In Vitro and *In Vivo* Blood-Brain Barrier Penetration Studies with the Novel Cyanide Antidote Candidate Dimethyl Trisulfide in Mice

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ABSTRACT

Recent *in vitro* and *in vivo* studies highlight the strong potential of dimethyl trisulfide (DMTS) as an antidote for cyanide (CN) intoxication. Due to its high oxygen demand, the brain is one of the main target organs of CN. The blood-brain barrier (BBB) regulates the uptake of molecules into the brain. In the literature, there is no data about the ability of DMTS to penetrate the BBB. Therefore, our aim was to test the *in vitro* BBB penetration of DMTS and its *in vivo* pharmacokinetics in blood and brain. The *in vitro* BBB penetration of DMTS was measured by using a parallel artificial membrane permeability assay (BBB-PAMPA), and a triple BBB co-culture model. The pharmacokinetics was investigated in a mouse model by following the DMTS concentration in blood and brain at regular time intervals following intramuscular administration. DMTS showed high penetrability in both *in vitro* systems (apparent permeability coefficients: BBB-PAMPA 11.8×10^{-6} cm/s; cell culture 158×10^{-6} cm/s) without causing cell toxicity and leaving the cellular barrier intact. DMTS immediately absorbed into the blood after the intramuscular injection (5 min), and rapidly penetrated the brain of mice (10 min). In addition to the observed passive diffusion in the *in vitro* studies, the contribution of facilitated and/or active transport to the measured high permeability of DMTS in the pharmacokinetic studies can be hypothesized. Earlier investigations demonstrating the antidotal efficacy of DMTS against CN together with the present results highlight the promise of DMTS as a brain-protective CN antidote.

INTRODUCTION

Cyanide (CN) is employed in industrial processes such as electroplating, and gold mining; released in the smoke of fires; being especially problematic when materials with high nitrogen content are burned; and naturally synthesized in certain plants such as cassava, and bitter almond. Inhalation or ingestion of CN causes severe intoxication. CN binds to and inhibits the cytochrome *c* oxidase, a member of the mitochondrial electron transport chain, thereby suppressing oxygen utilization and the aerobic ATP production (Vogel et al., 1981, Way et al., 1988). CN intoxication is followed by lactic acidosis (Baud et al., 2002) in anaerobic conditions. The organs most susceptible to CN poisoning are the brain and the heart, which use high amounts of ATP. Although intracellular enzymes, such as rhodanese (EC 2.8.1.1) or 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) convert CN to the less toxic thiocyanate, the efficacy of these enzymes are limited (Wrobel et al., 2004). The brain contains only small amounts of rhodanese and 3-mercaptopyruvate sulfurtransferase (Aminlari et al., 2002; Eskandarzade et al., 2012; Shahbazkia et al., 2009; Nagahara et al., 1999), therefore it is desirable that antidotes against CN rapidly reach the brain. Acute CN intoxication can be treated by available antidotes (Petrikovics et al., 2015) such as Nithiodote® (comprised of a combination of sodium thiosulfate and sodium nitrite) (Scottsdale, 2011) and Cyanokit® (hydroxocobalamin) (Borron et al., 2006; Zakharov et al., 2015), but each of these have their own limitations of requirement of intravenous administration that limits their application in mass scenarios (Maraffa et al., 2012). Additional limitation with Cyanokit is that it requires high injection volume >200 ml. The major limitation of sodium nitrite is the formation of excess amount of methemoglobin in certain individuals even at the recommended doses and causing methemoglobinemia (Klimmek et al., 1988). The antidotal activity of sodium thiosulfate has limitations due to its small volume of distribution, short biological half-life and high rhodanese dependence (Schulz, 1984; Way, 1988).

