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Antileukaemic activity of treosulfan in xenografted human acute lymphoblastic leukaemias (ALL)

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Abstract

Treosulfan (L-threitol-1,4-bis-methanesulphonate; Ovastat®) is a bifunctional alkylating drug indicated for the treatment of advanced ovarian carcinoma. Recent data revealed immunosuppressive characteristics and substantial haematopoietic stem cell toxicity after repeated dosing of mice. Therefore, treosulfan is considered to be an alternative conditioning agent to busulfan (for example) administered prior to allogeneic/autologous stem cell transplantation of patients with haematological malignancies. An antineoplastic activity for treosulfan has been previously shown in preclinical models of melanoma, breast, lung and renal-cell carcinomas. Here, *in vivo* antileukaemic activity of treosulfan is compared with the activity of equitoxic doses of cyclophosphamide or busulfan for the first time using human acute lymphoblastic leukaemia (ALL)-models of paediatric origin xenotransplanted into non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice. Treosulfan treatment achieved an optimum treated to control (T/C) value of 159% (survival time) against B-ALL-SCID 7 and a T/C value of 0% (tumour growth) against T-ALL-SCID 4 and proB-ALL-SCID 19, respectively. Complete regression of established subcutaneously (s.c.) growing nodules of ALL-SCID 4 and 19 was obvious and long-term survivors without tumour re-growth were observed. Equitoxic doses of busulfan (ALL-SCID 4, 7, 19) or cyclophosphamide (ALL-SCID 19) were less effective with regard to the numbers of complete regressions and the number of cured animals. Side-effects included myelotoxicity and a small reduction in body weight, but these were tolerable. Treosulfan can be considered a highly active antileukaemic drug whose corresponding clinical value is to be tested in appropriate protocols with leukaemic patients.

Keywords: Treosulfan; Acute lymphoblastic leukaemias; Xenografts; NOD/SCID mice

1. Introduction

Treosulfan (L-threitol-1,4-bis-methanesulphonate) is a bifunctional alkylating drug [1,2] indicated in the cytostatic treatment of patients with advanced ovarian carcinoma [3–5]. Antitumour activity was additionally shown in xenotransplanted breast- [6], lung- [7], renal-cell carcinoma [8] and melanoma [9,10].

More recently, data of myeloablative [11], immunosuppressive [11,12] and stem cell toxic [13] characteristics of treosulfan were reported. Therefore, treosulfan is currently considered an alternative, intravenously (i.v.) injectable conditioning agent prior to allogeneic transplantation of haematopoietic progenitor cells [14]. However, there are limited data available describing the antileukaemic potential of treosulfan. Treatment of chronic myeloic leukaemia (CML) patients with treosulfan capsules was found to be as active and showed superior tolerability than busulfan treatment as reported by Loeb in 1964 [15]. Current reports have demonstrated sufficient *in vitro* activity of the compound against chronic lymphocytic leukaemia (CLL) and multiple myeloma (MM) cell-lines of human origin [16,17]. Potent *in vivo* activity of treosulfan against human acute lymphoblastic leukaemia (ALL) models xenografted into non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice [18] is demonstrated here for the first time.

A single dose and a repeated dose regimen were used because it has previously been shown that a sufficient treosulfan-mediated stem cell toxicity is strongly

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dependent on a repeated treatment e.g. 3 consecutive days [11,13].

In addition, the antileukaemic activity of treosulfan is compared with busulfan and cyclophosphamide that are currently used in the conditioning of leukaemia patients.

2. Materials and methods

2.1. Substances

The test compound treosulfan (Ovastat®) was supplied by medac GmbH. The compound was dissolved in warm (30 °C) Aqua and vortexed for 5 min. The limit of solubility was reached at 100 mg/ml. Therefore, at higher doses increased volumes had to be administered (1500 mg/kg: 0.3 ml/20 g body weight; 3000 mg/kg: 0.6 ml/20 g body weight).

