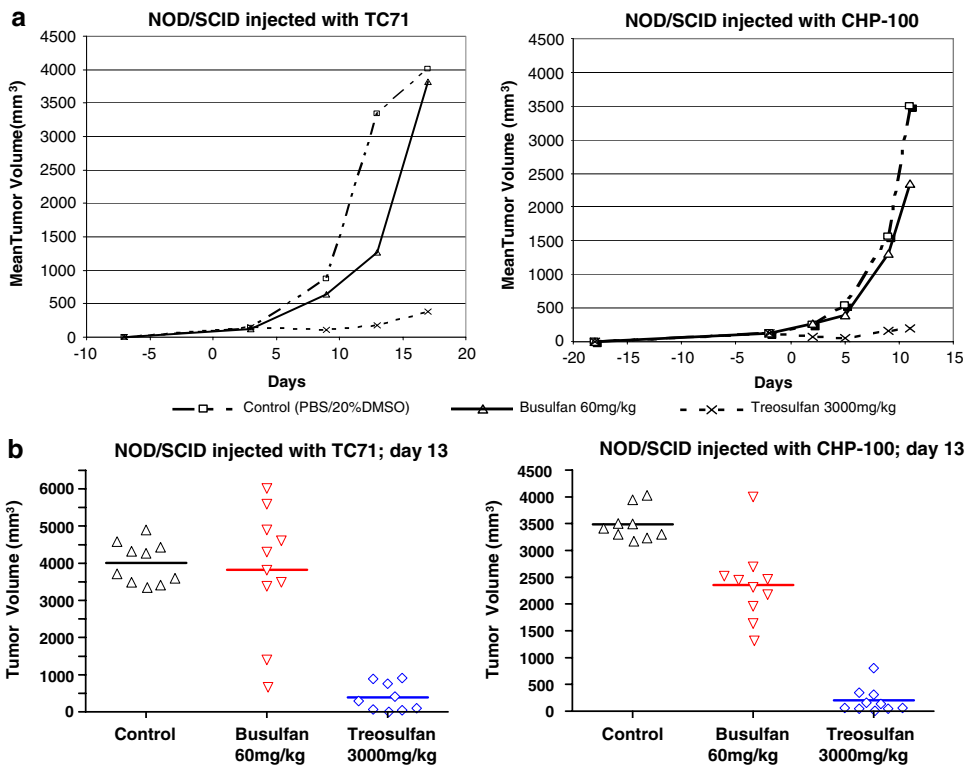


Fig. 9 Comparison of anti-tumor activity of treosulfan and busulfan in the orthotopic mouse xenograft model. NOD/SCID mice (10 per group) were either injected with ES sarcoma cell lines TC71 or CHP-100 in the left m. gastrocnemius. When tumors were palpable mice were either given treosulfan (3,000 mg/kg i.p.), busulfan (60 mg/kg i.p.) or vehicle (PBS/20% DMSO i.p.). Treosulfan markedly suppressed tumor growth in both cell lines, whereas busulfan only led to an initial inhibition of tumor growth in cell line TC71, and a moderate suppression of tumor growth in mice injected with cell line CHP-100 on day 13 (**a**, **b**). One experiment is shown. Experiments were repeated twice with similar results

IC_{50} ranged between 5 and 250 µmol/l, whereas maximum serum concentrations (C_{max}) in humans have been described up to 1,120 µmol/l at doses of 14 g/m² in a 2 h infusional regimen [13]. In contrast, in all cell lines examined, the IC_{50} for busulfan was above the C_{max} reported in humans [35]. Our results are in concordance with the data recently published by Lanvers-Kaminsky et al. who show a higher degree of growth inhibition by treosulfan in four ES cell lines examined compared to busulfan [21]. The IC_{50} for their cell lines were determined after 72 and 96 h of incubation in contrast to our studies, which were obtained after 48 h of incubation, therefore explaining higher IC_{50} values in our cell lines. When evaluating the effect of treosulfan in vitro, the airborne toxicity of its volatile metabolite diepoxybutane must be considered [39]. In order to minimize this effect, untreated cells were placed next to cells incubated with the two lowest concentrations of treosulfan, 1 and 5 µmol/l. There was no difference in the survival of untreated cells when placed either next to 1 or 5 µmol/l, suggesting that airborne toxicity was not a problem at lower concentrations. However, we can not rule out that airborne toxicity might have lowered survival at higher concentrations. Whereas the in vitro cytotoxicity of treosulfan could have been increased by airborne toxicity, the in vitro instability of busulfan due to hydrolyzation might have lowered its cytotoxic activity.

To further analyze the growth inhibitory effect of treosulfan, we investigated its influence on cell cycle and apoptosis. At low concentrations, the growth inhibitory effect was mainly due to the induction of a G2 arrest. Inhibition of cell cycle started at concentrations as low as 1 µmol/l with a peak at 5 µmol/l. This was observed in the wt p53 cell line TC32 as well as in the other p53-deficient cell lines. A similar G2 arrest was observed with busulfan starting at higher concentrations. G2 arrest has been previously described in several human cancer cell lines after incubation with busulfan and is also observed with other alkylating agents [40].

