Cytotoxicity of treosulfan and busulfan on pediatric tumor cell lines

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High-dose chemotherapy of solid tumors aims at eliminating residual or metastatic tumor cells, which remained after conventional treatment. Thus, anticancer drugs used for high-dose chemotherapy should display significant cytotoxicity against the respective tumors. As little data are available about the in-vitro toxicity of busulfan and treosulfan especially on pediatric tumor cell lines, we compared the cytotoxicity of treosulfan and busulfan on four Ewing tumor, four neuroblastoma, two osteosarcoma and two leukemia cell lines *in vitro*. Growth inhibition of tumor cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide test after 24, 48, 72 and 96 h. Treosulfan and busulfan reduced the growth of all tumor cell lines in a time-dependent and dose-dependent manner. *In vitro* treosulfan was consistently more cytotoxic than busulfan. Fifty percent growth inhibitions of 608–0.73 μmol/l were determined for treosulfan and of above 5000–2.81 μmol/l for busulfan. Both drugs exhibited similar cytotoxicity profiles. Busulfan-sensitive/resistant cell lines were also sensitive/resistant to treosulfan. Overall, the leukemia cell lines were most sensitive to busulfan and treosulfan. The Ewing tumor cell lines were the second most sensitive followed by the neuroblastoma cell lines. The osteosarcoma cell lines were the most resistant cell lines. Although the in-vitro stability of both drugs makes direct comparison of their in-vitro toxicity difficult and does not allow any estimation of dosages needed clinically, the in-vitro results indicate substantial cytotoxicity of both drugs on leukemias, Ewing tumors and neuroblastomas. These data suggest further evaluation of treosulfan for high-dose chemotherapy of advanced Ewing tumors, neuroblastomas and high-risk leukemias. *Anti-Cancer Drugs* 17:657–662

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Introduction

The prognosis of children with disseminated or relapsed solid tumors remains poor despite the adoption of multimodal combined treatment. High-dose chemotherapy followed by autologous stem cell rescue is increasingly used for the treatment of high-risk solid tumors in pediatric patients. Owing to varying eligibility criteria, conditioning regimens, patient selection, prior therapies and high-risk tumor characteristics, it is still difficult to draw clear conclusions with respect to benefit of high-dose chemotherapy for solid tumors. A group of children with advanced Ewing tumors and neuroblastomas, however, seemed to profit from high-dose chemotherapy followed by autologous stem cell rescue [1,2].

The alkylating agent busulfan (1,4-butanedioldimethylsulfonate) (Fig. 1) is widely used at high doses in conditioning regimens for hematological and non-hematological malignancies. Conditioning regimens with busulfan have been associated with favorable outcomes in terms of disease-free survival in patients with metastatic neuroblastoma and Ewing tumor [3,4]. Ewing tumor patients who received busulfan for high-dose chemotherapy had a 5-year overall survival of 44% in contrast to patients receiving high-dose chemotherapy without busulfan, who had a 23% 5-year overall survival [5,6].

Treosulfan (dihydroxybusulfan; L-threitol-1,4-bis-methanesulfonate; NSC 39069) (Fig. 1) is a structural analog of busulfan and is licensed for the treatment of patients with advanced ovarian carcinoma. While the methanesulfonyloxy groups of busulfan alkylate nucleophilic centers directly, treosulfan is transformed to monoepoxy (1,2-epoxy-3,4-butanediol-4-methanesulfonate) and diepoxy (L-(+)-diepoxybutane) intermediates, which are considered responsible for DNA alkylation [7–12] (Fig. 1). In preclinical trials, treosulfan showed antineoplastic activity

