Penetration of Treosulfan and its Active Monoepoxide Transformation Product into Central Nervous System of Juvenile and Young Adult Rats

Michał Romański, Joachim Baumgart, Sonja Böhm, and Franciszek K. Główka

Author Affiliations:
Department of Physical Pharmacy and Pharmacokinetics, Poznan University of Medical Sciences, Poznan, Poland (M.R., F.K.G); medac GmbH, Wedel, Germany (J.B., S.B)
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Address correspondence to:

Prof. Franciszek K. Główka, Department of Physical Pharmacy and Pharmacokinetics,
Poznan University of Medical Sciences, 6 Święcickiego Street, 60-781 Poznan, Poland. E-mail: glowka@ump.edu.pl

The number of text pages: 34
The number of tables: 4
The number of figures: 4
The number of references: 44
The number of words in the Abstract: 248
The number of words in the Introduction: 483
The number of words in the Discussion: 1497

Abbreviations

AUC, area under the concentration–time curve from zero to infinity; AUC_{last}, area under the concentration–time curve from zero to the time of the last concentration measured; BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; CNS, central nervous system; C_{last}, last concentration measured; C_{max}, maximum concentration; EMA, European Medicines Agency; f_u, unbound fraction; HPLC, high-performance liquid chromatography; HPLC-MS/MS, high-performance liquid chromatography with tandem mass spectrometry detection; HSCT, hematopoietic stem cell transplantation; IS, internal standard; JR, juvenile rats; k_{el},
elimination rate constant; MW, molecular weight; S.D., standard deviation; S.E., standard error; S.E.M, standard error of the mean; S,S-DEB, (2S,3S)-1,2:3,4-diepoxybutane; S,S-EBDM, (2S,3S)-1,2-epoxybutane-3,4-diol-4-methanesulfonate; t1/2, elimination half-life; tmax, time of maximum concentration; TREO, treosulfan; YAR, young adult rats.
Abstract

Treosulfan (TREO) is currently investigated as an alternative treatment for busulfan in conditioning prior to hematopoietic stem cell transplantation. The knowledge of the blood–brain barrier penetration of the drug is still scarce. In this paper penetration of TREO and its active monoepoxide (S,S-EBDM) and diepoxide (S,S-DEB) into CNS was studied in juvenile (JR) and young adult rats (YAR) for the first time. CD® rats of both sexes (n = 96) received an intravenous dose of TREO 500 mg/kg b.w. Concentrations of TREO, S,S-EBDM and S,S-DEB in rat plasma, brain and cerebrospinal fluid (CSF, in YAR only) were determined by validated bioanalytical methods. Pharmacokinetic calculations were performed in WinNonlin using a noncompartmental analysis and statistical evaluation was done in Statistica software. In male JR, female JR, male YAR and female YAR, the brain/plasma area under the curve (AUC) ratio for unbound TREO amounted 0.14, 0.17, 0.10 and 0.07, and for unbound S,S-EBDM 0.52, 0.48, 0.28 and 0.22, respectively. The CSF/plasma AUC ratio in male and female YAR was 0.12 and 0.11 for TREO, and 0.66 and 0.64 for S,S-EBDM, respectively. Elimination rate constants of TREO and S,S-EBDM in all the matrices were sex-independent with a tendency to be lower in the JR. No quantifiable levels of S,S-DEB were found in the studied samples. TREO and S,S-EBDM demonstrated poor and sex-independent penetration into CNS. However, the brain exposure was greater in juvenile rats, so very young children might potentially be more susceptible to high-dose TREO-related CNS exposure than young adults.
Introduction

In the last decade the anticancer drug treosulfan (TREO) has emerged as a promising myeloablative agent used prior to hematopoietic stem cell transplantation (HSCT) in pediatric as well as adult patients (Beier et al., 2013; Boztug et al., 2015; Casper et al., 2012; Dinur-Schejter et al., 2015; Główka et al., 2010; Lawitschka et al., 2015; Nemecek et al., 2011; Shimoni et al., 2012; Slatter et al., 2011; Strocchio et al., 2015; Wachowiak et al. 2011). Currently, various clinical phase II and one phase III trials are conducted aiming the registration of the myeloablative conditioning agent. Probably, TREO provides lower organ toxicity than busulfan, especially hepato-, pulmo-, and neurotoxicity. One hypothesis, that needs experimental verification, assumes that due to lower lipophilicity of TREO compared to busulfan, the former achieves lower concentrations in those key organs and, consequently, is less toxic (Danylesko et al., 2012; Główka et al., 2010; Shimoni et al., 2012). The underlying mechanism of potentially low neurotoxicity of TREO appears particularly important for infants, as maturity of their blood–brain barrier (BBB) is still unknown (Saunders et al., 2012). In general, clinical data regarding TREO application to this specific group of patients are limited. Noteworthy, Slatter et al. (2011) reported TREO-based conditioning in 70 young children with primary immunodeficiency among whom as many as 46 were infants. In this study 4 cases of seizures were noted and all of them occurred exceptionally in patients aged below four months. In view of the above facts, the present knowledge of neurotoxicity of TREO appears to be incomplete and requires more in-depth study. It should be borne in mind that TREO is a pro-drug that undergoes a non-enzymatic two-step transformation to biologically active epoxides, i.e. (2S,3S)-1,2-epoxybutane-3,4-diol-4-methanesulfonate (S,S-EBDM) and (2S,3S)-1,2:3,4-diepoxybutane (S,S-DEB) (Hartley et al., 1999) (Figure 1). Accordingly, one important shortcoming in understanding of potential TREO neurotoxicity is...
that distribution of neither the pro-drug alone nor its epoxy-transformers into central nervous system has been studied. For that purpose we have recently developed a new analytical method by using a small group of six male TREO exposed rats and confirmed that both TREO and S,S-EBDM were successfully analyzable in brain tissue (Romaniński et al., 2014).