The novel CN countermeasure dimethyl trisulfide (DMTS) showed remarkable anti-CN effects *in vitro* and *in vivo* (Rockwood et al., 2016; Kovacs et al., 2016a; Kovacs et al., 2016b). Comparing the sulfur donor efficacy of DMTS to sodium thiosulfate, the sulfur donor component of Nithiodote®, DMTS was three times more effective than thiosulfate *in vivo.* It also converts CN to thiocyanate 40 times faster than thiosulfate *in vitro* (Rockwood et al., 2016). These results indicate that DMTS is a promising agent against CN intoxication. As a highly lipophilic molecule, DMTS has low solubility in water and thus requires special formulation technology to obtain concentrations that are suitable for intramuscular administration. Such formulations have the potential for use in self-administration kits for mass casualty scenarios. A lipid based formulation was developed, when DMTS was encapsulated into micelles and

tested *in vitro* and *in vivo*, but the encapsulation efficacy of the product was not satisfying (Kovacs et al., 2016a). Various co-solvents and surfactants were tested for DMTS formulation (Kovacs et al., 2016b). Polysorbate 80 (Poly80) at 15 % (w/v) and 20 % (w/v) resulted the best solubilizing properties for DMTS and was further tested *in vivo* in mice (Kovacs et al., 2016b). The 15 % (w/v) Poly80 formulated DMTS provided over 3 times LD50 (Lethal Dose, 50%) protection at the DMTS dose of 100 mg/kg when the LD50 values were determined by the up-and-down method (Bruce, 1985). The antidotal protection was expressed as antidotal potency ratio (LD50 of CN with the antidote divided by the LD50 of CN without antidote (control)). Based on the works of Clark et al. (2003) and Lipinski et al. (2001) DMTS is expected to passively penetrate through the cellular membranes, because it is a highly lipophilic compound with a high logP value of 2.93 (predicted value: http://www.chemspider.com/Chemical-Structure.18219.html), it has small molecular weight (126.26 g/mol) and 76 Å polar surface area (predicted value: http://www.chemspider.com/Chemical-Structure.18219.html). Due to the brain is one of the most susceptible organ (as mentioned earlier) during CN intoxication, a potential CN antidote needs to cross the blood-brain barrier (BBB) and efficiently reach the brain. In case of the BBB the drug penetration is hindered not only by cellular membranes or tight junctions (in the paracellular space), but cells forming the BBB actively protect the brain against drugs and toxins by expressing efflux pumps and by their high metabolic activity. To the best of our knowledge there is no data in the literature about the permeability of the BBB to DMTS.

In this study, our aim was to test the BBB permeability and *in vivo* pharmacokinetics of DMTS in blood and brain. For permeability tests, two *in vitro* models of BBB were applied: 1) parallel artificial membrane permeability assay (PAMPA) and 2) brain endothelial cells co-cultured with pericytes and astrocytes. The effects of DMTS on cellular viability and on tight junctions were investigated. Furthermore, the pharmacokinetic profile in blood and brain for DMTS was determined on a mice model.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest purity commercially available. HPLC grade from J.T. Baker (Center Valley, PA, USA), HPLC grade acetonitrile and ethanol from Acros (Thermo Fisher Scientific, Geel, Belgium), Poly80 from Alfa Aesar (Ward Hill, MA, USA), DMTS, dimethyl disulfide (DMDS), sodium heparin from Sigma-Aldrich (SAFC, St Louis, MO, USA) and dibutyl disulfide (DBDS) from TCI America (Portland, OR, USA) were purchased. Hamilton gas tight luer-lock syringes (100 µL), micro

centrifuge tubes (1.7 mL), screw cap vials (2 mL and 5 mL) and needles (27G × ½) from VWR International (Radnor, PA, USA) and inserts (250 µL) with polymer feet were purchased from Agilent Technologies (Santa Clara, CA, USA). A 10 U/ml heparin solution was prepared by diluting a 10000 U/mL heparin stock solution with 0.9 % (w/v) saline solution. Aqueous Poly80 solutions were used at 5 and 15 % (w/v) concentrations. The DMTS stock solution (50 mg/ml) used for the *in vivo* studies was made by dissolving DMTS in 15 % (w/v) Poly80, and another DMTS stock solution (10 mg/ml) used for the BBB-PAMPA and the cell culture was prepared in 5 % (w/v) Poly80. Acetonitrile was used to prepare the 0.1 mg/ml DMDS solution, and ethanol for the 1 mg/ml DBDS solution. A twenty millimolar verapamil stock solution was prepared in DMSO (Sigma Aldrich). Ringer-HEPES (R-H) buffer was prepared by dissolving the components in distilled water resulting in the final concentrations of 150 mM for NaCl, 2.2 mM for $CaCl_2$, 0.2 mM for $MgCl_2$, 5.2 mM for KCl, 5 mM for HEPES, 6 mM for $NaHCO_3$, 3.3 mM for glucose. Poly80 (0.05 % (w/v)) in Prisma HT and Ringer-HEPES buffer was used in the BBB-PAMPA experiment and for the cell culture studies, respectively.