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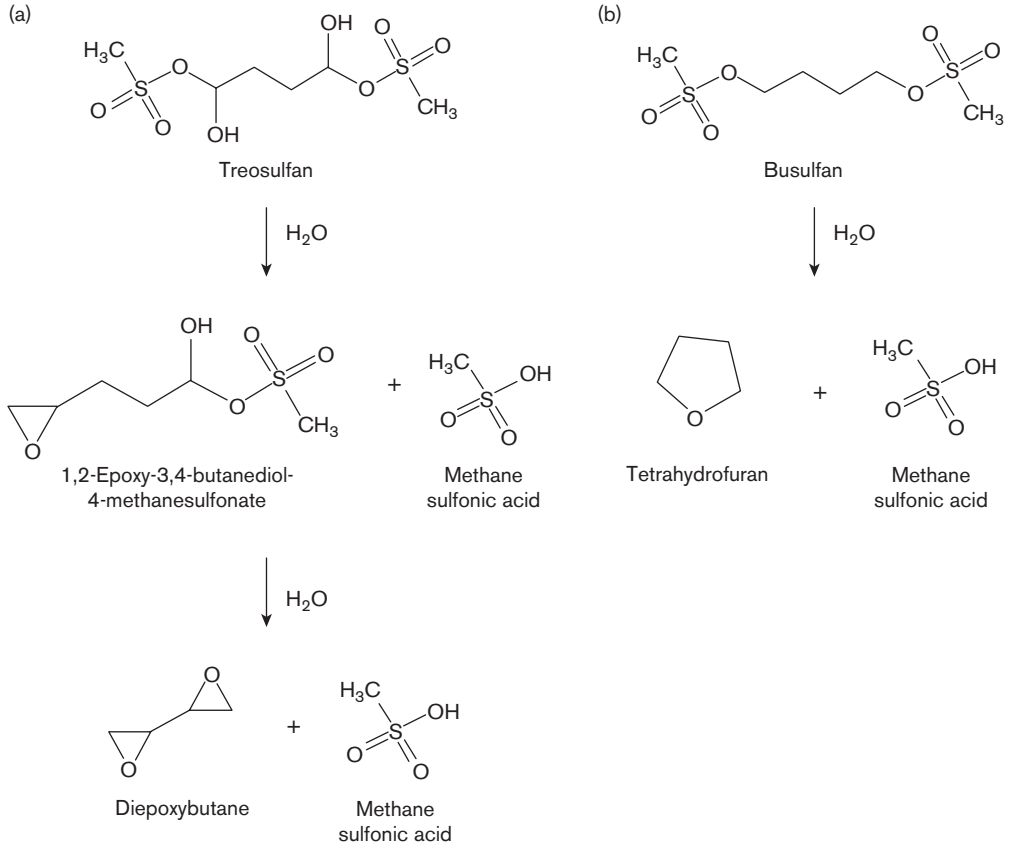
Fig. 1



Transformation of treosulfan (a) and busulfan (b).

against a number of adult cancers like renal carcinomas, breast cancer, small cell lung cancer, cutaneous and choroidal melanomas, chronic myelocytic leukemia, and malignant glioma [13–18]. Using conventional doses of treosulfan, myelosuppression with predominant thrombocytopenia is dose limiting. Combined with peripheral blood stem cell transfusion, dose escalation of treosulfan up to maximal tolerable doses of 47 g/m^2 was possible in adults [19]. Furthermore, all studies on high-dosed treosulfan in adults point out the remarkably safety of treosulfan with very low non-hematological toxicity in heavily pretreated patients who are at risk of treatment-related toxicity [20–22]. A few children at high-risk of treatment-related toxicity were also treated with high-dose treosulfan and they tolerated dosages of 30–42 g/m^2 very well with very low non-hematological toxicity. Thus, treosulfan, like busulfan, might be a candidate anticancer drug for high-dose chemotherapy.

High-dose therapy of solid tumors aims at eradicating residual or microscopic tumor cells that remain after conventional treatment and anticancer drugs used for high-dose chemotherapy of solid tumors have to possess substantial cytotoxicity against the respective tumors.

As little data are available on the in-vitro efficacy of treosulfan especially against pediatric tumors, we compared in-vitro toxicity of treosulfan and busulfan in 12 tumor cell lines including four Ewing tumors, four neuroblastomas, two osteosarcomas and two leukemias.

Materials and methods

Reagents

Treosulfan (Ovastat) was kindly supplied by medac (Hamburg, Germany). Busulfan was purchased from Sigma-Aldrich (Deisenhofen, Germany). Both busulfan and treosulfan are soluble in dimethylsulfoxide (DMSO), whereas only treosulfan is soluble in water. In order to provide comparable conditions for the in-vitro toxicity screens of treosulfan and busulfan, stock solutions of both anticancer drugs were prepared in DMSO and further diluted in complete cell culture medium.