In the present paper we describe bioanalytical results of TREO and both of its active epoxy-transformers in plasma, brain and cerebrospinal fluid (CSF) after i.v. TREO treatment of juvenile rats (JR) and young adult rats (YAR) of both sexes to reveal possible effect of age and sex on distribution of the analytes into central nervous system (CNS). This non-clinical study constituted one of the measures included in the pediatric investigation plan regarding TREO application in conditioning prior allogeneic HSCT in children, accepted by the Paediatric Committee of the European Medicines Agency (European Medicines Agency decision). The investigation revealed that BBB penetration of TREO and S,S-EBDM was rather weak and sex-independent, however, visibly higher in the juvenile than in the young adult animals.
Materials and Methods

Materials

A certified standard of TREO for preparation of the injection solution and also for analytical purposes was supplied by medac GmbH (Wedel, Germany). Citric acid of analytical grade was purchased from P.O.Ch. (Gliwice, Poland). Chemicals used in the quantitative chromatographic analysis of TREO and its epoxy-transformers were obtained as depicted in the previous papers (Główka et al., 2012; Romański et al. 2014). Drug-free rat plasma and brain tissue for preparation of the calibration standards was obtained from Laboratory of Pharmacology and Toxicology (Hamburg, Germany).

Animals

Animal experiments including blood, CSF and organ sampling were carried out by the GLP-certified facility of Laboratory of Pharmacology and Toxicology (Hamburg, Germany) in accordance with the ‘Good Laboratory Practice’ Regulations of the European Council and ‘OECD Principles of Good Laboratory Practice’. These principles are compatible with ‘Good Laboratory Practice’ regulations specified by regulatory authorities throughout the European Community, the United States and Japan. The animal procedures have been approved by the local government: Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz, Amt für Gesundheit und Verbraucherschutz, Billstraße 80, 20539 Hamburg, according to the German 'Tierschutzgesetz' (current version) and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Thirteen pregnant female CD® rats (approximate gestation day 15) and 24 male and 24 female young adult CD® rats aged approximately 4 weeks were supplied by Charles River
Laboratories (Sulzfeld, Germany). Animals were kept singly in Macrolon cages (39 × 23 cm in basal and 18 cm high) under controlled temperature (maximum range 22 ± 3 °C), humidity (maximum range 55 ± 15%) and lighting (12 h light/dark circle). Drinking water and the appropriate commercial feed (sniff, Soest, Germany) were offered to the dams and the YAR ad libitum. After the birth the pups were raised by their mothers until postnatal day 10. On that day, 24 male and 24 female pups with identical birth date were selected for the experiment by means of a computer randomization program. On day of TREO administration the JR and YAR were 10 and 34–35 days old, respectively. The body weight of the male JR, female JR, male YAR and female YAR that received TREO was 19.5 – 26.3, 18.0 – 26.0, 135 – 164 and 121 – 144 g, respectively.

**TREO administration and sample collection**

TREO solution was prepared by dissolving 1000 mg of the crystalline TREO powder in 20 mL sterile water for injection warmed to a maximum of 30 °C, according to the manufacturer instruction. The freshly prepared solution was administered to the CD® rats as a single intravenous bolus (about 15 sec/dose) into the tail vein at a volume of 10 mL/kg, resulting in TREO dose of 500 mg per kg body weight. Blood was withdrawn before (predose) and then 5 min, 0.5, 1.0, 2.0, 4.0, 6.0 and 24.0 h after drug injection, via heart puncture in JR (0.2 mL), and from retrobulbar venous plexus in YAR (2 mL), under isoflurane anesthesia. Immediately after blood sampling, the animals were sacrificed under ether anesthesia for withdrawal of a maximum possible volume of a blood-free CSF from Cisterna magna (YAR only) and collection of the brain (JR as well as YAR). Immediately after collection, the blood and cerebrospinal fluid were acidified by addition of 50 μL of 1 M citric acid per 1 mL of the sample in order to avoid the artificial *ex vivo* conversion of TREO and S,S-EBDM. Within the next 15 minutes the samples were centrifuged at 4000 × g over 10 min to obtain the plasma.
and clear CSF supernatant. The collected brains of the animals were immediately dissected, washed in 0.9% NaCl and divided along the longitudinal axis. One of the brain hemispheres were again rinsed three times with 5 mL of 0.9% NaCl, weighed and homogenized with 0.05 M citric acid (5 mL per 1 g of brain) in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 4000 × g over 10 min in order to obtain the solid particles free-supernatant. All the obtained samples of the rat plasma, CSF and brain homogenate supernatant were frozen at –80 °C, transported to the bioanalytical laboratory in dry ice (-78.5 °C), and stored at -80 °C until the HPLC analysis for not longer than 3 months.

**Preparation of plasma, CSF and brain samples for HPLC analysis**

Concentrations of TREO and S,S-EBDM in the rat plasma, CSF and brain tissue were determined using the validated high-performance liquid chromatography method with tandem mass spectrometry detection (HPLC−MS/MS) (Romański et al., 2014). Briefly, 52.5 μL of the acidified plasma and CSF or 100 μL of the brain homogenate supernatant was spiked with water and the solution of acetaminophen (internal standard, IS) and subjected to ultrafiltration (cut-off 30 kDa, 14,000 g at 20 °C over 20 min). If the volume of the plasma collected from the JR or of the CSF collected from the YAR was insufficient for the analysis, that is <52.5 μL, it was filled up with the drug-free rat plasma to the target value, as justified by the dilution integrity which was confirmed during the validation of the applied method. The obtained ultrafiltrate was appropriately diluted and applied to the HPLC-MS/MS system. The resolution of the analytes was performed in Zorbax Eclipse Plus C18 column using a mobile phase composed of the formate buffer pH 4.0 and acetonitrile (95:5, v/v).