Testing DMTS Permeability in the BBB-PAMPA System

BBB-PAMPA system was applied to model the passive permeability properties of BBB and to *in vitro* test the diffusion of DMTS through BBB (Di et al., 2003). Prisma HT buffer was used as a solvent in the donor compartment in the BBB-PAMPA. According to the manufacturer's instruction 6.25 ml of Prisma HT concentrate (PN 110151, pION, Billerica, MA, USA) was filled up with HPLC purity water to 250 ml and the pH was set to 7.4 by 0.5 M NaOH. The stock solution of 10 mg/ml DMTS in 5 % (w/v) Poly80 was diluted with Prisma HT buffer 100 times to get the working solution of 0.1 mg/ml DMTS in 0.05 % (w/v) Poly80. Verapamil (pION) was used as a control for the BBB-PAMPA experiments. The permeability profile of verapamil is known from the literature (Mensch et al., 2010). To test and exclude the effect of Poly80 on DMTS permeability, verapamil was used with and without Poly80. Two verapamil solutions with a concentration of 200 µM were prepared in Prisma HT buffer, one with 0.05 % (w/v) Poly80 and one without Poly80. Brain Sink Buffer (PN110674, pION) with or without 0.05 % (w/v) Poly80 was used in the acceptor compartment. The arrangement of the solutions in the donor and acceptor phases are shown in Table 1.

Before loading the solutions into the PAMPA plates, the concentrations of DMTS and verapamil were analyzed by HPLC or UV spectrophotometer, respectively. The bottom 96-well microplates (PN120551, pION) with magnetic discs served as the donor compartment for the BBB-PAMPA permeability experiments. The wells of the plates were filled with 180 µl of 0.1 mg/ml DMTS in 0.05 %

(w/v) Poly80, 200 µM verapamil with or without 0.05 % (w/v) Poly80 (Table 1). The BBB-PAMPA membranes on the acceptor plate (pION) were impregnated with 5 µl of BBB lipid cocktail (pION, PN 110672). The donor and acceptor plates were carefully mounted (PAMPA sandwich) to prevent any bubble formation between the compartments. The acceptor wells were filled with 200 µl Brain Sink Buffer with or without 0.05 % (w/v) Poly80 (Table 1). The mounted plates were placed on the PAMPA plate stirrer (Gut-Box, pION) and the donor phase was stirred with 40 µm Aqueous Boundary Layer. The DMTS samples from the acceptor phase were collected at 30 and 60 min. The DMTS and verapamil samples were collected from the donor and acceptor phases after 90-min incubation (Table 1). HPLC-UV was used for analyzing the DMTS samples and UV spectrophotometer for the verapamil samples. For each treatment group 5-10 replicates were measured. The clearance volume was calculated for DMTS at 30, 60, 90 min and the apparent permeability was derived from the line fit to the data of clearance (see in Supplementary Information) (Kiss et al., 2014). For verapamil the apparent permeability was calculated as described previously (Kiss et al., 2014, see in Supplementary Information).

