Cancer Chemother Pharmacol (2008) 62:821–830
DOI 10.1007/s00280-007-0669-3

ORIGINAL ARTICLE

Cytotoxic effects of treosulfan and busulfan against leukemic cells of pediatric patients

Doreen Munkelt · Ulrike Koehl · Stephan Kloess · Stefanie-Yvonne Zimmermann · Rabiá El Kalaäoui · Sibylle Wehner · Dirk Schwabe · Thomas Lehrnbecher · Ralf Schubert · Joerg Kreuter · Thomas Klingebiel · Ruth Esser

Received: 4 October 2007 / Accepted: 21 December 2007 / Published online: 2 February 2008

Abstract
Purpose The alkylating agent treosulfan exerts a high cytotoxic activity against various malignant cells. Due to limited non-hematological toxicity, treosulfan might be a promising compound in myeloablative therapy for hematopoietic transplantation in children. Since in vitro data regarding the activity of treosulfan against childhood leukemic cells are limited, we compared the effect of treosulfan and busulfan against pediatric leukemic and non-malignant cells.
Experimental design Both agents were tested alone and in combination with fludarabine by means of the MTT and/or a five color-flow cytometric assay. Moreover, the induction of apoptosis by treosulfan was investigated via regulation of the proteinase caspase 3.
Results Treosulfan was more active against leukemic cells of 20 children as well as against 3 leukemia-derived cell lines than busulfan, with increasing IC_{50} values from initial diagnosis to relapse. Overall purified stem cells were most sensitive, followed by $CD56^{+}CD3^{-}$ NK and $CD3^{+}$ T cells. The combination of treosulfan with fludarabine resulted in a synergistic effect against leukemic cells. In malignant cells, treosulfan induced rapid cell apoptosis measured by the activation of the centrally proteinase caspase 3.
Conclusion Our results indicate that treosulfan has activity against pediatric leukemic cells, myeloablative potential and immunosuppressive properties suitable for conditioning regimen in childhood malignancies.

Keywords Treosulfan · Busulfan · Leukemia

Doreen Munkelt and Ulrike Koehl have contributed equally to this work.

D. Munkelt · U. Koehl · S. Kloess · S.-Y. Zimmermann · R. E. Kalaäoui · S. Wehner · D. Schwabe · T. Lehrnbecher · T. Klingebiel · R. Esser
Pediatric Hematology and Oncology,
University Hospital, 60596 Frankfurt, Germany

R. Schubert
Pediatric Pneumology and Allergology,
University Hospital, 60596 Frankfurt, Germany

J. Kreuter
Department of Pharmaceutical Technology,
J. W. Goethe University, Biozentrum Niederursel,
60438 Frankfurt, Germany

U. Koehl (✉)
Pediatric Hematology and Oncology, Laboratory for Stem Cell Transplantation and Immunotherapy, Johann Wolfgang Goethe University, Theodor-Stern-Kai 7, 60596 Frankfurt, Germany
e-mail: koehl@em.uni-frankfurt.de

Introduction

Treosulfan is an alkylating agent that is structurally related to busulfan. It is used in the treatment of ovarian carcinoma and currently considered in clinical trials as a conditioning agent prior to autologous and allogeneic stem cell transplantation (SCT) [6, 26]. While high-dose busulfan is associated with severe dose-limiting adverse effects such as interstitial pneumonia, hemorrhagic cystitis, convulsion, mucositis [1, 19, 31, 32] and an increased risk for veno-occlusive disease, high-dose treosulfan has been found to be less toxic on non-hematological tissues [3, 26]. In addition, treosulfan combines favorably myeloablative and immunosuppressive efficacy and might be a preferential agent in combination with the immunosuppressive drugs fludarabine or cyclophosphamide for conditioning protocols. Therefore, it seems an ideal candidate to replace

busulfan in myeloablative therapy for allogeneic SCT. First successful results in adult hematological malignancies have been reported by Casper et al. [6]. Although childhood and adult leukemias are biologically different [13], treosulfan might be of interest for the treatment of childhood leukemia as well, because of both its very low non-hematological toxicity and its broad spectrum of antineoplastic activity against various cancers [9, 11, 14, 16, 20, 27]. To date, the prognosis of children with relapsed or therapy refractory leukemia remains still dissatisfactory despite multimodal chemotherapy and SCT. This is necessitating optimization of high-dose chemotherapy (HDC) protocols similar to the approach of Den Boer et al. [8] for patient stratification based on drug resistance profiles in children with leukemia. Accordingly, the development of new HDC protocols requires several stages of pre-clinical and clinical investigations including in vivo and in vitro studies.

There is little data available describing in vitro and in vivo efficacy of treosulfan compared to busulfan in leukemic cells, and literature lacks such in vitro data in leukemic cells of pediatric patients. Lanvers-Kaminsky et al. [18] show a high sensitivity of adult leukemic cell lines towards treosulfan, which has been superior, compared to busulfan incubation. Most importantly, xenograft mouse models described by Fichtner et al. [12] yielded promising in vivo results for three childhood leukemias when comparing treosulfan with busulfan.

We, therefore, sought to evaluate the cytotoxic effect and dose response of treosulfan and busulfan on both freshly isolated leukemic pediatric cell samples and normal hematological cells like stem cells, T cells and natural killer (NK) cells. The nucleoside-analogue fludarabine was added in some experiments to evaluate possible interactions, because fludarabine is commonly combined with busulfan. Additionally, the rapid cell apoptosis was accessed by increased cleavage of pro-caspase 3 in the high active parts of this proteinase to detect the apoptotic action in leukemic cells.