Quantification of S,S-DEB in the rat plasma, CSF and brain tissue was carried out using the validated HPLC method with UV detection described by Głównka et al.(2012), after its slight modification. Namely, 52.5 μL of the acidified plasma or CSF and 100 μL of the brain
homogenate supernatant were transferred into 1.5 mL HPLC screw cap glass vials, spiked with 50 μL of water and mixed. If the volume of the plasma or CSF sample was insufficient for the analysis, it was filled up to 52.5 μL with the drug-free rat plasma, as justified by the dilution integrity. The analyte was extracted from the samples with 1 mL of dichloromethane and acetonitrile mixture (9:1, v/v) containing 0.25 μM 2,2'-dinitrobiphenyl (IS) and then treated with 10 μL of 0.1 M solution of 3-nitrobenzenesulfonic acid (derivatizing agent). The excess of 3-nitrobenzenesulfonic acid was extracted from the post-derivatization solution with 200 μL of water. After evaporation of the organic layer the obtained residue was reconstituted in 100 μL of acetonitrile. A volume of 50 μL of the resulting solution was injected into the Agilent 1100 HPLC system with UV detector (Agilent Technologies, Waldbronn, Germany). The separation was accomplished at 25 °C in Nucleosil 100 C18 column (4.6 × 250 mm; 5 μm particle size) guarded by Nucleosil C18 (4.6 × 7.5 mm; 5 μm), both from Grace Davison Discover Science (Deerfield, USA), using a 1 mL/min flow rate of a mobile phase composed of water (A) and acetonitrile (B). The following gradient elution program was applied: 0–12 min linear from 40 to 80% B, 12–13 min 80% B, 13–15 min return from 80% to 40% B and the post time of 6 min with 40% B for the column equilibration.

Quantification of TREO and its epoxy-transformers was processed according to the current guidelines of EMA for bioanalytical methods (Guideline on bioanalytical method validation). The plasma and brain homogenate supernatant calibration standards and the quality control samples were prepared according to the procedure described above for the studied samples, except that the appropriate drug-free matrices were spiked with the standard solutions of the analytes instead of water. Linearity of the calibration curves and accuracy for determination of the quality control samples established during the analytical runs are presented in Table 1.
In none of the samples obtained from the TREO-treated rats the levels of S,S-DEB exceeded the LLOQ, that is 1 μM in plasma and CSF, and 0.5 μM in brain homogenate supernatant, therefore further analyses, described below, were performed only for TREO and S,S-EBDM.

**Determination of unbound fraction of TREO and S,S-EBDM in rat plasma and brain tissue homogenate**

Unbound fraction (f_u) of TREO and S,S-EBDM in rat plasma and brain tissue homogenate was determined by analysis of two series of samples (n = 5). In the first series, 237.5 μL of plasma separated from the drug-free rat blood acidified with 1 M citric acid (1:0.05, v/v) and 237.5 μL of the drug-free rat brain homogenate with 0.05 M citric acid (1:5, w/v) was spiked with 12.5 μL of the appropriate standard solutions of TREO and S,S-EBDM. The resulting concentrations of TREO in the plasma were 2.3, 57 and 2000 μM, and 2.3 and 17 μM in the brain homogenate, while the concentrations of S,S-EBDM in the plasma were 3.5 and 87 μM, and 3.5 and 8.7 μM in the brain homogenate. The samples were incubated at 37 °C over 1 h in a thermostaker and then the plasma and the supernatant obtained after centrifugation of the brain homogenate were filtered through Amicon Ultra-0.5 mL device with a cut-off 30 kDa (Millipore, Massachusetts, USA) applying 14,000 g over 20 min. Quantification of TREO and S,S-EBDM in the obtained filtrates was performed as described in the section ‘Preparation of plasma, CSF and brain samples for HPLC analysis’. The second series of samples were prepared exactly in the same manner as the first one except the standard solutions of the analytes were spiked into the protein-free rat plasma and brain homogenate supernatant which had been earlier prepared using the Amicon devices. Unbound fraction of the compounds was calculated using formula: f_u = P_I^{analyte/IS}/P_{II}^{analyte/IS}, where P_I^{analyte/IS} and P_{II}^{analyte/IS} denote the peak area of the given analyte to the internal standard (acetaminophen) from the first and the second series, respectively.
Calculations of the unbound analytes concentration in the studied samples

Total concentrations of TREO, S,S-EBDM and S-S-DEB in the studied plasma and brain homogenate supernatants were calculated from the equations of the matrix-specific calibration curves prepared in the same analytical run. Levels of the analytes in the brain tissue were calculated knowing that 1 μM in the brain homogenate supernatant was equivalent to 6 μmol/kg in the brain tissue. The total concentrations in brain tissue were corrected for the analytes present in residual brain blood using the method described by Fridén et al. (2010):

\[
C_{b,corr} = \frac{C_b - (f_{u,p} \cdot V_w \cdot C_p + (1 - f_{u,p}) \cdot V_{protein} \cdot C_p)}{1 - V_w}
\]

where \(C_{b,corr}\) denoted total concentration of the compound in the individual rat’s brain corrected for the residual blood; \(C_b\) – total concentration determined in the individual rat’s brain; \(C_p\) – mean of the total plasma concentration of the analyte observed in the rats of the given sex and age at the same time as in the brain; \(f_{u,p}\) – unbound fraction of the compound in plasma; \(V_w\) and \(V_{protein}\) – apparent brain vascular spaces of plasma water (10.3 μL/g) and plasma proteins (8.0 μL/g), respectively. Total concentrations of the analytes in the CSF samples were calculated on the basis of the calibration curves established for the plasma because validation of the analytical methods for the CSF was judged as not practicable due to the required volume of this unique matrix. If the plasma or CSF samples had been filled up to 52.5 μL with the drug-free rat plasma during their preparation, the dilution factor was used to calculate total concentration of the analytes in the original samples. Concentrations of unbound TREO, S,S-EBDM and S,S-DEB in plasma, brain tissue and CSF were calculated as a product of the total concentration and the unbound fraction factor \(f_u\). The value of \(f_u\) for CSF was calculated using the following formula (Fridén et al., 2010):
\[
f_{u,\text{CSF}} = \frac{1}{1 + 0.045 \times \left( \frac{1}{f_{u,p}} - 1 \right)}
\]

where the value of 0.045 stands for a ratio of protein concentration in CSF to plasma (Habgood et al., 1992).

