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

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

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ABSTRACT

Treosulfan (TREO) is currently investigated as an alternative treatment of busulfan in conditioning before 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 the 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.wt. 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 was 0.14, 0.17, 0.10, and 0.07 and for unbound S,S-EBDM, it was 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 before hematopoietic stem cell transplantation (HSCT) in pediatric and adult patients. 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. In general, clinical data regarding TREO application to this specific group of patients are limited. Noteworthy, reported TREO-based conditioning in 70 young children with primary immunodeficiency, among whom as many as 46 were infants. In this study, four cases of seizures were noted and all of them occurred exceptionally in patients aged less than 4 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 prodrug that undergoes a nonenzymatic 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). The distribution of epoxides in the brain and their transformation into biological active epoxides is crucial in understanding the potential neurotoxicity of TREO.

The studies were financially supported by medac GmbH (Wedel, Germany). The results of the work were presented in abstract form at the following conference: Główka F, Romaniški M, Baumgart J, Böhm S (2014) Blood-brain barrier penetration of treosulfan and its biologically active epoxides in juvenile and young adult rats. 40th Annual Meeting of the European Group for Blood and Marrow Transplantation; 2014 Mar 30–Apr 2; Milan, Italy. dx.doi.org/10.1124/dmd.115.066050.

ABBREVIATIONS: AUC, area under the concentration-time curve from zero to infinity; AUClast, 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; Cmax, last concentration measured; Cmax, maximum concentration; CSF, cerebrospinal fluid; fub, unbound fraction; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; IS, internal standard; JR, juvenile rats; k0, elimination rate constant; MW, molecular weight; 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; TREO, treosulfan; YAR, young adult rats.
bioanalytical results of TREO and both of its active epoxy-transformers in plasma, brain, and cerebrospinal fluid (CSF) after intravenous 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 nonclinical study constituted one of the measures included in the pediatric investigation plan into central nervous system (CNS) treated with TREO and its epoxy-transformers was obtained as depicted in the previous papers (Glówka et al., 2012; Romański et al., 2014). Drug-free rat plasma and brain tissue for preparation of the calibration standards were obtained from Laboratory of Pharmacology and Toxicology (Hamburg, Germany).

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 (Glówka et al., 2012; Romański et al., 2014). Drug-free rat plasma and brain tissue for preparation of the calibration standards were obtained from Laboratory of Pharmacology and Toxicology (Hamburg, Germany).

Animals. Animal experiments including blood, CSF, and organ sampling were carried out by the Good Laboratory Practice-certified facility of Laboratory of Pharmacology and Toxicology 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 "Tiererschutzgesetz" (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 hour light/dark circle). Drinking water and the appropriate commercial feed (Sniff, Soest, Germany) were offered to the dams and the YAR ad libitum. After 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 the 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 s/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 minutes, 0.5, 1.0, 2.0, 4.0, 6.0, and 24.0 hours 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 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 minutes 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 was 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 minutes 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 ultrfiltration (cut-off 30 kDa, 14,000 g at 20°C over 20 minutes). 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 that was confirmed during the validation of the applied method. The obtained ultrfiltrate 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 acetoniitrile (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 ultraviolet detection described by Glówka 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 was 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′-dinitrophenyl (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 postderivatization 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 ultraviolet 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 Sciences (Deerfield, IL), 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 minutes linear from 40 to 80% B, 12–13 minutes 80% B, 13–15 minutes return from 80% to 40% B and the post time of 6 minutes with 40% B for the column equilibration.

Quantification of TREO and its epoxy-transformers was processed according to the current guidelines of European Medicines Agency for bioanalytical methods (Guideline on Bioanalytical Method Validation, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf). 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 did the levels of S.S-DEB exceed the Lower Limit of Quantification, 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 (fu) 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, 57, and 17 µM in the brain homogenate, whereas 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 hour in a thermoshaker, 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, Bedford, MA) applying 14,000 g over 20 minutes. Quantification of TREO and S.S-EBDM in the obtained filtrates was performed as described in Preparation of Plasma, CSF, and Brain Samples for HPLC Analysis. The second series of samples was 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 that had been earlier prepared using the Amicon guarded by Nucleosil C18 (4.6 × 250 mm; 5 µm particle size) using the Amicon Ultra-0.5 ml device with a cut-off 30 kDa (Millipore, Bedford, MA) applying 14,000 g over 20 minutes. Quantification of TREO and S.S-EBDM in the obtained filtrates was performed as described in Preparation of Plasma, CSF, and Brain Samples for HPLC Analysis. The second series of samples was 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 that had been earlier prepared using the Amicon devices. Unbound fraction of the compounds was calculated using formula: 