Cell culture

The cytotoxicity of busulfan and treosulfan was analyzed in the Ewing tumor cell lines CADO-ES-1, RM-82, STA-ET-1 and WE-68; the neuroblastoma cell lines CHLA-90, KCN, SHEP-SF and SH-SY5Y; the osteosarcoma cell lines OST and MNNG-HOS; and the leukemia cell lines, HL-60 and MOLT-4. CADO-ES-1, SH-SY5Y, HL-60 and MOLT-4 were purchased from the German Collection of Microorganisms and Cell Culture (DMSZ, Braunschweig, Germany). RM-82, STA-ET-1 and WE-68 were kindly provided by F. van Valen (Department of Orthopedics, Münster, Germany) CHLA-90 was kindly provided by C.P. Reynolds (Division of Hematology–Oncology, Children's Hospital Los Angeles and Department of Pediatrics, University of Southern California Keck School of Medicine, Los Angeles, USA). MNNG-HOS, OST, SHEP-SF and KCN were kindly supplied by Professor C. Poremba (Institute of Pathology, University of Duesseldorf, Germany).

All cell lines were maintained in RPMI 1640 medium (Gibco/BRL cell culture, Invitrogen, Karlsruhe, Germany) supplemented with 200 mmol/l L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, 25 µg/ml amphotericin B and 10% fetal calf serum in 7.5-cm^2 tissue culture flasks in a humidified atmosphere of 5% CO_2 at 37°C. The Ewing sarcoma cell lines were grown on collagen-coated tissue culture flasks.

Cell viability assay

Chemosensitivity was evaluated by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay [23,24]. Cells were plated on 96-well flat-bottom microtiter plates (Becton Dickinson, Heidelberg, Germany), which were coated with collagen in the case of the Ewing sarcoma cell lines. One hundred microliters of cell suspension was incubated in a humidified atmosphere with 5% CO_2 at 37°C for 72 h in order to allow adhesion to the collagen matrix and resumption of exponential cell growth. After 72 h, 100 µl of complete cell culture medium containing the respective drugs at different concentrations was added. Ten drug concentrations (range 0.1–5000 µmol/l) were made by serial 1/2 log dilutions. Control cells were incubated with both drug-free complete cell culture medium and drug-free cell culture medium, which contained the volume of DMSO employed for the highest drug concentration tested. After 24, 48, 72 and 96, 20 µl of MTT reagent (Sigma-Aldrich; 5 mg/ml MTT dissolved in phosphate-buffered saline pH 7.4) was added to each well and the cells were incubated for another 4 h. Mitochondrial dehydrogenases of viable cells reduced the yellow soluble MTT to water insoluble blue formazan crystals, which were dissolved in a solution of sodium dodecylsulfate (20% w/v) solved in dimethylformamide and water (50% v/v). The absorbance of the dissolved formazan dye, which correlates to the amount of viable cells, was measured at 550 nm and a reference wavelength of 630 nm was found using an automated Dynatech MR (Dynatech Inc., Alexandria, Virginia, USA) 7000 micro plate reader.

Each drug concentration was tested in four replicates from which mean, standard deviation and coefficient of variation were calculated. Dose–response curves were plotted on a semilogarithmic scale with the percentage of viable cells compared with untreated controls versus drug concentrations. The drug concentration capable of 50% growth inhibition relative to untreated controls (GI_{50}) at the respective time points 24, 48, 72 and 96 h was calculated with the equation ([% viable cells (> 50%)] – 50)/([% viable cells (> 50%)] – [% viable cells (< 50%)]) × (drug concentration above 50% viable cells – drug concentration below 50% viable cells) + (drug concentration below 50% viable cells). Each experiment was carried out in duplicate at different times.