**Pharmacokinetic analysis**

Based on the concentrations of unbound TREO and S,S-EBDM in the rat plasma, CSF and brain tissue, the pharmacokinetic parameters were calculated using a non-compartmental analysis and sparse sampling technique in WinNonlin 6.2 (Pharsight, USA). \(C_{\text{max}}\) and \(t_{\text{max}}\) were read directly from the graphs of the mean concentration of the individual compound in the given matrix plotted against time. The elimination rate constant (\(k_{\text{el}}\)) was estimated from the slope of the terminal linear segment of the log mean concentration–time plot using the automatic best-fitting option in WinNonlin 6.2. The elimination half-life (\(t_{1/2}\)) was calculated from \(\ln 2/k_{\text{el}}\). The area under the concentration–time curve from zero to the time of the last concentration measured (AUC\(_{\text{last}}\)) was calculated by a linear trapezoidal rule and the residual area under the curve (AUC\(_{\text{res}}\)) was estimated by extrapolation from the last concentration measured (C\(_{\text{last}}\)) to infinity using \(C_{\text{last}}/k_{\text{el}}\) ratio. The area under curve from zero to infinity (AUC) was computed as a sum of AUC\(_{\text{last}}\) and AUC\(_{\text{res}}\).

**Data analysis and statistical procedures**

Standard error of estimate of the AUC\(_{\text{last}}\) was generated by WinNonlin 6.2 during the pharmacokinetic analysis. Additionally, standard error of the \(k_{\text{el}}\) was computed as a standard error of the slope of the best-fitted log mean concentration–time plot, using the regression tool in the Excel 2007 (Microsoft corp.). Standard errors of \(t_{1/2}\), AUC, and AUCs ratio were calculated using the differential calculus:
\[ S.E_{\text{e}_{1/2}} = \frac{\ln 2}{k_{el}} S.E_{k_{el}} \]

\[ S.E_{\text{AUC}} = S.E_{\text{AUC}_{\text{last}}} + S.E_{\text{AUC}_{\text{res}}} = S.E_{\text{AUC}_{\text{last}}} + \frac{1}{k_{el}} S.E.M_{\text{last}} + \frac{\text{Mean}_{\text{last}}}{k_{el}} S.E_{k_{el}} \]

\[ S.E_{\frac{\text{AUC}_1}{\text{AUC}_2}} = \frac{1}{\text{AUC}_2} S.E_{\frac{\text{AUC}_1}{\text{AUC}_2}} + \frac{\text{AUC}_1}{\text{AUC}_2} S.E_{\frac{\text{AUC}_1}{\text{AUC}_2}} \]

Statistical significance of the differences between the selected pharmacokinetic parameters was evaluated in Statistica 10 (StatSoft). Namely, the differences between the mean \( C_{\text{max}} \) values of TREO or S,S-EBDM in plasma, brain and CSF observed in the rat groups of different sex or age was evaluated by ANOVA after normal distribution of the data had been confirmed with the Shapiro-Wilk test. Effect of sex and age on the \( k_{el} \) of the compounds was studied by statistical comparison of the slopes of the terminal linear segments of the log concentration–time plots in GraphPad Prism 6.0 (GraphPad Software, Inc.) that uses the method equivalent to ANCOVA.
Results

Characteristics and health status of the animals

The pharmacokinetic studies were carried out in 96 JR and YAR, each including 6 pre-dose animals and 42 dosed ones. The number of the animals used resulted from the serial sacrifice design in which measurements were taken at 8 time points and at each time point the samples were collected from 6 animals (3 male and 3 female). During the nursing and 8-day adaptation period, the JR and YAR, respectively, were in a good health and demonstrated the usual body weight gain. After the treatment with 500 mg of TREO/kg b.w. none of the rats died prematurely. No signs of local intolerance reactions as well as systemic toxicity were noted in the YAR and the feces of all the animals were of a normal consistency. During the samples collections, no blood samples could be obtained from 2 male and 3 female JR. Therefore, 5 spare JR of the required age (postnatal day 10) taken from the surplus litters were used as replacement animals. The dosing and blood sampling were repeated for these animals on the next day but the blood sampling was again not possible in one animal. Nevertheless, these minor deviations did not affect the validity and integrity of the obtained results.

Unbound fraction of TREO and S,S-EBDM in the rat plasma, brain homogenate and CSF

Unbound fraction of TREO and S,S-EBDM determined in vitro in the rat plasma and brain homogenate was practically equal to 1, no matter of the analytes concentration applied (Table 2). The $f_u$ value of the compounds in the CSF, calculated on the basis of their $f_u$ in plasma (Fridén et al, 2010), also amounted to 1. As a consequence, the total concentrations of TREO and S,S-EBDM determined in the plasma, brain tissue and CSF of the rats which received TREO, exactly reflected the free drug concentration.
Concentration–time profiles of TREO and S,S-EBDM in the rat plasma, brain and CSF

Changes of the mean concentrations of unbound TREO and S,S-EBDM in plasma and brain of the JR and YAR as well as in CSF of YAR are presented in Figure 2. In none of the analyzed samples the levels of S,S-DEB exceeded the LLOQ, that is 1 μM in plasma and CSF, and 3 μM in brain tissue.