Materials and methods

Primary leukemic cells

Primary leukemic cells were obtained from 20 children at diagnosis ($n = 13$), during therapy ($n = 4$) or at relapse ($n = 6$). The mononucleated cells (MNC) from bone marrow aspirates, pleural effusions and/or peripheral blood samples were isolated by density gradient centrifugation (Biocoll, Biochrome, Berlin, Germany), with a median proportion of 82% leukemic cells. Immunophenotyping and immunological subclassification with four color-flow cytometry (FACSCalibur™, Beckton Dickinson, Heidelberg, Germany) according to EGIL criteria [4] showed cALL (common acute lymphoblastic leukemia) ($n = 12$), mature B-ALL ($n = 1$), mature T-ALL ($n = 5$), prae T-ALL ($n = 1$), AML (acute myeloid leukemia) ($n = 3$) and biphenotypical leukemia (AML/T-ALL, $n = 1$) (Table 1). Informed consent was given by the parents and the use of the samples has been approved by the University Hospital Ethics Committee (Frankfurt, Germany). Moreover, leukemic cells of three additional pediatric patients suffering from ALL were used to determine caspase 3.

Cell lines

For comparison with the data in the literature, three commercially available leukemic cell lines were used: H9 cells (T-ALL, ATCC No HTB-167), K562 cells (CML, ATCC No CCL-243) and Molt4/8 cells (T-ALL, ATCC No CRL-1582).

Purification of non-malignant cells from healthy donors

After informed consent, 20 ml blood or 5 ml of leukapheresis products from five healthy donors were used to isolate $CD3^+$ T cells, $CD56^+CD3^-$ NK cells or $CD34^+$ hematopoietic stem cells, respectively. Subpopulations were positively selected using either immunomagnetic microbeads on the MACS device (Miltenyi Biotec, Bergisch Gladbach, Germany) or the Rosette Sep enrichment Kits™ (CellSystems Biotechnology, St. Katharinen, Germany) as described previously [2]. Purity of the enriched cells was ≥93%.

Cell culture and expansion

Primary leukemic cells

Leukemic cells of the patients were cultivated in RPMI 1640 medium (Sigma, Poole, Hants, UK) supplemented with 20% FCS (Fetal Calf Serum Gold; PAA Laboratories GmbH, Austria) and 2 mM L-glutamine (Gibco, Paisley, UK) at 37°C in a humidified atmosphere with 5% CO_2. The viability was tested by flowcytometry using propidium iodide (PI) staining.

Cell lines

H9 and K562 cells were cultivated in RPMI 1640 medium, while Molt4/8 cells were maintained in IMDM (Iscoves

Table 1 IC_{50} values for treosulfan and busulfan in leukemic cells of pediatric patients suffering from leukemia, in leukemic cell lines and in purified cells of healthy donors

Material	Diagnosis	Disease status	IC_{50} treosulfan (μM)	IC_{50} busulfan (μM)	Bu:Treo ratio	Bu/Treo pre-treatment? [a]
Patients						
1	cALL	Initial	0.7	8.1	11.6	No/no
2	cALL	Initial	52.5	243.6	4.6	No/no
3	cALL	Initial	42.0	111.7	2.7	No/no
4	cALL	Initial	13.1	81.2	6.2	No/no
5	cALL	Initial	35.0	81.2	2.3	No/no
6	cALL	Initial	6.0	122.5	20.4	No/no
7	cALL	Progression during initial therapy	61.3	243.6	4.0	No/no
8	cALL	Progression during initial therapy	61.3	263.9	4.3	No/no
9	cALL	Relapse	245.0	446.6	1.8	No/no
10	cALL	Relapse	183.8	162.4	0.9	No/no
11	cALL	Relapse	122.5	162.4	1.3	No/no
12	cALL	Relapse	122.5	406.0	3.3	No/no
13	Mature T-ALL	Initial	13.1	60.9	4.6	No/no
14	Mature T-ALL	Initial	5.3	35.5	6.7	No/no
15a	Mature T-ALL	Initial	1.8	12.2	6.8	No/no
15b	Mature T-ALL	Relapse	1.8	52.8	29.3	No/no
15c	Mature T-ALL	Progression during relapse therapy	14.0	89.1	6.4	No/no
16	Pre T-ALL	Initial	5.3	126.0	23.8	No/no
17	Mature B-ALL	Initial	490.0	538.0	1.1	No/no
18	AML	Initial	9.8	140.0	14.3	No/no
19a	AML	Initial	30.6	365.4	11.9	No/no
19b	AML	d15 of initial therapy	30.6	365.4	11.9	No/no
20	AML/T-ALL biphenotypic	Relapse	280.0	284.2	1.0	Yes/no
Cell lines						
H9	T-ALL		55.0 ± 8.8	122.0 ± 5.4	2.2	
Molt4/8	T-ALL		9.8 ± 2.7	15.7 ± 2.1	1.6	
K562	CML		147.0 ± 22.3	540.0 ± 29.9	3.7	
Healthy donor cells						
T cells	Donor 1		12.3	122.0	9.9	
T cells	Donor 2		45.5	89.8	2.0	
T cells	Donor 3		49.7	144.6	2.9	
NK cells	Donor 4		1.8	9.0	5.0	
NK cells	Donor 5		4.6	12.0	2.6	
Stem cells	Donor 2[b]		<0.4	7.3	>18	

Cell survival of fresh leukemic cells of pediatric patients, of leukemic cell lines and purified donor cells was determined after incubation with various concentrations of busulfan and treosulfan for 72 h. IC_{50} values were determined by plotting the drug concentration versus cell viability. Surviving cells in patient samples and in samples of healthy donors were then analyzed by using a flowcytometric assay, which allows discrimination of leukemic cells and lymphocytes as described in materials and methods. In cells lines, both the MTT and the flow cytometric assay was used

Cells of two patients (No. 15 and 19) were investigated twice or three times initially, at the time point of relapse or during therapy, respectively (a–c)

Bu busulfan, *Treo* treosulfan, *AML* acute myeloid leukemia, *ALL* acute lymphoblastic leukaemia, *cALL* common acute lymphoblastic leukaemia, *CML* chronic myeloid leukemia, *NK cells* natural killer cells

[a] Cyclophosphamide was included as an alkylating agent on day 36 and 64 during the initial therapy

[b] CD34 selected stem cells were investigated in one single experiment in duplicate, only

modified Dulbeccos medium, Sigma–Aldrich, St. Louis-Missouri, USA). Both media were supplemented with 10% FCS and 2 mM L-glutamine. Cells were cultured at 37°C with 5% CO_2 to exponential growth phase.