\[ f_u = \frac{P_{analyte}^b}{P_{analyte}^I} \]

where \( P_{analyte}^b \) and \( P_{analyte}^I \) 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):

\[ C_{b,con} = \frac{C_p \cdot V_p \cdot F_C + (1 - f_u) \cdot V_{precess} \cdot C_p}{1 - V_w} \]

where \( C_{b,con} \) denoted total concentration of the compound in the individual rat’s brain corrected for the residual blood; \( C_p \) is total concentration determined in the individual rat’s brain; \( F_C \) is mean of the total plasma concentration of the analyte observable in the rats of the given sex and age at the same time as in the brain; \( f_u \) unbound fraction of the compound in plasma; \( V_p \) and \( V_{precess} \) 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 because of 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 (fu). The value of fu for CSF was calculated using the following formula (Fridén et al., 2010):

\[ f_u_{CSF} = \frac{1}{1 + 0.045 \times \left( \frac{1}{f_u_{plasma}} - 1 \right)} \]

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

Pharmacokinetic Analysis. On the basis of the concentrations of unbound TREO and S.S-EBDM in the rat plasma, CSF, and brain tissue, the pharmacokinetic parameters were calculated using a noncompartmental analysis and sparse sampling technique in WinNonlin 6.2 (Pharsight, Princeton, NJ). Concentration and time of maximum concentration 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 (ka) 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 (t1/2) was calculated from ln2/ka. The area under the concentration-time curve from zero to the time of the last concentration measured (AUC0inf) was calculated by a linear trapezoidal rule and the residual area under the curve (AUCres) was estimated by extrapolation from the last concentration measured (Clast) to infinity using Clast/ka ratio. The area under curve from zero to infinity (AUC) was computed as a sum of AUC0inf and AUCres.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>TREO Linearity of the Calibration Curves a (Accuracy/%Precision)</th>
<th>S.S-EBDM Linearity of the Calibration Curves a (Accuracy/%Precision)</th>
<th>S.S-DEB Linearity of the Calibration Curves a (Accuracy/%Precision)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.23–114 µM and 114–5720 µM (86.9–111.3%/8 and 2.5%)</td>
<td>0.87–174 µM (89.1–109.7%/12.2%)</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 (91.1–109.3%/7.4%)</td>
<td>0.35–44 µM (97.0–108.1%/10.4%)</td>
<td>0.50–10 µM (94.3–108.4%/10.3%)</td>
</tr>
</tbody>
</table>

aThe 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.

bThe 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.

cThe precision shown in the table was evaluated at the lower limit of quantitation.
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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 $k_d$ 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 Co., Redmond, WA). Standard errors of $t_{1/2}$, 
AUC, and $AUC$ ratio were calculated using the differential calculus:

$$S.E.\frac{t_{1/2}}{E}_{\text{el}} = \ln^2 S.E.\frac{k_d}{E}_{\text{el}}$$

$$S.E.\frac{AUC}{E}_{\text{el}} = S.E.\frac{AUC}{E}_{\text{rel}} + S.E.\frac{AUC}{E}_{\text{rel}} = S.E.\frac{AUC}{E}_{\text{rel}} + \frac{1}{k_d} S.E.\frac{M_{\text{rel}}}{E}_{\text{el}}$$

$$S.E.\frac{AUC}{E}_{\text{el}} = \frac{1}{AUC_2} S.E.\frac{AUC}{E}_{\text{el}} + \frac{AUC_2}{AUC_1} S.E.\frac{AUC}{E}_{\text{el}}$$

Statistical significance of the differences between the selected pharmacokinetic parameters was evaluated in Statistica 10 (StatSoft, Inc., Tulsa, OK). 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 analysis of variance after normal distribution of the data had been confirmed with the Shapiro-Wilk test. Effect of sex and age on the evaluated by analysis of variance after normal distribution of the data had been confirmed with the Shapiro-Wilk test. Effect of sex and age on the