TREO and S,S-EBDM exposure in plasma, brain and CSF

In order to measure TREO and S,S-EBDM exposure in rat plasma, brain and CSF, the C_max and AUC values were used. As presented in Figure 3A, the mean C_max of free TREO and S,S-EBDM in the plasma and CSF did not differ significantly between the studied groups, whether the effect of the rats’ sex or age was considered. However, the brain C_max of free TREO and S,S-EBDM in JR of both sexes were considerably higher than in YAR, though in the male specimens the difference in concentration of TREO and S,S-EBDM did not reach statistical significance (p = 0.052 and 0.054, respectively). Generally, the values of TREO C_max observed in brain and CSF were much lower in comparison to the plasma. Accordingly, the brain to plasma free TREO C_max ratio was about 0.05 and 0.02 in JR and YAR, respectively, and the CSF to plasma free TREO C_max ratio in YAR amounted approximately 0.04. Analogous results were obtained when analyzing the S,S-EBDM C_max in the three studied matrices though in this case the differences between the brain or CSF and plasma were not as huge. Namely, the brain to plasma free S,S-EBDM C_max ratio was about 0.4 and 0.25 in JR and YAR, respectively, and the YAR’s CSF to plasma free S,S-EBDM C_max ratio was close to 0.5. The results of the AUC, that in opposition to C_max shows a drug exposure over entire time of its residence in the given body compartment, are presented in Figure 3B. There were again no differences between males and females within the particular age group, and the AUC values designated for the brain tissue and CSF were visibly lower.
(approximately 6 – 14-fold for TREO and 2 – 4-fold for S,S-EBDM) when compared to the plasma. Moreover, the AUC of unbound TREO as well as S,S-EBDM in either plasma or brain was 2 to 4.5-fold higher in JR than in the YAR.

**Rate of elimination of TREO and S,S-EBDM from plasma, brain and CSF**

The results of the $k_{el}$ and $t_{1/2}$ presented in Table 3 show that in all the rats, TREO as well as S,S-EBDM were relatively fast eliminated from plasma, brain and CSF ($t_{1/2}$ range 0.6 - 3.3 h), yet there were some remarkable differences. In general, elimination of TREO from CSF and especially from brain tissue proceeded slower than from plasma. Contrary to TREO, S,S-EBDM was eliminated from brain at the similar rate as from plasma. Additionally, the testing of the slopes ($k_{el}$) of the terminal linear log concentration–time plots demonstrated that elimination of both TREO and S,S-EBDM from plasma were significantly slower in JR than in YAR ($p \leq 0.018$) with no effect of the animals’ sex. The same trend was noted for elimination of the compounds from the brain, yet the statistical significance was reached only for the difference in $k_{el}$ of S,S-EBDM between the JR and YAR male specimens. Moreover, the elimination of the prodrug and its epoxy-transformer from the YAR’s CSF did not depend on the sex (Table 3).

**Capability of TREO and S,S-EBDM to penetrate across the BBB and BCSFB**

To characterize penetration of TREO and S,S-EBDM across the BBB and the blood–cerebrospinal fluid barrier (BCSFB), the brain to plasma and CSF to plasma AUC ratios, respectively, were used as they are considered as the most accurate parameters for that purpose (Nau et al., 2010). As presented in Figure 4, the ratios obtained for S,S-EBDM were generally much higher in comparison to TREO. Additionally, no significant differences in either brain/plasma or CSF/plasma AUC ratios calculated for TREO and S,S-EBDM were
noted between the male and female specimens of the same age. However, the rats’ age did have a clear influence on capability of the studied compounds to cross the BBB. Accordingly, the brain/plasma AUC ratio of TREO was 0.14 ± 0.03 in the male JR, 0.17 ± 0.03 in the female JR, and 0.098 ± 0.008 in the male YAR, 0.073 ± 0.007 in the female YAR. The values obtained for S,S-EBDM were 0.52 ± 0.06 in the male JR, 0.48 ± 0.08 in the female JR, and 0.28 ± 0.03 in the male YAR and 0.22 ± 0.01 in the female YAR.
Discussion

High-dose TREO, currently investigated as a myeloablative conditioning agent, is believed to possess a more favorable toxicity profile than busulfan. Very young children, as inherently susceptible to toxicity of standard myeloablative agents – busulfan and total body irradiation, are the group of HSCT patients in which TREO is supposed to be particularly useful. So far, TREO-based conditioning has been applied in patients as young as one month old (Dinur-Schejter et al., 2015; Slatter et al., 2011, 2015). The present study was aimed at assessing penetration of TREO and its active epoxy-transformers across the BBB in 10-day old (JR) and 34-35-day old (YAR) rats and, in the latter, additionally across the BCSFB. Considering BBB development, it is established that tight junctions in cerebral vessel endothelium and key efflux transporters, ABCB1 (P-glycoprotein) and ABCG2 (breast cancer resistance protein, Bcrp), are present at birth in both humans and rats. Nevertheless, organization of the cell assemblies responsible for regulation of the transport of endogenous and exogenous substances at the blood–brain interface (neurovascular unit) is supposedly completed only after the first several postnatal months in humans and three months in rats (Saunders et al., 2012; Semple et al., 2013; Strazielle and Gersi-Egea, 2013, 2015). Moreover, contrary to ABCG2, expression of ABCB1 in brain microvessels is limited in newborns and increases during postnatal development. In humans, the immunohistochemical signal from ABCB1 in 0–3 and 3–6 postnatal months was about 60% and 90%, respectively, in relation to the adults (Lam et al., 2015). In 10-day and 35-day old rats the expression of this efflux transporter reached approximately 20–30% and 90–100%, respectively, in comparison to the adult animals (Gazzin et al., 2008; Ose et al., 2008). This study enabled us to better understand how TREO and its epoxides penetrate the CNS in HSCT pediatric patients with immature and mature BBB.
Brain exposure of TREO and S,S-EBDM