Cell Culture Conditions – BBB Model Construction

Primary cultures of rat brain endothelial cells, glia and pericytes were used in the construction of the *in vitro* blood-brain barrier (BBB) model as described previously (Nakagawa et al., 2009; Veszelka et al., 2013). Endothelial cells and pericytes were isolated from 3-weeks old Wistar rats, while mixed glial cultures (containing 90 % astrocytes) from neonatal Wistar rats. The detailed isolation procedure was published by Nakagawa et al. (2009). For the cell culture studies, all reagents were purchased from Sigma-Aldrich Kft., Hungary, unless otherwise indicated. Brain endothelial cells were cultured in DMEM F-12 (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 15 % plasma-derived bovine serum (First Link, UK), 100 µg/ml heparin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 1 ng/ml basic fibroblast growth factor (Roche, Switzerland) and 50 µg/ml gentamycin. For the establishment of the BBB model, brain microvascular pericytes were passaged to the bottom side of 12-well tissue culture inserts (Transwell, polycarbonate membrane, 0.4 µm pore size, Corning Costar, USA) coated with collagen IV at a density of 1.5×10^4 cells/cm^2. After attachment of the pericytes, brain endothelial cells (8×10^4 cells/cm^2) were seeded to the upper side of the fibronectin and collagen IV coated membranes. Primary cultures of rat glial cells were passaged to the bottom of 12-well dishes (Corning, Costar, New York, NY) coated with 100 mg/mL collagen type IV in sterile distilled water and cultured for 2 weeks before using for the triple co-culture model. Pericytes and glial cells were cultured in DMEM/HAM's F-12 supplemented with 10 % fetal bovine serum (Pan-Biotech GmbH) and

50 µg/mL gentamycin. To construct the in vitro BBB co-culture model Transwell culture inserts were placed into 12-well plates containing glial cells with endothelial culture medium in both compartments. After two days of co-culture leading to the formation of a confluent monolayer of brain endothelial cells, 550 nM hydrocortisone was added to the culture medium to tighten junctions (Deli et al. 2005; Walter et al., 2015).

Cellular Viability - MTT Dye Conversion Assay

For the cell viability assay, the isolated rat brain primary endothelial cells were seeded to 96-well plates at a density of 6×10^3 cells/well and cultured for 3-5 days until confluency (Kiss et al., 2013). To test the dose-dependent toxicity of DMTS the monolayers were treated with DMTS solution in the concentration range of 1-300 µg/ml in 0.05 % (w/v) Poly80 and phenol red free DMEM (Life Technologies, Gibco, Carlsbad, CA, USA) for 10 min. Cells which received only culture medium served as a negative control group (100 % viability), while cells treated with 1 mg/ml Triton X-100 detergent served as a positive control (100 % toxicity). The viability of cells treated with DMTS was compared to these positive and negative controls. After the 10-min incubation time the DMTS containing treatment medium was removed, the cells were washed with phosphate buffer saline (PBS) and 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) containing fresh medium was added to the culture and kept for 3 h in a CO_2 incubator. The conversion rate of the yellow MTT dye to the purple formazan reflects the metabolic activity of the cells. The formazan crystals were dissolved in DMSO and the amount of the converted dye was determined by measuring absorbance at 595 nm in a microplate reader (Fluostar Optima, BMG Labtechnologies, Ortenberg, Germany). The cell metabolic activity (viability) was calculated as a percentage of the dye conversion by the non-treated cells.

Integrity of the Paracellular Barrier

Transendothelial electrical resistance (TEER) were used to determine the tightness of the intercellular junctions in the BBB model (Deli et al. 2005). TEER measurements were made by an EVOM Volt/Ohm Meter (World Precision Instruments, USA) combined with STX-2 electrodes and expressed relative to the surface area of the monolayers ($\Omega \times cm^2$). The resistance of cell-free inserts (130 $\Omega \times cm^2$) was subtracted from the measured values. The BBB model showed a TEER value of 386 ± 67 $\Omega \times cm^2$ indicating tight barrier properties necessary for the permeability assay.