Results

Characteristics and Health Status of the Animals. The pharmacokinetic studies were carried out in 96 JR and YAR, each including 6 predose animals and 42 dosed ones. The number of the animals used resulted from the serial sacrifice design in which measurements were taken at eight 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.wt. none of the rats died prematurely. No signs of local intolerance reactions or systemic toxicity were noted in the YAR and the feces of all the animals were of a normal consistency. During the sample 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, regardless 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 that 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 Fig. 2. In none of the analyzed samples did the levels of S,S-DEB exceed the Lower Limit of Quantification that is 1 $\mu$M in plasma and CSF and 3 $\mu$M in brain tissue.

TREO and S,S-EBDM Exposure in Plasma, Brain, and CSF. To measure TREO and S,S-EBDM exposure in rat plasma, brain, and CSF, the $C_{\text{max}}$ and AUC values were used. As presented in Fig. 3A, the mean $C_{\text{max}}$ 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 $C_{\text{max}}$ of free TREO and S,S-EBDM in JR of both sexes was considerably higher than in YAR, although 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 $C_{\text{max}}$ observed in brain and CSF were much lower in comparison with the plasma. Accordingly, the brain-to-plasma free TREO $C_{\text{max}}$ ratio was about 0.05 and 0.02 in JR and YAR, respectively, and the CSF-to-plasma free TREO $C_{\text{max}}$ ratio in YAR amounted to approximately 0.04. Analogous results were obtained when analyzing the S,S-EBDM $C_{\text{max}}$ in the three studied matrices, although 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 $C_{\text{max}}$ ratio was about 0.4 and 0.25 in JR and YAR, respectively, and the YAR’s CSF-to-plasma free S,S-EBDM $C_{\text{max}}$ ratio was close to 0.5. The results of the AUC, that in opposition to $C_{\text{max}}$ shows a drug exposure over entire time of its residence in the given body compartment, are presented in Fig. 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- to 14-fold for TREO and 2- to 4-fold for S,S-EBDM) when compared with 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 $k_d$ and $t_{1/2}$ presented in Table 3 show that in all the rats, TREO and S,S-EBDM were eliminated relatively fast from plasma, brain, and CSF ($t_{1/2}$ range 0.6–3.3 hours), 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 ($k_d$) 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 $k_d$ 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 Blood-Cerebrospinal Fluid Barrier. 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, because they are considered as the most accurate parameters for that purpose (Nau et al., 2010). As presented in Fig. 4, the ratios obtained for S,S-EBDM were generally much higher in comparison with 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 $0.14 \pm 0.03$ in the male JR, $0.17 \pm 0.03$ in the female JR, and $0.098 \pm 0.008$ in the male YAR, $0.073 \pm 0.007$ in the female YAR. The values obtained for S,S-EBDM were $0.52 \pm 0.06$ in the male JR, $0.48 \pm 0.08$ in the female JR, and $0.28 \pm 0.03$ in the male YAR and $0.22 \pm 0.01$ 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 1 month old (Slatter et al., 2011, 2015; Dinur-Schejter et al., 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- to 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), 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 3 months in rats (Saunders et al., 2012; Semple et al., 2013; Strazielle and Ghersi-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- and 35-day-old rats the expression of this efflux transporter reached approximately 20–30% and 90–100%, respectively, in comparison with 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.wt., the exposure of the rat brain to TREO and S,S-EBDM was strikingly lower in comparison with the plasma (systemic) exposure (Fig. 3) despite very low plasma protein binding (Table 2). In comparison with YAR, the JR’s brain tissue was about twofold more exposed to TREO and fourfold more to S,S-EBDM.