Non-malignant cells

Purified stem cells were cultivated in X-Vivo 10 (Bio Whittacker Europe, Verviers, Belgium) complete medium supplemented with 5% fetal bovine serum (FBS, Sigma–Aldrich, Steinheim, Germany), 2 mM L-glutamine (Gibco, Paisley, UK), and the following recombinant human proteins: flt3-ligand and stem cell factor (100 ng/ml each) and interleukin-3 and -6 (20 ng/ml each) (Cellsystems Biotechnology, St. Katharinen, Germany).

T cells and NK cells were cultivated in X-Vivo 10 supplemented with recombinant human interleukin-2 (IL-2, Chiron, Ratingen, Germany) at 100 or 1,000 U/ml for T cells or NK cells, respectively. In addition, T cells were stimulated with soluble antibodies against CD3 (20 ng/ml, Orthoclone®OKT3, Janssen Cilag, Neuss, Germany) and CD28 (1 μg/ml, Beckman Coulter Immunotech, Krefeld, Germany). The activating agents were added to keep cells alive. Drug sensitivity was tested directly after isolation (T cells) or after 3–5 days of culture (NK cells), when cells were stable but more or less non-proliferating [15].

Drugs

Treosulfan was kindly provided by medac GmbH (Hamburg, Germany). A stock solution of 50 mg/ml was prepared with acidic media and stored at −20°C until use. Busulfan (Busilvex, Pierre Fabre, Boulogne, France) and fludarabine (Fludara, medac Schering GmbH, Munich, Germany) stock solutions (busulfan: 6,000 μg/ml and fludarabine: 10,000 μg/ml) were obtained as freshly prepared clinical grade pharmacy preparations for each experiment.

Drug sensitivity tests

Cells were incubated for 72 h in culture flasks with concentrations of 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1,000 μmol/l of busulfan or treosulfan, respectively, either alone or in combination with fludarabine at 2.5, 6.25 and 12.5 μmol/l. An incubation period of 72 h was considered necessary, because treosulfan is only meaningful in long-term drug incubation due to its action via non-enzymatic formation of alkylating epoxides. Cells in the corresponding media but without drugs added served as controls. All experiments were performed in triplicate.

MTT assay

The MTT assay was used as an in vitro drug sensitivity test for the experiments with leukemic cell lines. The assay measures the ability of viable cells to convert the soluble tetrazolium salt 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium-bromide (MTT) into the insoluble formazan precipitate [21]. A volume of 10^5 cultured cells was transferred to a 96 well plate, 500 μg/ml MTT/well was added and incubated for 4 h at 37°C. In order to lyse cells and solubilize the formazan precipitate, isopropanol + 2% 2N HCl was added. The optical density of the formazan solutions was measured on the Tecan Spectra-mini (Crailsheim, Germany) using a 540 nm filter.

Flow cytometric assay

To determine the cytotoxicity of drugs against leukemic cells in patient samples containing >20% of normal peripheral blood cells, we have used a modified flow cytometric assay as described previously [35]. Because lymphocytes may differ from leukemic cells in their susceptibility to cytotoxicity of treosulfan and busulfan, a differentiation of both the cell types seemed to be essential. Briefly, the assay is based on differential immunostaining of both leukemic and normal cells in combination with propidium iodide (PI) and Annexin. PI and Annexin V (Annexin V-FITC Apoptosis-Detection-Kit, BD Bioscience Pharmingen, San Diego, USA) were used to stain apoptotic and necrotic cells, respectively. Phycoerythrine (PE) and phycoerythrine-cyanine 7 (PC7) conjugated monoclonal antibodies, possible suited to distinguish the leukemic cells from lymphocytes, were chosen according to the known immunologic phenotype of the leukemia for optimal staining of the leukemic cells. In most cases, the antibodies were directed against CD10, CD19, CD34, CD13 and/or CD133. The percentage of leukemic cells in the patient samples ranged from 41−98%. Five color-flow cytometric analysis was performed with a Cytomics FC500 flow cytometer (Beckman Coulter, Krefeld, Germany).

Statistics and calculations

Cytotoxicity was calculated for both the MTT assay and flow cytometric assay with the following equation:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental percentage}}{\text{Control percentage}} \times 100$$

IC_{50} values (the drug concentrations that kill 50% of the cell population) were determined by plotting the drug concentration versus cell viability.

Synergistic and antagonistic effects of treosulfan or busulfan and fludarabine were evaluated with median effect analysis according to Chou and Talalay [7]. Thereby, the dose-effect curve was plotted for each agent and the indicated ratio-combination using the equation:

$$f_a/f_u = (D/D_m)^m$$

In this equation, D is the dose administered, D_m is the dose required for 50% inhibition of growth, f_a is the fraction affected by dose D, f_u is the unaffected fraction and m is the coefficient denoting the sigmoidicity of the dose-effect curve. In a computerized program (CalcuSyn software, Biosoft, Cambridge, UK) the f_a values from each single concentration as well as from their mixture from our dose-response curves were entered in the CalcuSyn software and the combination index (CI) was calculated. CI values >1 indicate synergism, values >1 show antagonism and values of 1 indicate additive effects. The CI values obtained from the classical and the conservative isobologram equations are presented in this report.

For statistical analysis, we used GraphPadPrism version 4-00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com. Data were compared by the nonparametric Mann–Whitney rank sum test. Two-sided $P < 0.05$ was considered to be statistically significant.