Permeability Experiments on the BBB Culture Model

For permeability studies in cell culture 0.1 mg/ml working concentration of DMTS was selected, which was approximately 100 times higher concentration than the detection limit in Ringer-HEPES buffer (1.4 µg/ml) and was still non-toxic to the cells. In contrast to a PAMPA system, in the culture BBB model the membrane of the inserts is thin (10 µm) and highly permeable, therefore log phase is negligible for a lipophilic compound, like DMTS. A short time period, 10-min for the measurement of P_{app} in culture condition was selected based on these characteristics and to limit any potential effect of cellular metabolism of DMTS. Inserts were transferred to 12-well plates containing 1.5 ml Ringer-HEPES buffer in the acceptor (abluminal) compartments. Culture medium was replaced in the donor (luminal) compartment for freshly prepared 0.1 mg/ml DMTS solution containing 0.05 % (w/v) Poly80. After a 10-min incubation on a horizontal shaker, solutions from both compartments were collected and prepared for HPLC measurement. DMTS concentrations from the luminal and abluminal compartments were determined by HPLC-UV. After the penetration assay the integrity of brain endothelial barrier was verified by the permeability of the permeability marker molecule fluorescein (Mw: 376 Da; 10 µg/ml final concentration in Ringer-Hepes buffer). The assay with fluorescein was 15 min long. Concentrations of fluorescein in samples were determined by a fluorescence microplate reader (Fluostar Optima, BMG Labtechnologies, Germany; excitation wavelength: 485 nm, emission wavelength: 535 nm). The apparent permeability coefficients (P_{app}) for DMTS and fluorescein and transfer and recovery (mass balance) of DMTS were calculated as described in previous papers (Kürti et al., 2012; Kiss et al., 2014; Hellinger et al., 2012).

Immunohistochemistry

Morphological changes in brain endothelial monolayers were followed by immunostaining for junctional proteins claudin-5, zonula occludens protein-1 (ZO-1), and β-catenin. After the transport studies, inserts were washed with PBS and the cells were fixed with 3 % paraformaldehyde solution for 30 min at room temperature and incubated in 0.2 % TX-100 solution for permeabilization. Non-specific binding sites were blocked with 3 % bovine serum albumin in PBS. Cells were incubated with primary antibodies rabbit anti-ZO-1, rabbit anti-β-catenin and mouse anti-claudin-5 (Life Technologies, Carlsbad, CA, USA) overnight. Incubation with Alexa Fluor-488-labeled anti-mouse and anti-rabbit secondary antibodies (Life Technologies, Invitrogen, USA) lasted for 1 hour. Nuclei of the living cells were stained with ethidium homodimer-1 and Hoechst dye 33342 for 5 min before fixation. After mounting the

samples (Fluoromount-G; Southern Biotech, Birmingham, USA) staining was visualized by a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Animals

Animal studies were conducted using CD-1 male mice (18 – 28 g; Charles River Breeding Laboratories, Inc., Wilmington, MA). Animal procedures were conducted in accordance with the guidelines by *The Guide for the Care and Use of Laboratory Animals* (National Academic Press, 2010), accredited by AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care, International). The mice were fed with 4 % Rodent Chow (Teklad HSD, Inc., Madison, WI) and water *ad libitum,* and were housed at 21°C in light-controlled rooms (12-h light/dark, full-spectrum lighting cycle with no twilight). At the termination of the experiments animals were euthanized in accordance with the *AVMA Guidelines for the Euthanasia of Animals*: 2013 Edition (AVMA Guidelines). The Institutional Animal Care and Use Committee (IACUC) permission number is 15-09-14-1015-3-01.

Pharmacokinetics of DMTS in Mouse Blood and Brain

For the absorption kinetics experiment, the DMTS concentration in blood and brain was measured from samples taken 0, 5, 10, 20, 60 and 120 min after intramuscular injection. DMTS stock solution at 50 mg/ml concentration was prepared by dissolving DMTS in 15 % (w/v) Poly80 solution (Petrikovics and Kovacs, 2016). A 200 mg/kg DMTS dose was applied by injecting the DMTS stock solution intramuscularly. The injection volume (approximately 90 - 110 μl) was halved and both rear legs were injected. For the control mouse, the 15 % (w/v) Poly80 solution without DMTS was injected. The mice were anesthetized by inhalation of isoflurane before the blood and the brain samples were taken. Right after anesthesia blood samples were collected from the heart into heparinized tubes. The rest of the blood was washed out from the circulation by performing cardiac perfusion with approximately 8 ml physiological saline containing 10 U/ml heparin under the deep terminal anesthesia. The brain was quickly removed from the skull and divided into two parts. The measurement of the DMTS concentrations in the blood and brain samples was initiated immediately following collection to minimize the effect of any post collection reactions of DMTS with the sample (Kiss et al. 2017). The volume of distribution and the tissue partitioning coefficient ($Kp_{brain:blood}$) for DMTS were calculated (see in Supplementary Information).