Table 3

The $k_d$ describing the elimination of TREO and S,S-EBDM from the rat plasma, brain and CSF with the corresponding $t_{1/2}$ values

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Rat Group</th>
<th>TREO $k_d \pm S.E.$</th>
<th>$t_{1/2} \pm S.E.$</th>
<th>S,S-EBDM $k_d \pm S.E.$</th>
<th>$t_{1/2} \pm S.E.$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</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></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>Brain</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>CSF</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>

Data are presented as the estimate ± S.E. The results of evaluation of the statistical difference in the $k_d$ 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 $k_d$ of TREO and S,S-EBDM in plasma, brain and CSF between the male and female rats from the same age groups ($P > 0.05$).
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 (Fig. 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). In that 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- to 35-day-old YAR (Gazzin et al., 2008; Ose et al., 2008), is not surprising. Because of age-dependent kidney function development, renal clearance mechanisms might also 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 g/m². Consequently, the plasma drug C_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_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 hematologic malignancies that spread to the brain. On the other hand, brain exposure to drugs could be associated with neurologic 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 with high-dose busulfan, requiring anticonvulsive prophylaxis (Wachowiak et al., 2011; Casper et al., 2012; Danylesko et al., 2012; Shimonii et al., 2012; Boztug et al., 2015). 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.

### Table 4

Comparison of brain penetration of TREO, S,S-EBDM, and busulfan

<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*</td>
</tr>
<tr>
<td>Brain/plasma AUC ratio in rats</td>
<td>0.040–0.060 (JR)</td>
<td>0.38–0.44 (JR)</td>
<td>0.75*</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%a</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^e</td>
<td>15–25 μM × h^f</td>
</tr>
<tr>
<td>Expected brain AUC in HSCT patients</td>
<td>230–1500 μM × h</td>
<td>4–47 μM × h</td>
<td>11–19 μM × h</td>
</tr>
</tbody>
</table>

*Results obtained in male Sprague-Dawley rats (250–300 g), Hassan et al., 1988.
*aGłówka et al., 2012.
*cMean 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.
*dPlasma AUC of S,S-EBDM is about 100–200 times lower than of TREO, Główka et al., 2012, 2015.
*eTherapeutic window established for i.v. busulfan dose of 0.8 mg/kg is 900–1500 μM × min (Hoy and Lyseng-Williamson, 2007).
*fCalculated as a product of "Brain/plasma AUC ratio in rats" and "Plasma AUC in HSCT patients."
in relation to those above 1 year of age, similar to when comparing the JR to the YAR used in our study (Gazzin et al., 2008; Ose et al., 2008; Saunders et al., 2012; Semple et al., 2013; Strazielle and Gherzi-Eega, 2013, 2015; Lam et al., 2015). One may hypothesize that the seizure 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.** In the YAR, TREO and S,S-EBDM demonstrated higher penetration into the CFS than into the brain (Fig. 4). It is not surprising taking into account the anatomic 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; Tam and Watts, 2010; Partridge, 2012). As TREO may be a substrate for the efflux transporters, it is also worth mentioning 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 $C_{\text{max}}$ 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 of crossing 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; Tam and Watts, 2010; Partridge, 2012; Strazielle and Gherzi-Eega, 2013). 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 is in accordance with their ability to penetrate the BBB (Westerhof et al., 2000; Glówka et al., 2013).

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 versus 0.8 mg/kg) and achieves much higher plasma concentrations (Hoy and Lyseng-Williamson, 2007; Glówka et al., 2010, 2015). As shown in Table 4, despite TREO and S,S-EBDM penetrating across the BBB weaker than busulfan, in the brain of patients conditioned before HSCT, TREO is supposed to achieve much higher AUC compared with busulfan, whereas S,S-EBDM is expected to have a similar AUC. Therefore, lower clinical neurotoxicity of TREO seems not to be caused by smaller, in comparison with busulfan, accumulation in the brain, but rather different pharmacodynamics.

**Conclusion**

By using the rat model, it was demonstrated that TREO and its active transformer, S,S-EBDM, crossed the BBB and BCSFB to a 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 neurologic side-effects in very young patients after TREO-based conditioning before HSCT. Additionally, our results indicate that rather pharmacodynamic not pharmacokinetic reasons account for lower clinical neurotoxicity of TREO compared with busulfan. Current posology of TREO-based conditioning in clinical trials reflects pharmacokinetic differences between pediatric and adult patients (van den Berg et al., 2014).

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**Authorship Contributions**

**Conducted research in: Romáski, Baumgart, Böm, and Glówka.**

**Performed data analysis:** Romáski.

**Wrote or contributed to the writing of the manuscript:** Romáski, Baumgart, Böm, and Glówka.

**References**