Caspase 3 determination

An absolute cell number of 20×10^6 cells was used for Western blot protein preparation. First, the cells were incubated for 40 h with increasing treosulfan concentrations. Then, the total protein was precipitated (1.5 ml of 100% isopropanol) from a phenol–ethanol supernatant, and the precipitate was dissolved in 1% SDS. The protein (40 µg per lane) was fractionated on Laemmli gels and electroblotted onto nitrocellulose filters (Protrans; Schleicher & Schuell). After blocking overnight at 4°C, the blots were incubated for 2 h at room temperature with a rabbit antibody (IgG) directed against caspase 3 in a 1:100 dilution with blocking buffer (Santa Cruz Biotechnology Inc., Heidelberg, Germany). The blots were washed and developed with a peroxidase A-conjugated goat-anti rabbit-IgG (1:10,000) (Merck Biosciences, Darmstadt, Germany). Immunoreactive peptides were visualized by chemiluminescence and exposure to X-ray film. The autoradiographs were analyzed by scanning densitometry. Equal protein loading and blotting was verified by α -tubulin immunostaining (Santa Cruz Biotechnology Europe, Heidelberg, Germany).

Results

Correlation between the MTT and flowcytometric assays for evaluation of cytotoxicity

The MTT assay provides an easy method to measure cytotoxic effects in cell lines but does not allow distinguishing the effect on leukemic and non-malignant cells contained in the same sample. Percentage of leukemic cells in the 23 samples of our pediatric patients ranged from 41–98%. Therefore, our newly established four and five-color flowcytometric assay, which allows a distinct differentiation between normal and leukemic cells [35], was applied to the patients' samples. To compare the results obtained with both the methods, we conducted a series of experiments with leukemic cell lines. The experiments yielded a good correlation ($r > 0.95$) as shown exemplarily for Molt4/8 cells (Fig. 1a). As measured by flow cytometry, the percentage of apoptotic and dead cells increased substantially with rising concentrations of treosulfan in Molt4/8 cells (Fig. 1b, c). Similar results were obtained from K562 and H9 cells (data not shown).

Cytotoxicity of treosulfan and busulfan against leukemic and non-malignant cells

Both treosulfan and busulfan IC_{50} values varied widely in 23 samples of primary childhood leukemia (Table 1). Overall, treosulfan was more effective against the malignant cells than busulfan (median four times). Moreover, the median IC_{50} values of treosulfan and busulfan increased from diagnosis to relapse in samples (including progression under therapy), from 13.1 to 91.9 µmol/l for treosulfan and 111.7 to 253.8 µmol/l for busulfan, respectively. Significant differences between initial and relapsed leukaemia could be shown for treosulfan ($P = 0.02$; Mann–Whitney rank sum test), only, but not for busulfan.

Interestingly, initial T-ALL samples were more sensitive to treosulfan compared to cALL samples. In initial T-ALL samples, treosulfan was nine times more active compared to busulfan, while in initial and relapsed cALL samples the median activity was only four and two times higher. Thereby, sensitivity of T-ALL compared to cALL cells has been significantly different for both treosulfan and busulfan, respectively ($P = 0.02$ and 0.03). Similarily, a wide variation was found in the cell lines as well, with median IC_{50} values ranging from 9.8 to 147 µmol/l (treosulfan) and 15.7 to 540 µmol/l (busulfan). In addition, we found that NK cells were more sensitive to either drug as compared to T cells of healthy donors with a three times lower median IC_{50} value for treosulfan versus busulfan (Table 1). In a single experiment, purified stem cells seemed to be most sensitive to both