Results

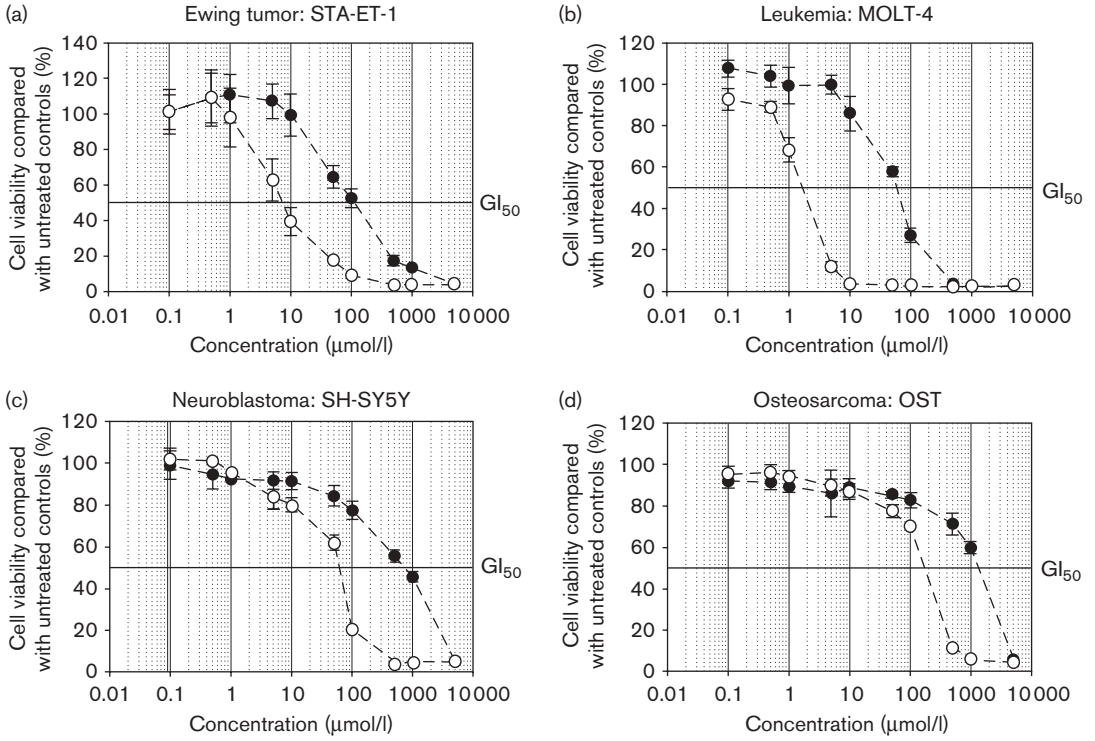
Both treosulfan and busulfan showed logarithmic cytotoxicity in proportion to dose. In all cell lines, treosulfan was more cytotoxic than busulfan after 24, 48, 72 and 96 h. Figure 2 represents the dose–response curves of treosulfan and busulfan for the most sensitive cell lines of each tumor type. With regard to all GI_{50}s determined *in vitro*, treosulfan was, in mean, about 15 times more cytotoxic than busulfan. The sensitivity profiles of busulfan and treosulfan on the 12 cell lines were comparable. Cell lines sensitive to treosulfan were also sensitive to busulfan and cell lines with a treosulfan-resistant phenotype were also resistant to busulfan.

Out of the different tumor types tested, the leukemia cell lines were most sensitive to busulfan and treosulfan. The Ewing tumor cell lines were second most sensitive followed by the neuroblastoma cell lines. The osteosarcoma cell lines showed the lowest sensitivity to treosulfan and busulfan (Fig. 3).

Out of all cell lines, the acute lymphatic leukemia cell line MOLT-4 was the most sensitive one. For MOLT-4, the GI_{50}s of treosulfan declined from 26.9 µmol/l after 24 h to 0.73 µmol/l after 96 h and the GI_{50}s of busulfan dropped from 389 µmol/l after 24 h to 2.81 µmol/l after 96 h.

Among the Ewing tumor cell lines, WE-68 was the most sensitive cell line followed by STA-ET-1. Sensitivity of WE-68 and STA-ET-1 to treosulfan was comparable to that of the HL-60 leukemia cell line. RM-82 was about 2 times more resistant to treosulfan compared with STA-ET-1 and WE-68. CADO-ES-1 was the cell line most resistant to treosulfan. The GI_{50}s of treosulfan ranged

Fig. 2



Dose–response curves determined for busulfan (●) and treosulfan (○) after 48 h in selected cell lines of the different tumor types studied. (a) Ewing tumor, (b) leukaemia, (c) neuroblastoma and (d) osteosarcoma.

from 253 (determined in CADO-ES-1 after 24 h) to 0.91 μmol/l (determined in WE-68 after 96 h).

Among the neuroblastoma cell lines, the highest GI_{50} of treosulfan was determined in SHEP-SF after 24 h (593 μmol/l) and the lowest GI_{50} was calculated 2.54 μmol/l for SH-SY5Y after 96 h. For the osteosarcoma cell lines, the GI_{50}s of treosulfan ranged from 608 (determined in MNNG-HOS after 24 h) to 15.3 μmol/l (determined in OST after 96 h).