Cyclophosphamide (Asta Medica GmbH, Frankfurt, Germany), Daunorubicin (Pharmacia Upjohn, Erlangen, Germany), Vincristine (Jenapharm, Jena, Germany), Cytarabine (H. Mack, Illertissen, Germany), Asparaginase and Methotrexate (medac GmbH, Hamburg, Germany) and Busulfan (Sigma, Taufkirchen, Germany) were used as reference compounds. Clinical formulations were dissolved as prescribed for clinical use. Busulfan was dissolved in dimethyl sulphoxide (DMSO) (5%) and diluted with saline. The maximum tolerated doses (MTDs) of cyclophosphamide and busulfan had been previously determined as 100 (single intraperitoneal (i.p.) treatment) and 20 mg/kg (3 times i.p. treatment), respectively.

2.2. Mice and tumour models

We have recently established in our laboratory a panel of different paediatric leukaemias as xenografts in NOD/SCID mice [18].

From that panel, three ALL models were selected for this treosulfan study. The characteristics of these models are indicated in Table 1. The ALL-SCID 4 originated from bone marrow blasts of a 15-year old male patient primarily diagnosed with a T-cell leukaemia. The ALL-SCID 7 was established from a bone marrow sample of a 5-year old female patient in secondary relapse with a B-cell leukaemia. The ALL-SCID 19 originated from a 1-year old boy and was characterised as pre-B-cell leukaemia.

In the first passage, blasts were transplanted i.p., i.v. and s.c. into 2–3 NOD/SCID mice for each. Further passages were performed so that a stable tumour take rate and growth could be observed within one month. The procedure led to the establishment of transplantable lines (ALL-SCID 4 and 19 s.c., ALL-SCID 7 i.v.) with an acceptable take rate (>90%) and growth. All ALL-SCID models were used between the 6th and 10th *in vivo* passage. The immunophenotype and genotype (fingerprint) coincided with the original patient sample, an advantage of this approach when compared with the use of cell line-derived ALL. The three models used were all *BCR/ABL* and *TEL/AML* negative.

In the models ALL-SCID 4 and ALL-SCID 19, treosulfan treatment was started when the tumour sizes were palpable (approximately 5 mm in diameter). Doses and schedules are shown in the figures. One group of mice received solvent (negative controls), other groups of mice were treated with cyclophosphamide (100 mg/kg i.p.) or busulfan in doses of 10 or 20 mg/kg i.p. as positive controls. Tumour diameters were measured twice weekly with callipers. Tumour volumes were calculated according to the formula $V = (\text{length} \cdot (\text{width})^2)/2$. The tumour volumes at each daily measurement were related to that on the first day of treatment (relative tumour volume (RTV)). Treated to control (T/C) values in percent were calculated for each daily measurement, the optimum (lowest) T/C value obtained during the experimental period was evaluated.

In the i.v. model ALL-SCID 7, treatment was initiated on day 3 after the transplantation. Doses and schedules are on the corresponding figure. Mice were sacrificed in the moribund stage, mean survival times (MST) (in days) and T/C values (in percent) were calculated.

The body weight of the mice was determined in all of the experiments twice a week and mean body weight change (BWC) was calculated as a percentage per group.

All NOD/SCID mice (Jackson Lab., Bar Harbor, USA) were maintained under sterile and standardised environmental conditions (20±1 °C room temperature, 50±10% relative humidity, 12 h light-dark-rhythm). They received autoclaved food and bedding (ssniff, Soest, Germany) and acidified (pH 4.0) drinking water *ad libitum*. All animal experiments were performed according to the German Animal Protection Law and with permission from the responsible authorities.