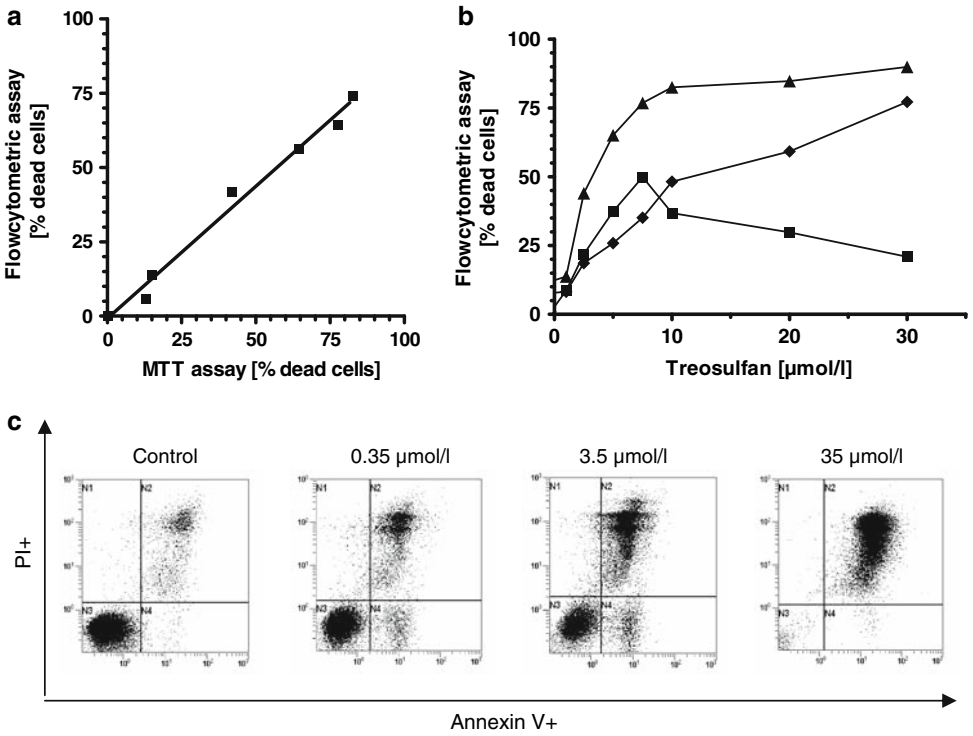

Fig. 1 **a**. Comparison of the flowcytometric and MTT assay for the evaluation of cytotoxicity. The percentage of dead cells was determined with both 5-color flowcytometric assay and MTT assay in Molt4/8 cells for 72 h after incubation with increasing concentrations of treosulfan; $r = 0.98$. **b**. Differentiation of apoptotic and dead cells via staining with Annexin V and PI. Apoptotic as well as dead cells were evaluated in Molt4/8 cells using the 5-color flowcytometric assay during incubation with increasing concentrations of treosulfan. *Filled square* Annexin V^+ PI^- cells (early apoptotic cells), *Filled diamond* PI^+ Annexin V^+ cells (late apoptotic/necrotic cells), *Filled triangle* Σ whole amount of apoptotic and dead cells. **c**. Flowcytometric dot plots of apoptotic and dead cells. Molt4/8 cells were incubated with no (control) or increasing concentrations of treosulfan and cytotoxicity was measured using Annexin V and PI staining. PI^+/Annexin V^- dead cells via non-apoptotic mechanism. PI^+/Annexin V^+ late apoptotic/necrotic cells. PI^-/Annexin V^- living cells. PI^-/Annexin V^+ early apoptotic cells. With increasing drug concentrations, a slide of viable cells from the living cells to the early apoptotic cells appears, which ends up in the quarter "late apoptotic/necrotic cells" because of cell death. If cells are going into the apoptotic pathway, phosphatidylserine is switched to the cellsurface, visualized by Annexin V

the agents. However, sensitivity of lymphocytes against both the cytostatic drugs was nonsignificant compared to the leukemic cells of our patients.

Interaction of fludarabine with treosulfan and busulfan

We saw a highly synergistic effect of a combination of treosulfan with fludarabine for concentrations of treosulfan higher than 1 µmol/l in combination with either 2.5, 6.25 or 12.5 µmol/l fludarabine (Fig. 2a). At lower concentrations, synergism was converted to antagonism. In contrast, the combination of busulfan and fludarabine resulted in antagonism (Fig. 2b).

Caspase 3 activity in treosulfan-treated leukemic cells

The apoptosis was investigated in freshly isolated ALL cells of three pediatric patients and in the T-cell derived leukemic cell line Molt4/8. Treosulfan induced rapid cell apoptosis enhanced cleavage of the inactive pro-caspase 3 into the high active effector elements of the active caspase 3 (17 and 11 kDa) in a dose-dependent manner (Fig. 3).

Discussion

The alkylating agent treosulfan, structurally related to busulfan, is known for its favorable side-effect profile. This has led to its high-dose use in pediatric solid tumors with acceptable organ toxicity [10]. Whereas treosulfan has been used for both myeloablative and non-myeloablative conditioning therapy in adult allogeneic SCT for hematological malignancies [3, 6, 17, 25], literature lacks equivalent data on children. While on one hand the scarce pharmakodynamic data on adult leukemic cells seem to favor the use of treosulfan as antileukemic drug, on the other hand, childhood acute leukemias are known to be biologically different from that of adults [13]. Therefore,

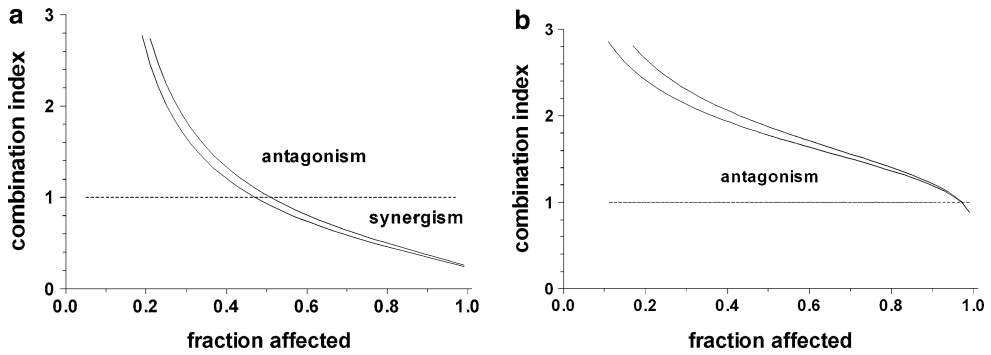

Fig. 2 Interaction between treosulfan or busulfan and fludarabine. Molt4/8 cells were incubated for 72 h with treosulfan or busulfan (0–1,000 µmol/l), alone or in combination with various concentrations of fludarabine (2.5; 6.25 and 12.5 µmol/l). Cytotoxicity was determined by the 5-color flowcytometric assay. Combination Index (CI) plots were generated by computerized median-effect analysis using the mutually non-exclusive isobologram equation (*upper curves*) and the mutually exclusive isobologram equation (*lower curves*), which resulted in highly synergistic effect of treosulfan with fludarabine for concentrations of treosulfan higher than 1 µmol/l in combination with fludarabine (**a**). In contrast, the combination of busulfan and fludarabine resulted in antagonism (**b**). The values of combination index higher than 1 represents antagonism and below than 1 synergism as described in "Material and methods"

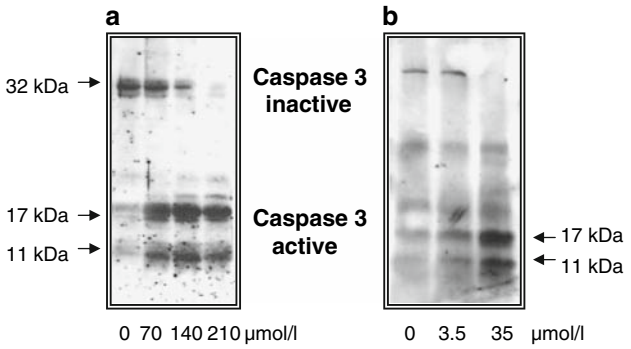

Fig. 3 Induction of apoptosis via caspase 3 activation by treosulfan. Molt4/8 cells (**a**) and cultured leukemic cells of a pediatric patient suffering from ALL (**b**) were incubated with increasing concentrations of treosulfan for 72 h compared to control cells without drugs. Pro-caspase 3 and its active cleavage products were monitored by immunoblot method. Representative Western blot analysis from one of three independent cell line (Molt4/8) experiments (*left side*) and from one of three different pediatric patients suffering from ALL are displayed

our study aimed at gathering data on the in vitro efficacy of treosulfan against childhood leukemic cells and at comparing treosulfan with busulfan, which to date is used for conditioning therapy prior to allogeneic SCT.