After the intravenous bolus administration of TREO 500 mg/kg b.w., the exposure of the rat brain to TREO and S,S-EBDM was strikingly lower in comparison to the plasma (systemic) exposure (Figure 3) despite very low plasma protein binding (Table 2). In comparison to YAR, the JR’s brain tissue was about 2-fold more exposed to TREO and 4-fold more to S,S-EBDM over time, as expressed by the brain AUC of the compounds. This difference in the brain exposure between the two age groups correlated with higher TREO and S,S-EBDM systemic exposure and brain penetration (Figure 4), as well as slower elimination from the JR brain than the YAR. Therefore, a hypothesis can be given that clearing mechanisms present in the endothelial cells of the BBB may have had lower activity in the juveniles. Recently, in an in vitro BBB model consisting of porcine brain capillary endothelial cells transport of TREO in basolateral-to-apical (efflux) direction turned out to be higher than in apical-to-basolateral (influx) direction. However, the efflux ratio for TREO (1.88) did not reach the threshold for recognition of P-gp substrates, that is 2, and no concentration dependency of the drug distribution was observed. Therefore, involvement of the active efflux mechanisms in elimination of TREO from brain in vivo remains an open question (Linz et al., 2015). If that is the case, the higher brain exposure to TREO in the 10-day old JR, in which the P-gp expression at BBB could be several times lower than in the 34-35-day old YAR (Gazzin et al., 2008; Ose et al., 2008), is not surprising. Because of age-dependent kidney function development, also renal clearance mechanisms might contribute to the higher AUCs of TREO in plasma of the JR, which results in higher AUCs in brain (Fleck, 1999).

Low penetration of TREO and S,S-EBDM across BBB reported here may explain why in the patients with glioblastoma a combination of TREO at the dose of 3.5 g/m² with gemcitabine followed by radiotherapy provided no advantage over radiotherapy alone (Wick et al., 2002). However, HSCT patients receive much higher TREO doses than 3.5 g/m², namely 10 – 14
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g/m². Consequently, the plasma drug C_{\text{max}} reaches 1 – 3 mM (Główka et al., 2010, 2015), which is similar to that observed in our study in the rats’ plasma. Additionally, TREO C_{\text{max}} found in the rats’ brain (about 120 μM in the JR and 60 μM in the YAR) is higher than the concentrations reported to efficiently inhibit a viability of leukemia cells obtained from pediatric patients (Munkelt et al., 2008). Therefore, a hypothesis may be offered that, although TREO penetrates across BBB weakly, its myeloablative doses can indeed provide cytotoxic effects in leukemia and other hematological malignancies that spread to the brain. On the other hand, brain exposure to drugs could be associated with neurological adverse effects, for instance seizures. Beneficially, in clinical trials high-dose TREO demonstrated low neurotoxicity in adults as well as children, at least in comparison to high-dose busulfan requiring anticonvulsive prophylaxis (Boztug et al., 2015; Casper et al., 2012; Danylesko et al., 2012; Shimoni et al., 2012; Wachowiak et al. 2011). Nevertheless, in one pediatric study (n = 70, including 46 infants), seizures occurred in four infants aged ≤ 4 months (Slatter et al., 2011). In such young children, organization of the neurovascular unit and expression of ABCB1 in the brain capillary endothelium is expected to be diminished in relation to those above one year of age, similarly when comparing the JR to the YAR used in our study (Gazzin et al., 2008; Lam et al., 2015; Ose et al., 2008; Saunders et al., 2012; Semple et al., 2013; Strazielle and Gersi-Egea, 2013, 2015). One may hypothesize that the seizures appearance in those youngest patients could be related, at least in part, from the higher brain penetration of TREO and/or S,S-EBDM. Therefore, the data presented here may corroborate a special care of very young children against seizures, including consideration of prophylactic use of antiepileptic drugs, as proposed by Slatter et al. (2011).

CSF exposure of TREO and S,S-EBDM

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In the YAR TREO and S,S-EBDM demonstrated higher penetration into the CFS than into
the brain (Figure 4). It is not surprising taking into account the anatomical differences
between the BBB and BCSFB that result in the latter being permeable to drugs. In fact, all
molecules enter from blood to CSF at a rate inversely related to molecular weight (MW),
which explains why S,S-EBDM (MW 182 Da) penetrated into the rat CSF better than TREO
(MW 278 Da) (Nau et al., 2010; Pardridge, 2012; Tam and Watts, 2010). As TREO may be a
substrate for the efflux transporters, it is also worth to mention that P-gp substrates which
cannot be found in appreciable concentrations in brain typically penetrate into CSF (Linz et
al., 2015).

**TREO and S,S-EBDM versus busulfan penetration across the BBB**

Comparing the rat brain/plasma C<sub>max</sub> and AUC ratios obtained for TREO and S,S-EBDM
with those reported previously for busulfan (Table 4), it can be concluded that TREO, in
particular, and S,S-EBDM are less capable to cross the BBB than busulfan. As far as lipid-
mediated free transcellular diffusion is considered, the BBB is generally permeable for
protein unbound species with a MW <400 Da and sufficient lipophilicity (Nau et al., 2010;
Pardridge, 2012; Strazielle and Gersi-Egea, 2013; Tam and Watts, 2010). All TREO, S,S-
EBDM and busulfan have MW <400 Da and bind to human plasma proteins weakly (Table
4), so their lipophilicity arises here as a factor that determines the differences in brain
penetration. Indeed, the lipophilicity increases in order TREO < S,S-EBDM < busulfan (log n-
octanol/water partition coefficient is −1.58, −1.18, and −0.58, respectively) which accords
with their ability to penetrate the BBB (Główka et al., 2013; Westerhof et al., 2000).

Comparison of brain exposure to TREO and busulfan in HSCT patients should not be based
on the rat rough brain/plasma AUC ratios because TREO is administered in much higher
doses than busulfan (12 – 14 g/m² vs 0.8 mg/kg) and achieves much higher plasma
concentrations (Główka et al., 2010; 2015; Hoy and Lyseng-Williamson, 2007). As shown in Table 4, despite TREO and S,S-EBDM penetrate across the BBB weaker than busulfan, in the brain of patients conditioned prior to HSCT TREO is supposed to achieve much higher AUC when compared to busulfan, while S,S-EBDM a similar one. Therefore, lower clinical neurotoxicity of TREO seems not to be caused by smaller, in comparison to busulfan, accumulation in the brain, but rather different pharmacodynamics.