Table 1
Human ALL-models used

Code	Description	Inoculation	Host	Evaluation parameter
ALL-SCID 4	Human T-cell leukaemia	Fragments s.c.	NOD/SCID mice	Tumour growth
ALL-SCID 7	Human B-cell leukaemia	10^7 Cells i.v.	NOD/SCID mice	Survival time
ALL-SCID 19	Human pre-B-cell leukaemia	10^7 Cell s.c. in matrigel	NOD/SCID mice	Tumour growth

s.c., subcutaneous; ALL-SCID, acute lymphoblastic leukaemia-severe combined immunodeficiency; i.v. intravenous.

The injection volume was—if not otherwise mentioned—0.2 ml/20 g body weight.

Statistical evaluation was performed with the U-test of Mann and Whitney using the Windows program STATISTICA 5.1.

Haematotoxicity (white blood cells (WBC)), red blood cells (RBC), haemoglobin, haematocrit, platelets) of treosulfan and cyclophosphamide dosing was assessed in female DBA/2 mice (Charles River, Sulzfeld, Germany). We decided to use immunocompetent instead of NOD/SCID mice for that investigation because of the better tolerability of these mice to most cytostatics. For instance, the LD_{10} for cyclophosphamide in DBA/2 mice is 200 mg/kg, while in NOD/SCID mice only half of that dose is possible without causing lethality. Blood was taken from the retrobulbar venous plexus of 6 mice every four days after a single i.v. treatment with treosulfan (500, 1000, 1500, 2000, 3000 mg/kg body weight) or cyclophosphamide (200 mg/kg body weight). Blood parameters were determined using a Coulter counter (model A^C-T diff, Beckmann Coulter GmbH, Krefeld, Germany with a special veterinary oftware).

3. Results

The s.c. inoculation of the human ALL-SCID 19 leukaemic cells into immunodeficient NOD/SCID mice led to palpable tumour nodules after a latency period of 18 days (Fig. 1). At that time point, a single i.p. treatment was performed. While tumours in the control group developed to approximately 8-fold of their initial size during a period of 10 days, treosulfan treatment induced a clear dose-dependent antileukaemic effect. The lowest dose used (1000 mg/kg) induced a retardation of tumour growth. The intermediate dose (2000 mg/kg) caused 100% complete remission by day 25 with a re-growth of tumours in 2/7 mice at day 42 and of 7/7 mice by the end of experiment. The highest dose used (3000 mg/kg) cured 7/8 mice by day 55 (complete remission without re-growth). One mouse died tumour-free at day 74 from thymoma (a frequent cause of death in NOD/SCID mice). Only 1 mouse of the 8 mice in this group developed a suspicious nodule, being 7×9 mm in size at the end of experiment (day 55). Cyclophosphamide treatment induced complete tumour regressions at day 25. However, re-growth of all leukaemia nodules in 7/7 mice was observed by the end of experiment.

The treatment-related reduction in body weight was mild to moderate in all groups (0, −3, −7% for treosulfan doses of 1000, 2000, 3000 mg/kg, respectively), no mouse died of toxicity.

In a second experiment, performed with the same leukaemia model ALL-SCID 19 (Fig. 2), we wanted to compare a repeated dose regimen using a single bolus injection of treosulfan on 3 consecutive days. In this experiment, the latency period until detection of palpable s.c. leukaemia nodules was 23 days. At that time point, i.p. treatment with treosulfan or busulfan was started according to schedule outlined in the legend of Fig. 2. Treosulfan administered on 3 consecutive days led to a dose-dependent antileukaemic effect. In the high-dose group (3×1500 mg/kg), 6/7 mice remained tumour-free (cured) by day 84, while in the intermediate dose group (3×1000 mg/kg), an initial 100% complete remission was followed by a re-growth of the tumours in 6/6 mice by day 50. A sub-optimal dose of 3×500 mg/kg revealed only a transient antileukaemic effect. Treosulfan given as a single bolus injection of 1×3000 mg/kg had a comparable efficacy to the 3×1500 mg/kg schedule leading to 5/7 tumour-free mice at the end of