Because patient samples frequently contain non-malignant cells that can distort the results unless excluded from analysis, the patient samples were analyzed by our recently established flow cytometric assay [35]. This allowed discrimination of dead as well as apoptotic leukemic cells from normal cells. First, this method was compared with the simpler MTT assay with the cell lines K562, Molt4/8 and H9. Our results obtained with both the methods after 72 h of drug incubation were similar resulting in IC_{50} values in the range of 10–147 µmol/l and 16–560 µmol/l for treosulfan and busulfan, respectively. This was in accordance with data in these cell lines reported in the literature [18, 24]. Similar differences between both the drugs were seen for several tumor cell lines, where treosulfan showed a range of IC_{50} values from 1 to 600 µmol/l [9, 16, 18, 33]. For busulfan, IC_{50} values from 3–5,000 µmol/l for several tumor- and leukemia cell lines were given [18, 23, 24, 33, 34]. Of note, comparison of these studies is hampered due to various incubation periods ranging from 24 to 96 h.

However, our major goal was the comparison of treosulfan and busulfan in freshly isolated leukemic cells of pediatric patients because of both the necessity of in vitro data prior to optimization of new chemotherapy protocols and the relevance of in vitro data about cytotoxic drugs for tailored therapy in childhood leukemia as demonstrated by Den Boer et al. [8]. In our 23 childhood ALL and AML samples, IC_{50} values were four times lower in the primary leukemic cells for treosulfan than for busulfan (median IC_{50}: 31 and 126 µmol/l). Moreover the median IC_{50} values of treosulfan and busulfan increased from diagnosis (treosulfan: 13 µmol/l; busulfan 92 µmol/l) to relapse (treosulfan: 112 µmol/l; busulfan 254 µmol/l) in samples. Similarly, for treosulfan, Styczynski et al. [30] described a median IC_{50} value of 6 and 77 µmol/l for leukemic cells from children with AML who relapsed after hematopoietic stem cell transplantation (HSCT) compared to those who stayed in remission. Schmidmaier et al. [27, 30] reported in vitro IC_{50} values of approximately 10 µmol/l in leukemic cells of five adult patients with AML. Interestingly, in our pediatric samples, where the majority has been ALL samples, treosulfan was nine times more active compared to busulfan in initial T-ALL cells, which has been significantly different.

In summary, this suggests that in vitro treosulfan is more efficacious at lower doses than busulfan. In accordance

with data acquired by Scheulen et al. and Hilger et al. [14, 26], the concentrations shown to be effective in our experiments were about tenfold lower than those that can be achieved in the blood (>1 mmol/l) of patients [3, 14]. Plasma levels after busulfan treatment are two-tenths power lower compared to treosulfan [31]. Interestingly, although only one of our patients had received busulfan and no treosulfan, IC_{50} values seemed to be increased in the overall samples from diagnosis to relapse, which might be due to cross-resistance with other drugs. Alternatively, this may be a statistical phenomenon due to limited and heterogeneous sample numbers or the higher instability of busulfan. To exclude airborne influence of treosulfan [5], in our experiments single cell culture flasks for each drug concentration has been used.

In clinical treatment protocols, the maximum tolerated dose of treosulfan has been determined to be 47 g/m^2 body surface area [26] in adults when combined with SCT. This is almost 100 times more than the maximally accepted dose of 16 mg/kg body weight [1] of busulfan. This corroborates the results from a NOD/SCID xenograft mouse model using leukemic cells of children ($n = 3$) suffering from T-ALL, B-ALL and pre-B-ALL. In this setting, treosulfan could also be administered in a 100 times higher concentration ($1 \times 3{,}000$ or $3 \times 1{,}500$ mg/kg) compared to busulfan (3×20 mg/kg) [12]. Taken together these clinical differences between busulfan and treosulfan regarding plasma level as well as maximum tolerated dose and our in vitro IC_{50} values in leukemic cells of pediatric patients may suggest that treosulfan is efficious at lower doses than busulfan.

Beside the use of treosulfan in autologous SCT as reported by Drabko et al. [10] in two pediatric patients, treosulfan has been noted for its immunosuppressive potential in adult allogeneic SCT [3, 6, 17, 26]. From the results of studies in mice, this has been attributed to its toxicity against T cells [34] and a preclusive effect on the "cytokine storm" that precedes the development of graft-versus-host disease and is involved in the pathogenesis of veno-occlusive disease [29]. Ploemacher et al. [22] showed that non-myeloablative conditioning regimen using treosulfan led to enhanced chimerism and immunologic tolerance in a mouse model. We demonstrate an increasing effect of treosulfan and busulfan against purified T cells and NK cells. In a single experiment, stem cells were most sensitive. Again, in the lymphocyte subsets, treosulfan was threefold and in stem cells 20 times more effective than busulfan.

Schmidmaier et al. [28] could show that treosulfan can induce considerable cell death in the human bone marrow stromal cell line HS-5 and in primary bone marrow stromal cells. Impaired stromal cell viability could reduce the integrin binding of malignant and stromal cells and diminished cell adhesion mediated drug resistance (CAM-DR) so that enhanced tumor cell apoptosis should be expected after HSCT.

In our experiments, we could show that treosulfan acts via apoptosis by increasing activation of caspase 3 by cleavage into the active products. Schmidmaier [27] as well as Meinhardt [20] also demonstrated the induction of the apoptosis pathway like Bcl2 during treosulfan incubation.