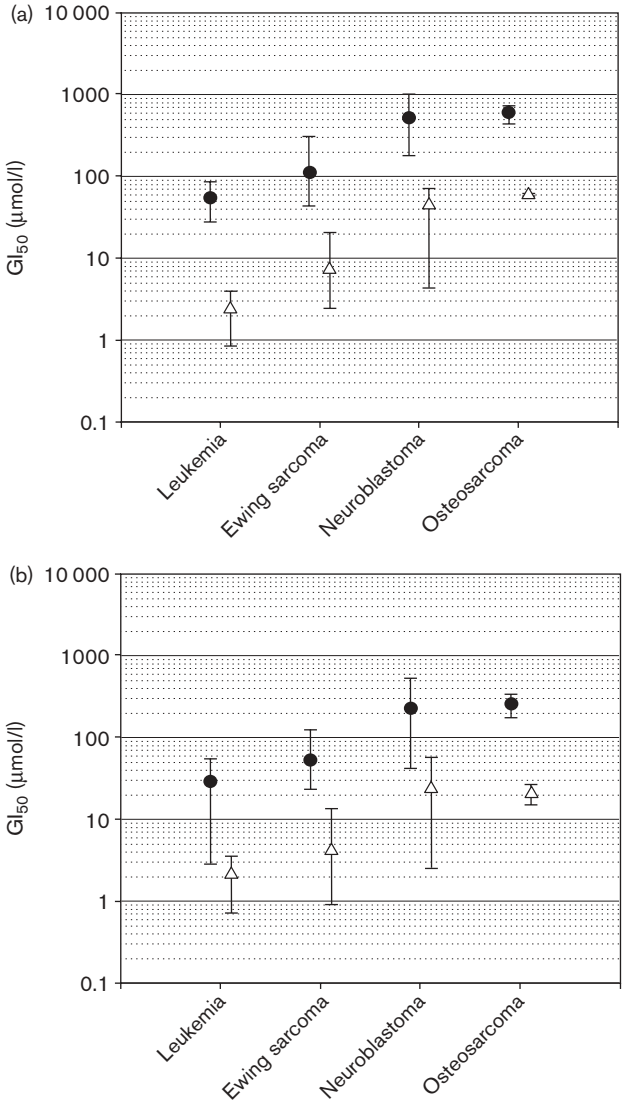
For busulfan, GI_{50}s from 688 (determined in HL-60 after 24 h) to 2.81 μmol/l (determined in MOLT-4 after 96 h) were determined in the leukemia cell lines. Among the Ewing tumor cell lines, the growth of RM-82 was not reduced by 50% after 24 h with the highest busulfan concentration tested (5000 μmol/l) and the lowest GI_{50} of busulfan was calculated for STA-ET-1 after 96 h (22.5 μmol/l). The neuroblastoma cell lines SHEP-SF and CHLA-90 were more resistant to busulfan, whereas KCN and SH-SY5Y were more sensitive. The GI_{50}s ranged from 4899 (determined in SHEP-SF after 24 h) to 41.8 μmol/l (determined in SH-SY5Y after 96 h). Among the osteosarcoma cell lines, the highest GI_{50} of busulfan was determined in MNNG-HOS after 24 h (3433 μmol/l) and the lowest in MNNG-HOS (178 μmol/l) after 96 h.

Discussion

While busulfan is well established in high-dose chemotherapy of hematological and solid tumors, its structural analog treosulfan has not been established for high-dose chemotherapy so far despite limited organ toxicity. As high-dose chemotherapy of solid tumors requires anticancer drugs with substantial tumor cytotoxicity in order to target residual or metastatic tumor cells, we evaluated the preclinical activity of busulfan and treosulfan on 12 tumor cell lines, four neuroblastomas, four Ewing tumors, two osteosarcomas and two leukemias.

On all tumor cell lines, treosulfan and busulfan showed significant cytotoxicity in a time-dependent and dose-dependent manner. *In vitro*, treosulfan was always more cytotoxic than busulfan. Both drugs displayed the same cytotoxicity profiles. Busulfan alkylates DNA directly, while treosulfan is transformed to alkylating mono-intermediates and diepoxy-intermediates by a non-enzymatic, pH-dependent and temperature-dependent

Fig. 3



Mean 50% growth inhibitions (GI_{50}s), lowest and highest GI_{50}s determined after busulfan (●) and treosulfan (△) exposure for 72 (a) and 96 h (b) in the leukemia, Ewing tumor, neuroblastoma and osteosarcoma cell lines studied. The dots represent the mean GI_{50}s for each tumor type. The range bars represent the lowest and the highest GI_{50}s determined after 72 (a) and 96 h (b).

intramolecular nucleophilic substitution. Despite the different alkylation mechanisms, the sites of DNA alkylation are obviously identical [25,26], which corresponds to the similar cytotoxicity profiles observed for treosulfan and busulfan.