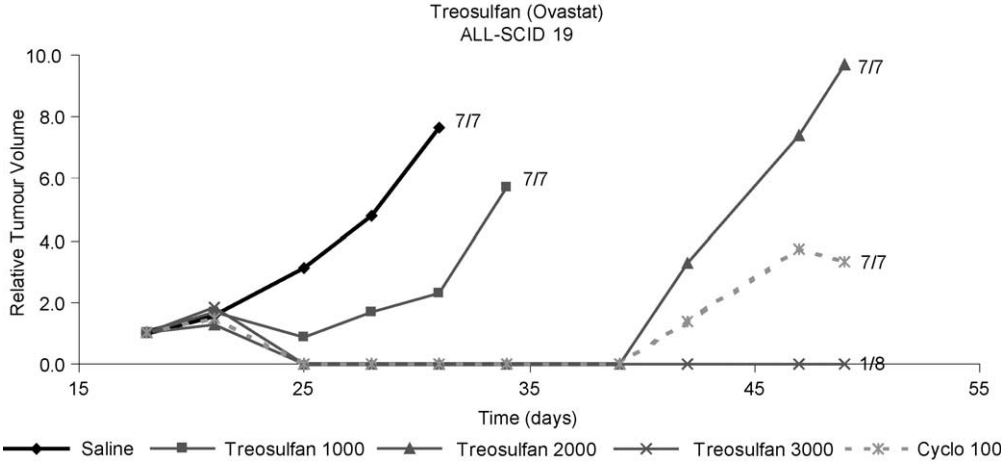

Fig. 1. Antileukaemic efficacy of treosulfan in preB-cell leukaemia acute lymphoblastic leukaemia, ALL-SCID is a special code which was defined earlier. ALL-SCID 19 model. 10^7 ALL cells admixed with matrigel were inoculated subcutaneously (s.c.) into NOD/SCID mice at day 0. At day 18, mice were treated once intraperitoneally (i.p.) with 1×1000, 1×2000 or 1×3000 mg/kg treosulfan. One group of mice received 100 mg/kg cyclophosphamide (Cyclo) i.p. The number of mice/total which showed leukaemia re-growth are indicated.

experiment. No lethality and only a slight, but dose-dependent, reduction in body weight (0 to −5%) was noticed in the treosulfan-treated mice.

Busulfan led to a moderate and transient antileukaemic effect comparable with the lowest dose of treosulfan used. Thus, it appears that dosing with 3×10 mg/kg busulfan was not in the range of the MTD. Therefore, in further experiments a higher dose of busulfan (3×20 mg/kg) was administered.

Results of treatment of ALL-SCID 4 mice are summarised in Fig. 3. Treatment was started after a relatively long latency period of 41 days. Treosulfan showed an equal activity after 3×1000 mg/kg or 1×3000 mg/kg treatment schedules, inducing 100% cure of mice by day 105. Again, no drug-related lethality was observed, but a significant reduction in body weight of −18% was noticed in the highest dose group.

Busulfan led to a dose-dependent antileukaemic activity. The increased dose of 3×20 mg/kg induced complete remissions with a re-growth of 4/6 tumours by day 70. No lethality and only a slight reduction in body weight (−4%) were observed in both of the busulfan-treated dose groups.

Leukaemic cells of the ALL-SCID 7 model were inoculated i.v. and treatment started 3 days later (Fig. 4). In all mice, a severe splenomegaly (approximately