The nucleoside analogon fludarabine is an established antimetabolic drug in conditioning therapy for allogeneic SCT, especially in low toxicity regimens and haploidentical transplantation. We, therefore, investigated the interaction between treosulfan or busulfan and fludarabine in vitro. Treosulfan showed a synergistic effect with fludarabine, while busulfan did not show any notable synergism.

In summary, treosulfan showed better in vitro activity against the patients' leukemic cells at concentrations below those achieved in a clinical setting and was also effective against lymphocytes. Together with its already known favorable clinical side-effect profile, it seems a promising agent for myeloablative therapy in childhood allogeneic transplantation for leukemia.

Acknowledgments This project was supported by "Hilfe für Krebskranke Kinder Frankfurt e.V.", "Frankfurter Stiftung für Krebskranke Kinder" and medac GmbH, Hamburg. The authors thank Regine Quaritsch, Andrea Brinkmann, Carla Kramm, Stephanie Grohal and Frauke Röger for technical support.

References

1. Blaise D, Maraninchi D, Archimbaud E, Reiffers J, Devergie A, Jouet JP, Milpied N, Attal M, Michallet M, Ifrah N (1992) Allogeneic bone marrow transplantation for acute myeloid leukemia in first remission: a randomized trial of a busulfan-cytoxan versus cytotxan-total body irradiation as preparative regimen. Blood 79:2578–2582
2. Beck O, Seidl C, Lehrnbecher T, Kreyenberg H, Schwabe D, Klingebiel T, Seifried E, Bader P, Koehl U (2006) Quantification of chimerism within peripheral blood, bone marrow and purified leukocyte subsets: comparison of singleplex and multiplex PCR amplification of short tandem repeat (STR) loci. Eur J Haematol 76:237–244
3. Beelen DW, Trenschel R, Casper J, Freund M, Hilger RA, Scheulen ME, Basara N, Fauser AA, Hertenstein B, Mylius HA, Baumgart J, Pichlmeier U, Hahn JR, Holler E (2005) Dose-escalated treosulphan in combination with cyclophosphamide as a new preparative regimen for allogeneic haematopoietic stem cell transplantation in patients with an increased risk for regimen-related complications. Bone Marrow Transplant 35:233–241
4. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, vańt Veer MB (1995) Proposals for the immunological classification of acute leukemias. European group for the immunological characterization of leukemias (EGIL). Leukemia 9:1783–1786
5. Bosanquet AG, Burlton AR (1994) Airborne cytotoxicity in the DiSC assay caused by solutions of treosulfan but not busulphan. Cytotechnology 16:131–136