Preparation of DMTS Samples for HPLC Analysis

DMTS Samples Originated from the BBB-PAMPA Measurements

The DMTS concentrations from the Ringer-HEPES solutions in the BBB-PAMPA system were determined by HPLC-UV. A 60 µl aliquot of internal standard solution (0.05 mg/ml DMDS in acetonitrile) was transferred to 250 µl glass inserts within the HPLC glass vials (Agilent Technologies), and 40 µl of the BBB-PAMPA DMTS samples were added to each insert. The vials were hand-vortexed for 10 s, followed by auto-vortexing for 5 min at room temperature, and loaded into the HPLC instrument for measurement.

DMTS Samples Collected from the Cell Culture Measurements

A 300 µl aliquot of internal standard solution (0.05 mg/ml DMDS in acetonitrile) was mixed with 200 µl of the DMTS containing samples. The solutions were hand-vortexed for 10 s, auto-vortexed for 5 min at room temperature, and centrifuged for 5 min at 4°C with 14000 RCF. The supernatant (150µl) was transferred to 250 µl glass inserts in the HPLC glass vials, and loaded into HPLC instrument for measurement.

Blood Samples Containing DMTS Collected from Animals

The method developed for DMTS detection from blood was applied in this study (Kiss et al., 2017). Briefly, immediately after collection of the DMTS-exposed blood (80 µl), 200 µl of the internal standard (0.1 mg/ml DMDS) in ice cold acetonitrile was added. Then the micro-centrifuge tubes with the solutions were hand-vortexed for 10 s, auto-vortexed for 10 min at room temperature, and centrifuged for 5 min at 4°C, and 14000 RCF. The supernatants (80µl) were transferred to 250 µl glass inserts in the HPLC glass vials, and loaded into HPLC instrument for measurement.

DMTS Analysis by HPLC-UV

A Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA) HPLC-UV instrument was used in the analysis (Kiss et al., 2017). Forty microliters of DMTS containing samples were injected onto a guard column connected to a 250 × 4.60 mm non-polar C-8 analytical column having a Phenomenex Luna stationary phase (consisting of bonded octane units coated on silica support particles, pore size of 100Å, outer diameter of 5 µm). Isocratic elution was employed. A 35 : 65 v/v mixture of water and acetonitrile flowing at rate of 1 mL/min served as the mobile phase. The column backpressures ranged from 1430 to 1450 psi. The analyte absorbance at 215 nm was monitored by a UV detector. The

calibration curve used for BBB-PAMPA and cell culture studies is shown on Figure 1. This method was also used for experiments with blood, where the limit of detection (LOD) and limit of quantification (LOQ) were 1.46 and 4.45 µg/ml, respectively. Furthermore, the intra- and inter-day precisions varied from 6.9 to 12.2 CV% (relative standard deviation), while the intra- and inter-day accuracies varied from -3.7 to -14.8 %. (Kiss et al., 2017).

DMTS Analysis by GC-MS

GC-MS was used to measure DMTS concentration from the brain samples with a previously developed method (Kiss et al, 2017). To every 220-mg mouse brain tissue 1 ml ethanol was added. The brains were homogenized by a Precellys 24 tissue homogenizor (Bertin Technologies, Montigny-le-Bretonneux (France), Precellys vials with 1.4 mm ceramic beads, 6500 RPM, 3 times 1 min). The brain homogenate (475 µl) was added to 25 µl of 1 mg/ml DBDS (in ethanol) solution. A magnet bar was placed into the vials and the sample was stirred for 5 min. PDMS fiber (Agilent Technologies) was inserted into the headspace of the vials and were incubated for 10 min. To detect the molecules from the fiber GC-MS was used. For sample analysis an Agilent Model 6890A gas chromatograph and Agilent Model 5973C mass selective detector was used. The GC column was an Agilent DB-5MS (30 m × 0.25 mm with 0.1 µm film). The chromatographic method parameters were: 40ºC for 1 min, 60ºC/min to 280ºC, and 280ºC for 3 min with a He flow rate of 1 ml/min. The temperature of the inlet was 250ºC. The source temperature for MS was 230ºC, while the quadrupole temperature was 150ºC. Both scan mode and single ion monitoring (SIM) were applied for detection, in the scan range between 30 and 200 m/z. The following ions were selected for the SIM detection with 5ms dwell time for DMTS: 44.9, 45.0, 63.9, 64.0, 78.9, 79.0, 110.8, 111.0, 125.9, 126.0 m/z. The ions used for DBDS quantification included: 178 and 178.1 m/z. For data processing Agilent ChemStation version E.02.02.1431 software was used. Concentrations were determined by the calibration curve published earlier, and the LOD and LOQ were determined to be 213 and 645 ng DMTS/g brain, respectively (Kiss et al., 2017). The intra- and inter-day precisions were below 24.3 CV %, while the accuracy was between -1.3 and +2.4 %.