Incubation with higher concentrations of treosulfan led to marked cell death via apoptosis in all cell lines examined except of cell line JR. Apoptosis was accompanied by a decrease in caspase-9 suggesting activation of the intrinsic apoptotic pathway, which is initiated by the activation of the initiators caspase-9 [41]. Since cell line A4573 does not express caspases-8 and -10 and treosulfan does not induce their expression (data not shown), activation of the extrinsic pathway in which caspases-8 and/or -10 function as initiator caspases, does not appear to play a major role in treosulfan-induced apoptosis. In cell line JR, treosulfan induced apoptosis at 1,000 µmol/l only in a minority of cells as shown by nuclear fragmentation analysis of sub-G1 content, annexin V/propidium iodide and nuclear condensation by Hoechst 33258 staining, whereas more than 80% of cells were demonstrated to be dead by the trypan-blue exclusion method. This underlines the notion that treosulfan is able to induce cell death even in cells resistant to apoptosis at concentrations achievable in vivo.

Treosulfan was able to suppress the growth of Ewing's sarcoma cell lines in vivo in an orthotopic mouse xenograft model. Pharmacokinetic analysis of treosulfan administered i.p. at doses of 3,000 mg/kg in mice reflect a concentration versus time curve (AUC) calculated by a simulation of an intravenous administration of 30 g/m² treosulfan in humans based on the data of Hilger et al. and Scheulen et al using TOPFIT 2.0 [32–34]. The dose of 3,000 mg/kg has been described as the maximal tolerated dose in mice [38, 42], whereas in a phase I/II trial in humans dosages of up to 47 g/m^2 have been tolerated when bone marrow rescue with autologous stem cells was done [34]. The dose limiting toxicities in humans have been diarrhea grade III/IV, mucositis grade III and toxical epidermal necrolysis [34]. Preliminary data in patients with primary metastatic or relapsed Ewing's sarcoma and previous central axis radiotherapy show that high-dose chemotherapy with treosulfan at a dose of 3 × 12 g/m^2, together with melphalan and followed by autologous stem cell rescue, is well tolerated [22]. Since pharmacokinetic studies in humans have demonstrated identical AUCs for single dose (1 × 39 g/m^2 iv) and split dose (3 × 13 g/m^2/d iv on 3 consecutive days) [34], one can assume that at least in the adult patients of the study an AUC similar to the one in mice had been achieved. Pharmacokinetics often differ between children and adults. Since a large percentage of patients with Ewing's sarcoma are children [43, 44], such studies in children and adolescents are needed. In contrast to treosulfan, treatment of mice with busulfan at a concentration of 60 mg/kg only weakly inhibited tumor growth. There has been only one other report published so far, describing the effect of busulfan on the growth of Ewing's sarcoma cells in mice [45]. In that report, busulfan did not suppress tumor growth in athymic mice bearing tumors of the Ewing's sarcoma cell line SK-N-MC at dosages of 50 or 62.5 mg/kg, whereas it was able to induce growth delay in 3/3 neuroblastoma and 1/3 medulloblastoma xenografts. Pharmacokinetics done in mice at a dose of 50 mg/kg i.p., which was the maximal tolerated dose in this study, have shown similar total exposure levels when compared to children who got busulfan at a dose of 37.5 mg/m^2 po every 6 h for 16 dosages [45]. Since the focus of our paper is on treosulfan, we have not done pharmacokinetics for busulfan and therefore can not completely rule out that the weak effect of busulfan on the growth of Ewing's saroma cells in our xenograft model might have been due to low plasma levels. Busulfan is poorly soluble in water and requires the addition of an organic solvent, usually DMSO [45]. In the literature different ways of preparing busulfan for xenograft experiments have been reported. In our in vivo studies, busulfan was

first dissolved in 100% DMSO and then diluted to a 20% solution in PBS as described by Ashizuka [46]. In other xenograft studies, final concentrations of busulfan in DMSO ranged between 5 and 100% [38, 45]. In a mouse xenograft model for acute lymphoblastic leukaemia (ALL), busulfan was dissolved in 5 % DMSO and inhibited the growth of ALL xenografts [38]; in the xenograft model of different solid tumors by Boland busulfan was dissolved in 100% DMSO [45]. It is therefore unlikely, that the weak anti-tumor effect of busulfan observed here was due to its preparation.

In summary, we demonstrate that the alkylating agent treosulfan inhibits the growth of ES cell lines in vitro and in vivo in a mouse xenograft model and that this growth inhibitory effect is mainly due to a G2 cell cycle arrest at low concentrations and due to apoptosis at higher concentrations. In addition, we show that treosulfan was able to induce cell death in an apoptosis-deficient cell line at concentrations achievable in vivo. Although chemosensitivity in vitro and in a xenograft model can not be directly transferred to the clinics, our data clearly demonstrates that treosulfan is effective against ES cells and establishes a foundation for clinical trials using treosulfan in patients with Ewing's sarcoma.

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