6. Casper J, Knauf W, Kiefer T, Wolff D, Steiner B, Hammer U, Wegener R, Kleine HD, Wilhelm S, Knopp A, Hartung G, Dölken G, Freund M (2004) Treosulfan and fludarabine: a new toxicity-reduced conditioning regimen for allogeneic haematopoietic stem cell transplantation. Blood 103:725–731
7. Chou TC, Talalay P (1988) Quantitative analysis of dose-effect relationship: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
8. Den Boer M, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, Graubner U, Haas RJ, Jorch N, Spaar HJ, Kaspers GJ, Kamps WA, Van der Does-Van den Berg A, Van Wering ER, Veerman AJ, Janka-Schaub GE (2003) Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol 21:3262–3268
9. Di Nicolantonio F, Knight LA, Di Palma S, Sharma S, Whitehouse PA, Mercer SJ, Charlton PA, Norris D, Cree IA (2004) Ex vivo characterization of XR11576 (MLN576) against ovarian cancer and other solid tumors. Anticancer Drugs 15:849–860
10. Drabko K, Zawitkowska-Klaczynska J, Wojcik B, Choma M, Zaucha-Prazmo A, Kowalczyk J, Gorczynska E, Toporski J, Kalwak K, Turkiewicz D, Chybicka A (2005) Megachemotherapy followed by autologous stem cell transplantation in children with Ewing's sarcoma. Pediatr Transplant 9:618–621
11. Feyerabend S, Feil G, Krug J, Kassen A, Stenzl A (2007) Cytotoxic effects of treosulfan on prostate cancer cell lines. Anticancer Res 27:2403–2408
12. Fichtner I, Becker M, Baumgart J (2003) Antileukaemic activity of treosulfan in xenografted human acute lymphoblastic leukaemias (ALL). Eur J Cancer 39:801–807
13. Gutiérrez MI, Siraj AK, Ibrahim MM, Hussain A, Bhatia K (2005) Childood and adult ALL: differences in epigenetic lesions associated with cell cycle genes. Am J Hematol 80:158–160
14. Hilger RA, Harstrick A, Eberhardt W, Oberhoff C, Skorzec M, Baumgart J, Seeber S, Scheulen ME (1998) Clinical pharmacokinetics of intravenous treosulfan in patients with advanced solid tumours. Cancer Chemother Pharmacol 42:99–104
15. Koehl U, Esser R, Zimmermann S, Tonn T, Kotchetkov R, Bartling T, Sörensen J, Grüttner HP, Bader P, Seifried E, Martin H, Lang P, Passweg J, Klingebiel T, Schwabe D (2005) Ex vivo expansion of highly purified NK-cells for immunotherapy after haploidentical stem cell transplantation in children. Klin Padiatr 217:345–350
16. Kopf- Maier P (1998) The alkylator treosulfan shows activity towards human renal-cell carcinoma in vivo and in vitro. In vivo 12:275–288
17. Kröger N, Shimoni A, Zabelina T, Schieder H, Panse J, Ayuk F, Wolschke C, Renges H, Dahlke J, Atanackovic D, Nagler A, Zander A (2006) Reduced-toxicity conditioning with treosulfan, fludarabine and ATG as preparative regimen for allogeneic stem cell transplantation (alloSCT) in elderly patients with secondary acute myeloid leukemia (sAML) or myelodysplastic syndrome (MDS). Bone Marrow Transplant 37:339–344
18. Lanvers-Kaminsky C, Bremer A, Dirksen U, Jurgens H, Boos J (2006) Cytotoxicity of treosulfan and busulfan on pediatric tumor cell lines. Anticancer Drugs 17:657–662
19. Litzow MR, Prez WS, Klein JP, Bollwell BJ, Camitta B, Copelan EA, Gale RP, Giralt SA, Keating A, Lazarus HM, Marjus DI, McCarthy PL, Miller CB, Milone G, Prentice HG, Russel JA, Schultz KR, Trigg ME, Weisdorf DJ, Horowitz MM (2002) Comparison of outcome following allogeneic bone marrow transplantation with cyclophosphamide-total body irradiation versus busulfan-cyclophosphamide conditioning regimen for acute myelogenous leukaemia in first remission. Br J Haematol 119:1115–1124
20. Meinhardt G, Dayyani F, Jahrsdörfer B, Baumgart J, Emmerich B, Schmidmaier R (2003) Treosulfan is an effective inducer of cell death in myeloma cell lines and primary myeloma cells from patients. Br J Haematol 122: 892–899
21. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
22. Ploemacher RE, Johnson KW, Rombouts EJC, Kenol E, Westerhof GR, Baumgart J, White-Scharf ME, Down JD (2004) Addition of Treosulfan to a nonmyeloablative conditioning regimen results in enhanced chimerism and immunologic tolerance in an experimental allogeneic bone marrow transplant model. Biol Blood Marrow Transplant 10:236–245
23. Sanderson BJ, Johnson KJ, Henner WD (1991) Dose-dependent cytotoxic and mutagenic effects of antineoplastic alkylating agents on human lymphoblastoid cells. Environ Mol Mutagen 17:238–243
24. Sanyal U, Nanda R, Samanta S, Pain A, Dutta S, Verma AS, Rider BJ, Agrawal KC (2000) Evaluation of dimethylaminosulfonates of alkane diols as a novel group of anticancer agents. Cancer Lett 155:89–97
25. Sauer M, Zeidler C, Meissner B, Rehe K, Hanke A, Welte K, Lohse P, Sykora K-W (2007) Substitution of cyclophosphamide and busulfan by fludarabine, treosulfan and melphalan in a preparative regimen for children and adolescents with Shwachman-Diamond syndrome. Bone Marrow Transplant 39:143–147
26. Scheulen ME, Hilger RA, Oberhoff C, Casper J, Freund M, Josten KM, Bornhauser M, Ehninger G, Berdel WE, Baumgart J, Harstrick A, Bojko P, Wolf HH, Schindler AE, Seeber S (2000) Clinical phase I dose escalation and pharmacokinetic study of high-dose chemotherapy with treosulfan and autologous peripheral blood stem cell transplantation in patients with advanced malignancies. Clin Cancer Res 6:4209–4216
27. Schmidmaier R, Oellerich M, Baumgart J, Emmerich B, Meinhardt G (2004) Treosulfan-induced apoptosis in acute myeloid leukemia cells is accompanied by translocation of protein kinase C delta and enhanced by bryostatin-1. Exp Hematol 32:76–96
28. Schmidmaier R, Baumann P, Emmerich B, Meinhardt G (2006) Evaluation of chemosensitivity of human bone marrow stromal cells—differences between common chemotherapeutic drugs. Anticaner Res 26:347–350
29. Sjoo F, Hassan Z, Abedi-Valugerdi M, Griskevicius L, Nilsson C, Remberger M, Aschan J, Concha H, Gaughan U, Hassan M (2006) Myeloablative and immunosuppressive properties of treosulfan in mice. Exp Hematol 34:115–121
30. Styczynski J, Toporski J, Wysocki M, Debski R, Chybicka A, Boruczkowski D, Wachowiak J, Wojcik B, Kowalczyk J, Gil L, Balwierz W, Matysiak M, Krawczuk-Rybak M, Balcerska A, Sonta-Jakimczyk D (2007) Fludarabine, treosulfan and etoposide sensitivity and the outcome of hematopoietic stem cell transplantation in childhood acute myeloid leukemia. Anticancer Res 27:1547–1552
31. Vassal G, Koscielny S, Challine D, Valteau-Couanet D, Boland I, Deroussent A, Lemerle J, Gouyette A, Hartmann O (1996) Busulfan disposition and hepatic veno-occlusive disease in children undergoing bone marrow transplantation. Cancer Chemother Pharmacol 37:247–253
32. Vergnon JM, Boucheron S, Riffat J, Guy C, Blanc P, Emonot A (1988) Interstitial pneumopathies caused by busulfan. Histologic, developmental and bronchoalveolar lavage analysis of 3 cases. Rev Med Interne 9:377–383
33. Werner S, Mendoza A, Hilger RA, Erlacher M, Reichardt W, Lissat A, Konanz C, Uhl M, Niemeyer CM, Khanna C, Kontny U (2007) Preclinical studies of treosulfan demonstrate potent activity in Ewing's sarcoma. Cancer Chemother Pharmacol. (Epub ahead of print)

34. Westerhof GR, Ploemacher RE, Boudewijn A, Blokland I, Dillingh JH, McGown AT, Hadfield JA, Dawson MJ, Down JD (2000) Comparison of different busulfan analogues for depletion of hematopoietic stem cells and promotion of donor-type chimerism in murine bone marrow transplant recipients. Cancer Res 60:5470–5478

35. Zimmermann SY, Esser R, Rohrbach E, Klingebiel T, Koehl U (2005) A novel four-colour flowcytometric assay to determine natural killer cell or T cell-mediated cellular cytotoxicity against leukaemic cells in peripheral or bone marrow specimens containing greater than 20% of normal cells. J Immunol Methods 296:63–76