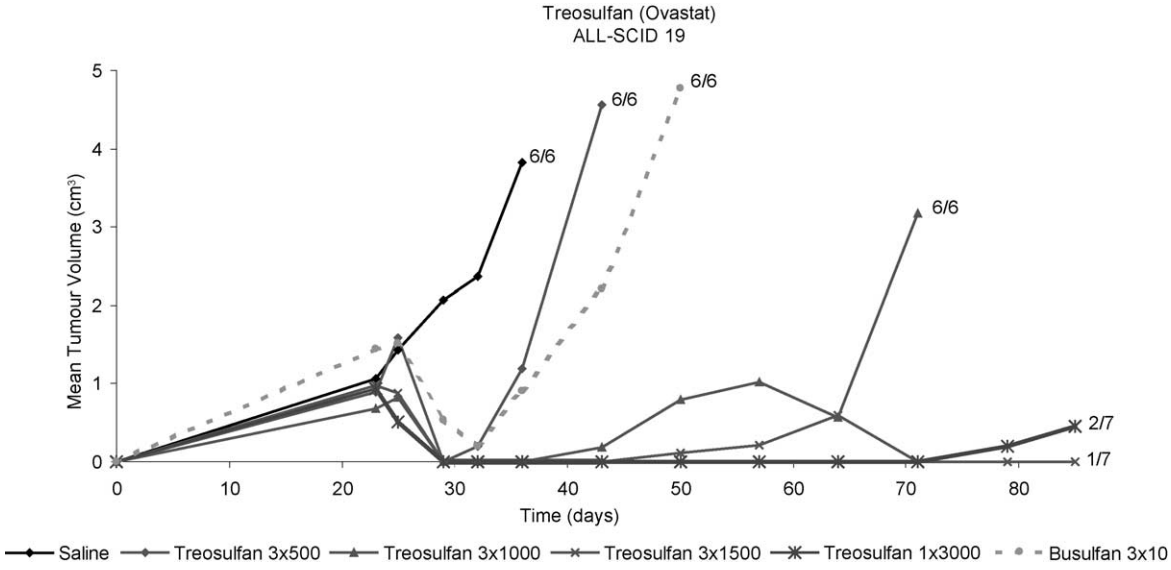

Fig. 2. Schedule-dependent efficacy of treosulfan in preB-cell leukaemia ALL-SCID 19 model. 10^7 ALL cells admixed with matrigel were inoculated s.c. into NOD/SCID mice at day 0. At day 23, treatment was started and performed either on 3 consecutive days (3×500, 3×1000, 3×1500 mg/kg/day) or as single bolus injection (1×3000 mg/kg) i.p. Busulfan was administered on 3 consecutive days i.p. (3×10 mg/kg/day). The number of mice/total which showed leukaemia re-growth is indicated.

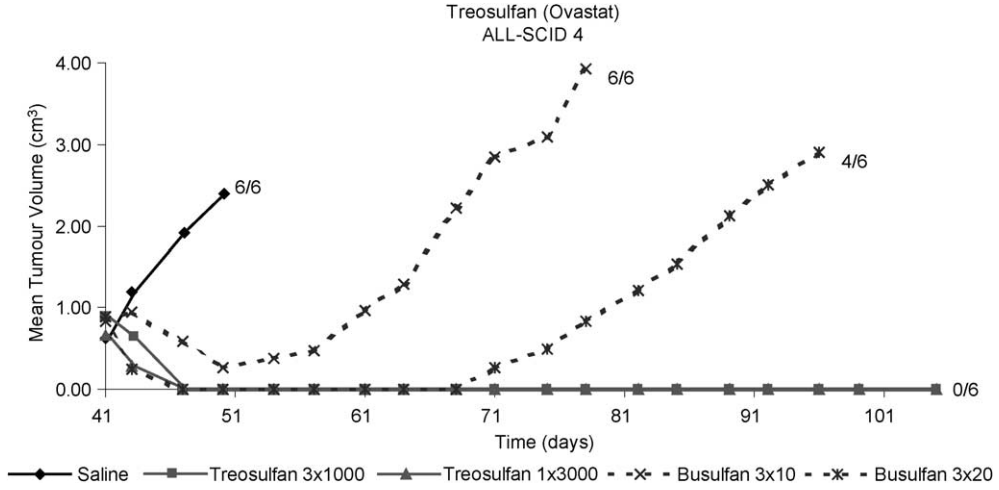

Fig. 3. Antileukaemic efficacy of treosulfan in T-cell leukaemia ALL-SCID 4 model. Tumour fragments (3×3 mm) of an s.c. growing leukaemia were transplanted s.c. into NOD/SCID mice at day 0. After 41 days, treatment with treosulfan was initiated and was performed either on 3 consecutive days (3×1000 mg/kg/day) or once (1×3000 mg/kg) i.p. Busulfan was administered on days 41, 42, 43 i.p. in dose groups of 10 or 20 mg/kg/day. The number of mice/total which showed leukaemia re-growth are indicated.