UV Spectrophotometric Method for Verapamil Analysis

Samples containing verapamil were collected from the BBB-PAMPA measurements. To the micro-centrifuge tubes filled with 300 µl of 200 mg/ml DMSO in water, 140 µl of the collected samples were added. After vortexing 400 µl of the mixture was transferred to a narrow quartz cuvette (VWR). Absorbances were measured in the range of 210 - 400 nm by a Shimadzu UV2101 (Shimadzu Corp., Kyoto, Japan) spectrophotometer. The absorbances measured at 278 nm were used for the calculation.

Statistical Analysis

All plotted values represent the means ± standard deviations (SD). Unpooled Student t-tests were used to assess the significance of changes in the permeabilities of verapamil in the presence and absence of Poly80. To evaluate the toxic effect of DMTS on endothelial cells one-way ANOVA followed by Dunnett's test (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA) was used. The effect of the variable under study was considered statistically significant, if the random probability (p) of the observed change in signal associated with a specific treatment was less than 0.05. The number of replicate samples varied from 3 to 10.

RESULTS

Permeability of DMTS in the BBB-PAMPA System

The BBB-PAMPA system is a widely used surrogate model of BBB permeability, therefore the passive penetration of DMTS was tested on it (Di et al., 2003 and 2009; Mensch et al., 2010; Müller et al., 2015). The clearance volume of DMTS was determined at 30, 60, and 120 mins after injection and the permeability was calculated from the data shown in Figure 2A. In the BBB-PAMPA system the P_{app} of DMTS was 11.8×10^{-6} cm/s. A lag time of 6.41 mins in DMTS appearance in the acceptor compartment was measured. Verapamil, used as control, showed a P_{app} of 25.7×10^{-6} cm/s in the absence of Poly80. In the presence of 0.05 % (w/v) Poly80, the permeability of verapamil decreased by 16 %, to 21.5×10^{-6} cm/s.

Effect of DMTS on Viability of Cultured Brain Endothelial Cells

The viability of brain endothelial cells in the presence of DMTS at different concentrations was tested by the MTT dye conversion assay (Figure 3). A 10-min treatment with DMTS in a concentration range of 1–300 µg/ml did not significantly decrease the viability compared to the non-treated control group. However, at the concentrations of 30, 100 and 300 µg/ml DMTS enhanced the metabolic activity of the cells. Triton X-100 was the positive control for cell death and resulted in complete toxicity within 10 min.

Permeability of DMTS Through the Triple Co-Culture Model of BBB

A very high permeability was measured for DMTS in the BBB culture model ($158 \pm 14 \times 10^{-6}$ cm/s) indicating a complete distribution between the two compartments (Table 2). The permeability of DMTS in the cell free inserts ($159 \pm 7 \times 10^{-6}$ cm/s) was similar to those measured with cell culture. The 70 % recovery of the highly lipophilic DMTS is in good accordance with the recovery of more than 100 tested

compounds on BBB culture models in our previous study (Hellinger et al., 2012). The passive paracellular permeability of hydrophilic fluorescein through the cellular barrier remained low (P_{app}: 4.1 ± 0.9 × 10^{-6} cm/s) following a 10-min DMTS treatment, indicating that the barrier integrity was not compromised by DMTS treatment. The DMTS permeability was ~40 times higher than that of fluorescein.