**Conclusion**

Using the rat model, it was demonstrated that TREO and its active transformer, S,S-EBDM, crossed the BBB and BCSFB to rather small extent. However, the juvenile animals experienced greater brain exposure of the studied compounds than the young adults. This shows that age-related difference in distribution of TREO and S,S-EBDM into CNS may cause higher risk of neurological side-effects in very young patients after TREO-based conditioning prior to HSCT. Additionally, our results indicate that not pharmacokinetic but rather pharmacodynamic reasons account for lower clinical neurotoxicity of TREO when compared to busulfan. Current posology of TREO-based conditioning in clinical trials reflects pharmacokinetic differences between pediatric and adult patients (van den Berg et al, 2014).
Acknowledgments

The authors appreciate Laboratory of Pharmacology and Toxicology in Hamburg (Germany) carrying out \textit{in vivo} experiment with TREO in rats.
Authorship Contributions

Participated in research design: Romański, Baumgart, Böhm, and Główka

Conducted experiments: Romański and Główka

Performed data analysis: Romański

Wrote or contributed to the writing of the manuscript: Romański, Baumgart, Böhm, and Główka.
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References


treosulfan in children prior to allogeneic HCT. Bone Marrow Transplant 49 (Suppl. 1): S380–S381.


preparative regimen for allogeneic hematopoietic SCT. *Bone Marrow Transplant* **42**: S67–S70.


Footnotes

The studies were financially supported by medac GmbH (Wedel, Germany).

The results of the work were presented in abstract form during the 40th Annual Meeting of the European Group for Blood and Marrow Transplantation, Milan, Italy, 30 March – 2 April 2014 (Bone Marrow Transplant. 2014: Vol. 49, suppl. 1, p. S46).

For reprints, please contact Prof. Franciszek K. Główka, Department of Physical Pharmacy and Pharmacokinetics, Poznan University of Medical Sciences, 6 Swiecickiego Street, 60-781 Poznan, Poland. E-mail: glowka@ump.edu.pl
Figure Legends

Figure 1. Nonenzymatic conversion of TREO to biologically active epoxides.

Figure 2. Changes of the mean concentration of unbound TREO and S,S-EBDM in the JR and YAR’s plasma and brain, and in the YAR’s CSF after the intravenous bolus of TREO 500 mg/kg b.w. The whiskers show the standard deviation of the concentrations (n = 3).

Figure 3. Values of C_{max} (mean ± S.E.M., n = 3) (A) and AUC (estimate ± S.E.) (B) obtained for unbound TREO and S,S-EBDM in the JR and the YAR after the intravenous bolus of TREO 500 mg/kg b.w. The asterisks in (A) show statistically significant differences in the C_{max} related to the rat’s age (*** p < 0.01, ANOVA test).

Figure 4. Brain/plasma and CSF/plasma AUC ratios (estimate ± S.E.) obtained for unbound TREO and S,S-EBDM in the JR and the YAR after the intravenous bolus of TREO 500 mg/kg b.w. The linkers show where the age-related difference between the estimated values is greater than 1.96 square root of the sum of the standard errors squares.
Table 1 Validation parameters established for the analytical runs in which the studied rat samples were processed

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Linearity of the calibration curves&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(accuracy&lt;sup&gt;b&lt;/sup&gt; / precision&lt;sup&gt;c&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREO S,S-EBDM S,S-DEB</td>
<td>0.23 – 114 μM and 114 – 5720 μM</td>
<td>0.87 – 174 μM (89.1 – 109.7% / 12.2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 – 20 μM (95.8 – 112.7% / 15.0%)</td>
</tr>
<tr>
<td>Brain homogenate supernatant</td>
<td>0.23 – 28 μM and 114 – 5720 μM</td>
<td>0.35 – 44 μM (91.1 – 109.3% / 7.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 – 10 μM (97.0 – 108.1% / 10.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(94.3 – 108.4% / 10.3%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The calibration curves were established using the calibration standards at a minimum of six concentration levels including the lower and upper limit of quantitation. For analysis of TREO in plasma, two calibration curves were prepared that covered low and high concentrations. <sup>b</sup> The accuracy ranges shown in the table include the results obtained for at least three levels of the quality control samples (low, medium and high) in duplicate or triplicate. <sup>c</sup> The precision shown in the table was evaluated at the lower limit of quantification.
Table 2 Unbound fraction of TREO and S,S-EBDM in rat plasma and brain homogenate

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Concentration ( ^a )</th>
<th>Unbound fraction ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>TREO</td>
<td>2.3 ( \mu M )</td>
<td>0.962 ± 0.137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 ( \mu M )</td>
<td>0.940 ± 0.061</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 ( \mu M )</td>
<td>0.960 ± 0.163</td>
</tr>
<tr>
<td></td>
<td>S,S-EBDM</td>
<td>3.5 ( \mu M )</td>
<td>1.083 ± 0.211</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87 ( \mu M )</td>
<td>0.986 ± 0.119</td>
</tr>
<tr>
<td>Brain homogenate</td>
<td>TREO</td>
<td>2.3 ( \mu M )</td>
<td>1.063 ± 0.172</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.7 ( \mu M )</td>
<td>0.999 ± 0.081</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.2 ( \mu M )</td>
<td>1.000 ± 0.108</td>
</tr>
<tr>
<td></td>
<td>S,S-EBDM</td>
<td>3.5 ( \mu M )</td>
<td>1.092 ± 0.079</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.7 ( \mu M )</td>
<td>0.974 ± 0.075</td>
</tr>
</tbody>
</table>