10-fold of normal weight) was observed as the main leukaemic manifestation. Saline-treated control mice had a median survival time of 28±5 days. Treatment with treosulfan, either 3×1000 mg/kg or 1×3000 mg/kg, led to a significant prolongation of survival to 139% or 159% T/C, respectively. The bolus injection of 1×3000 mg/kg induced 1/8 toxic deaths and therefore has to be considered as the MTD in this experiment. The reduction in body weight observed after treosulfan treatment was only moderate (−6%).

Busulfan administrations of 3×10 or 3×20 mg/kg led to 125% or 136% T/C of survival times, respectively, without any toxic deaths or significant reductions in body weight.

Table 2 gives an overview with regard to the chemosensitivity of the three ALL models to clinically used antileukaemic drugs. The ALL-SCID 4 and ALL-SCID 19 are relatively chemo-sensitive models responding to almost all of the compounds tested, including treosulfan. On the contrary, the ALL-SCID 7 model has to be considered as a relatively drug-resistant leukaemia, being responsive only to cyclophosphamide and vincristine. However, even in this model treosulfan showed convincing activity.

The determination of blood parameters in the NOD/SCID mice is questionable because of the relative constitutive myeloablation and depression of the immune system. Therefore, the influence of treosulfan on different blood parameters was investigated in immunocompetent DBA/2 mice at doses corresponding to the MTD in this strain. Treosulfan induced a dose-dependent significant leucopenia ($1.0\pm1.2\cdot10^9$/l WBC) in the highest dose used (1×3000 mg/kg i.v.) compared with saline-treated controls ($5.3\pm1.1\cdot10^9$/l WBC). Cyclophosphamide administered at the MTD of 200 mg/kg decreased WBC counts to a nadir of $0.6\pm0.8\cdot10^9$/l.

4. Discussion

Treosulfan was tested in three human ALLs xenotransplanted into NOD/SCID mice. It showed a significant and striking antileukaemic effect in all three models. In the s.c. growing leukaemias (ALL-SCID 4 and 19), almost 100% long-term cured animals could be observed. There was no major difference between treatment results after repeated dosing at three

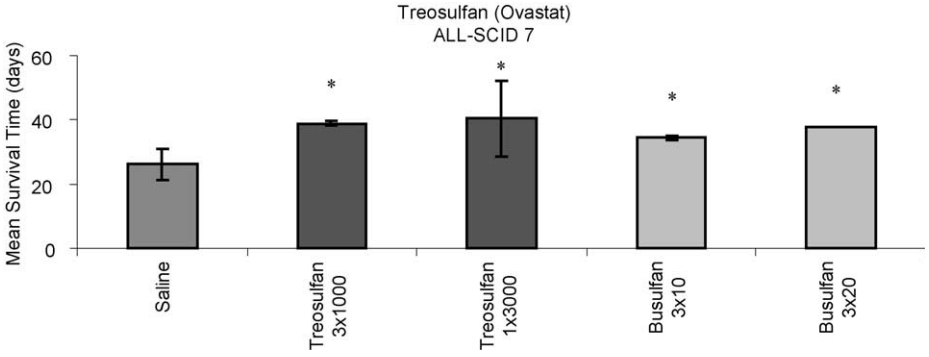

Fig. 4. Antileukaemic efficacy of treosulfan in B-cell leukaemia ALL-SCID 7 model. 10^7 leukaemic cells were inoculated i.v. into the tail vein of NOD/SCID mice at day 0; 8 mice/group. Treatment with treosulfan started at day 3 and was performed either for 3 consecutive days (3×1000 mg/kg/day) or once (1×3000 mg/kg) i.p. Busulfan doses of 10 or 20 mg/kg were administered i.p. on days 3, 4, 5. *significant vs. saline-treated control ($P<0.05$).