At pH > 6 treosulfan is rapidly hydrolyzed to alkylating mono-derivatives and diepoxy-derivatives, which can act directly on tumor cells *in vitro*. In addition, diepoxybutane is volatile and was reported to cause airborne cytotoxicity of treosulfan *in vitro*. Tetrahydrofuran and methane sulfonic acid, which are released by hydrolyzation of busulfan, did not cause airborne cytotoxicity (Fig. 1) [27]. In preliminary tests, we also observed airborne cytotoxicity of treosulfan, which affected untreated controls incubated beside wells with high concentrations of treosulfan. To minimize the effect of airborne cytotoxicity by treosulfan, we incubated untreated controls beside the lowest concentration of treosulfan and then treosulfan concentrations were increased with each new column on the 96-well plate. In addition, only incubations with either treosulfan or busulfan were carried out on one 96-well plate in order to avoid the interference of treosulfan cytotoxicity with busulfan effects. Nevertheless, volatile diepoxybutane from adjacent columns with higher treosulfan concentrations might still have increased the treosulfan cytotoxicity of the neighboring column with the next lower concentration of treosulfan. Thus, GI_{50}s of treosulfan might be overestimated by about 1/2 log concentration. Moreover, hydrolyzation of the alkylating busulfan to tetrahydrofuran and methane sulfonic acid might have reduced in-vitro toxicity of busulfan compared with that of treosulfan. At 37°C, about 50% of busulfan was reported to degrade within 16 h in an aqueous solution [28].

So far little in-vivo data are available which compares the cytotoxicity of treosulfan and busulfan. These data, however, indicate that higher dosages of treosulfan are needed to achieve effects comparable to those obtained by busulfan. To treat leukemia xenografts, dosages of 3000 mg/kg treosulfan had to be administered to achieve a better antileukemic effect than 30 mg/kg busulfan [29]. The treatment of chronic myelocytic leukemia patients with treosulfan was found to be as active as that with busulfan, when applied at higher dosages [30].

On the other hand, the in-vivo data also indicate that treosulfan is much better tolerated than busulfan. For high-dose chemotherapy, busulfan is applied at dosages of 16 mg/kg body weight. Higher dosages were associated with an increased risk of severe hepatotoxicity, especially life-threatening venoocclusive disease, which was reported to affect about 20% of patients treated with high-dose busulfan [31–33]. In a phase I study in adults, the maximal tolerable dose of high-dose treosulfan was 47 g/m^2 body surface area, which was about 100 times higher than the doses of busulfan used for high-dose chemotherapy. Diarrhea, mucositis/stomatitis and epidermal necrolysis were reported to be dose limiting. After retransfusion of autologous peripheral blood stem cells, peripheral white blood cells and thrombocytes recovered within a few days. The nadir of thrombocytes took a median of 2 days (range 1–3) and the nadir of peripheral white blood cells a median of 4 days (range 3–4) [19]. Moreover, in contrast to busulfan, treosulfan is soluble in water and can be easily administered intravenously.

Intravenous administration of busulfan is only possible with the aid of organic solvents, like DMSO or *N*,*N*-dimethylacetamide. *N*,*N*-dimethylacetamide displays neurotoxic and hepatotoxic effects, which may add to the neurotoxicity and hepatotoxicity of busulfan [34–36].

The in-vitro results showed substantial cytotoxicity of busulfan and treosulfan on leukemia, Ewing tumor and neuroblastoma cell lines. For Ewing tumors and neuroblastomas, benefits of busulfan-containing regimens in terms of event-free survival were reported. Like busulfan, treosulfan targets quiescent tumor cells, is not used for conventional chemotherapy and possesses limited organ toxicity. Moreover, it is much better tolerated than busulfan. Thus, treosulfan seems a candidate anticancer drug for high-dose chemotherapy of solid tumors which might be further explored for patients with advanced neuroblastomas, Ewing tumors and high-risk leukemias.

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