\( ^a \) The used concentrations covered the range observed in the JR and the YAR which received TREO 500 mg/kg b.w. as an intravenous bolus. \( ^b \) At each concentration level of TREO or S,S-EBDM the unbound fraction was determined using five lots of plasma and brain homogenate obtained from different 2-month old rat donors.
Table 3 The \( k_{el} \) describing the elimination of TREO and S,S-EBDM from the rat plasma, brain and CSF with the corresponding \( t_{1/2} \) values \(^{a}\)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Rat group</th>
<th>TREO ( k_{el} ) [h(^{-1})]</th>
<th>( t_{1/2} ) [h]</th>
<th>S,S-EBDM ( k_{el} ) [h(^{-1})]</th>
<th>( t_{1/2} ) [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JR male</td>
<td>0.708 ± 0.046</td>
<td>0.98 ± 0.06</td>
<td>0.593 ± 0.090</td>
<td>1.17 ± 0.18</td>
</tr>
<tr>
<td>Plasma</td>
<td>JR female</td>
<td>0.587 ± 0.036</td>
<td>1.18 ± 0.07</td>
<td>0.511 ± 0.051</td>
<td>1.36 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>YAR male</td>
<td>1.012 ± 0.020</td>
<td>0.68 ± 0.01</td>
<td>1.039 ± 0.033</td>
<td>0.67 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>YAR female</td>
<td>1.066 ± 0.028</td>
<td>0.65 ± 0.02</td>
<td>0.969 ± 0.023</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>JR male</td>
<td>0.274 ± 0.074</td>
<td>2.52 ± 0.68</td>
<td>0.451 ± 0.030</td>
<td>1.54 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>JR female</td>
<td>0.213 ± 0.003</td>
<td>3.26 ± 0.05</td>
<td>0.535 ± 0.029</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>YAR male</td>
<td>0.289 ± 0.0007</td>
<td>2.39 ± 0.006</td>
<td>1.106 ± 0.064</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>YAR female</td>
<td>0.331 ± 0.034</td>
<td>2.09 ± 0.21</td>
<td>0.881 ± 0.086</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>YAR male</td>
<td>0.489 ± 0.010</td>
<td>1.42 ± 0.03</td>
<td>0.740 ± 0.050</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>YAR female</td>
<td>0.528 ± 0.014</td>
<td>1.31 ± 0.03</td>
<td>0.735 ± 0.010</td>
<td>0.94 ± 0.01</td>
</tr>
</tbody>
</table>

\(^{a}\) Data are presented as the estimate ± S.E. The results of evaluation of the statistical difference in the \( k_{el} \) observed between the JR and YAR were as follows: plasma TREO: \( p = 0.0010 \) (male), 0.0009 (female); plasma S,S-EBDM: \( p = 0.0179 \) (male), 0.0022 (female); brain TREO: \( p = 0.8581 \) (male), 0.0731 (female); brain S,S-EBDM: \( p = 0.01332 \) (male), 0.0582 (female). There were no significant differences in the \( k_{el} \) of TREO and S,S-EBDM in plasma, brain and CSF between the male and female rats from the same age groups (\( p > 0.05 \)).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>TREO</th>
<th>S,S-EBDM</th>
<th>busulfan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/plasma $C_{max}$ ratio in rats</td>
<td>0.013 – 0.025 (YAR)</td>
<td>0.22 – 0.30 (YAR)</td>
<td>0.74 $^a$</td>
</tr>
<tr>
<td></td>
<td>0.040 – 0.060 (JR)</td>
<td>0.38 – 0.44 (JR)</td>
<td></td>
</tr>
<tr>
<td>Brain/plasma AUC ratio in rats</td>
<td>0.07 – 0.10 (YAR)</td>
<td>0.22 – 0.28 (YAR)</td>
<td>0.75 $^a$</td>
</tr>
<tr>
<td></td>
<td>0.14 – 0.17 (JR)</td>
<td>0.48 – 0.52 (JR)</td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>278</td>
<td>182</td>
<td>246</td>
</tr>
<tr>
<td>Unbound fraction in human plasma</td>
<td>100% $^b$</td>
<td>80% $^b$</td>
<td>68 – 93% $^c$</td>
</tr>
<tr>
<td>Plasma AUC in HSCT patients</td>
<td>3200 – 9000 μM × h $^d$</td>
<td>16 – 90 μM × h $^c$</td>
<td>15 – 25 μM × h $^f$</td>
</tr>
<tr>
<td>Expected brain AUC in HSCT patients $^g$</td>
<td>230 – 1500 μM × h</td>
<td>4 – 47 μM × h</td>
<td>11 – 19 μM × h</td>
</tr>
</tbody>
</table>

$^a$ Results obtained in male Sprague-Dawley rats (250 – 300 g), Hassan et al., 1988; $^b$ Główka et al., 2012; $^c$ Hoy and Lyseng-Williamson, 2007; $^d$ Mean AUC range observed in patients after 2 h i.v. infusion of 12 or 14 g/m² of TREO, data taken from Beelen et al., 2005; Chiesa et al., 2014; Główka et al., 2008, 2012, 2015; Nemecek et al., 2011; ten Brink et al., 2014; $^e$ Plasma AUC of S,S-EBDM is about 100 – 200 times lower than of TREO, Główka et al., 2012, 2015; $^f$ Therapeutic window established for i.v. busulfan dose of 0.8 mg/kg is 900 – 1500 μM × min (Hoy and Lyseng-Williamson, 2007); $^g$ Calculated as a product of ‘Brain/plasma AUC ratio in rats’ and ‘Plasma AUC in HSCT patients’. MW, molecular weight; logP, logarithm of n-octanol/water partition coefficient.
Figure 1
Figure 2
**Figure 3**

A

**C$_{\text{max}}$ of TREO**

![Graph showing $C_{\text{max}}$ of TREO in Plasma, Brain, and CSF for JR male, JR female, YAR male, and YAR female.]

**C$_{\text{max}}$ of S,S-EBDM**

![Graph showing $C_{\text{max}}$ of S,S-EBDM in Plasma, Brain, and CSF for JR male, JR female, YAR male, and YAR female.]

B

**AUC of TREO**

![Graph showing AUC of TREO in Plasma, Brain, and CSF for JR male, JR female, YAR male, and YAR female.]

**AUC of S,S-EBDM**

![Graph showing AUC of S,S-EBDM in Plasma, Brain, and CSF for JR male, JR female, YAR male, and YAR female.]

JR male, JR female, YAR male, YAR female

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Figure 4