Table 2
Antileukaemic efficacy of clinically used drugs

Drug	Dose (mg/kg/inj.)	Treated/control (T/C)		
		ALL-SCID 4 s.c.	ALL-SCID 7 i.v.	ALL-SCID 19 s.c.
Daunorubicin	1×4 i.v.	+ + +	–	-
Cyclophosphamide	1×100 i.p.	+ + + +	+ + +	+ + + +
Vincristine	1×1 i.p.	+ +	+ +	+ + + +
Cytarabine	10×40 i.p.	+ + + +	–	+ +
Asparaginase	4×2000 IU i.v.	+ + +	–	+ + + +
Methotrexate	2×40 i.p.	+ + + +	n.t.	+ + + +
Busulfan	3×20 i.p.	+ + + +	+ +	+ + + +°
Treosulfan	1×3000 i.p.	+ + + +	+ + +	+ + + +

T/C evaluation (tumour growth): − = > 50%, + = 36–50%, + + = 21–35%, + + + = 5–20%, + + + + = < 5%, n.t. = not tested, °only 3×10 mg/kg tested. T/C evaluation (survival): − = < 120%, + + + > 150, + + = 120–149%, T/C, treated to control values.

consecutive days compared with a single bolus injection. Antileukaemic activity of treosulfan was at least comparable to the activity of busulfan or cyclophosphamide.

In the i.v. inoculated leukaemia (ALL-SCID 7), both treosulfan and busulfan treatment resulted in a moderate, but significant, prolongation of survival time.

The maximum tolerable (sublethal) dose of treosulfan is 1×3000 mg/kg i.p. and this dose induced a slight to moderate reduction in body weight and significant haematotoxicity.

Obviously, the use of a split dosing regimen may increase the MTD to a certain extent, if myelosuppression is the dose-limiting toxicity.

A clinical phase I trial revealed a MTD of 1×10 g/m^2 treosulfan i.v. with thrombocytopenia and leucocytopenia as the dose-limiting toxicities [19]. These clinical data are in accordance with the maximum tolerated single dose of approximately 9 g/m^2 body surface in mice [7].

However, further dose escalation of treosulfan was possible, and doses up to 49 g/m^2 were administered in several clinical phase I protocols, where patients were rescued with autologous peripheral haematopoietic stem cell transplantations [20–22].

Recently, a substantial *in vitro* [23] and *in vivo* [13] activity of treosulfan with regard to the depletion of primitive and committed stem cells in the bone marrow of mice was reported. In consequence, a successful H2-matched allogeneic transplantation of bone marrow cells was possible after treosulfan conditioning of the recipients. In contrast to previous reports with single dose treatments, sufficient stem cell toxicity of treosulfan is obviously dependent on repeated treosulfan administrations [23]. However, the antileukaemic activity of treosulfan seems to be more or less independent of the treatment regimen as shown here. Moreover, potent immunosuppressive and myeloablative characteristics of repeated treosulfan administrations were reported [11,12]. Therefore, the compound is currently considered a potential alternative conditioning agent prior to allogeneic haematopoietic stem cell transplantation.

Encouraging clinical results for treosulfan/fludarabine conditioning prior to allogeneic transplantation of patients with haematological malignancies have been reported [14]. These data demonstrate rapid engraftment and stable donor-type chimerism in patients after matched related and matched unrelated allogeneic transplantation. In addition, the simple i.v. infusion of treosulfan, its linear pharmacokinetic characteristics and the comparably low non-haematological toxicity of this conditioning regimen were emphasised.

Therefore, the strong antileukaemic activity of treosulfan reported here suggests it may have potential clinical value.

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