Effect of DMTS on Cell Junctions

Following the transport experiments, the cells on the inserts were stained for junctional proteins. ZO-1 and β-catenin stainings show that the monolayer integrity is not disturbed in agreement with the fluorescein permeability values (Figure 4). The cellular distribution of claudin-5 was slightly changed, with more cytoplasmic staining. The cell nuclei were stained for both ethidium homodimer-1 (red) and Hoechst dye 33342 (blue). No red labeling of nuclei was observed, denoting intact cell membranes after the DMTS treatment. The untreated cells showed similar morphology following each immunostaining treatment.

Pharmacokinetics of DMTS

The pharmacokinetics of DMTS was investigated *in vivo* in blood and brain (Figure 5A and B). The concentrations of DMTS were determined at time intervals of 0, 5, 10, 20, 60, 120 min after the intramuscular injection of 200 mg/kg dose of DMTS using previously developed methodology (Kiss et al., 2017). A comparison of the first three time points in Figures 5A and 5B, shows that DMTS uptake into the blood is largely complete by 5 min, while uptake into the brain is still climbing strongly between the 5 and 10 min sampling points. For both samples, uptake is rapid, and c_{max} is achieved in less than 10 min in blood, and very close to 10 min in the brain. The volume of distribution (V_D) of DMTS was calculated after 10 min treatment, where the c_{max} was measured in blood, and resulted 0.21 ± 0.03 L. The tissue partitioning coefficient for brain ($Kp_{brain:blood}$) was 0.0179 (1.79 %).

DISCUSSION

The present studies focus on the BBB permeability and pharmacokinetics of the promising CN antidote candidate DMTS. To the best of our knowledge this is the first publication investigating the absorption of DMTS into blood, the *in vitro* BBB permeability and the *in vivo* distribution in the brain.

The penetrability of DMTS through the BBB was measured in both a BBB-PAMPA system and in a cell culture model of BBB. Both these systems are widely used by pharmaceutical companies to screen the permeability profile of drug candidates (Mensch et al., 2010; Di et al., 2009; Hellinger et al., 2012;

Helms et al., 2016). Compound lipophilicity is an important physicochemical property which has an impact on the penetration to the central nervous system, the partitioning into other organs, the membrane permeability and the plasma protein binding *etc*. (Lipinski et al., 2001). The octanol/water partitioning coefficient (log*P*) describes the lipophilicity. The log*P* for DMTS is 2.93 (http://www.chemspider.com/Chemical-Structure.18219.html) and for verapamil 3.79 (http://www.drugbank.ca/drugs/DB00661). Lipophilicity is a determining factor for the penetration ability of DMTS and verapamil in the BBB-PAMPA system. Verapamil was used as a high permeability standard in BBB-PAMPA experiments. Our permeability result with verapamil (25.7×10^{-6} cm/s) as a control in the BBB-PAMPA is comparable with the literature data (16.0×10^{-6} cm/s; 18.5×10^{-6} cm/s; 23.7×10^{-6} cm/s; Di et al., 2003; Chlebek et al., 2016; Mensch et al., 2010). Although verapamil has higher permeability (P) and log*P*, these values of DMTS are large enough to categorize it as a molecule with high permeability in the BBB-PAMPA system.

The presence of Poly80 was required to achieve sufficiently high concentrations of DMTS. Due to its surfactant properties Poly80 may indirectly influence the lipid membrane permeability in BBB-PAMPA. To check its effect on the lipid membranes, a verapamil control was applied in the system with and without Poly80. One would expect a permeability enhancement effect for verapamil in the presence of Poly80 due to its absorption enhancer properties (Kiss et al., 2014; Rege et al., 2002). However, a slight decrease in the permeability of verapamil through the BBB-PAMPA model was observed in the presence of 0.05 % (w/v) Poly80 (Figure 2B). This decrease in permeability may be due to the association between the Poly80 and the BBB-PAMPA membrane. This effect is not expected to be significant *in vivo*, because (1) Poly80 will be diluted upon entering the circulation and will have many competing opportunities to partition out of blood into tissues before reaching the BBB; (2) earlier studies demonstrated that Poly80 at 0.1 % (w/v) (1 mg/ml) did not cause any cell damage after 1 hour in Caco-2 and RPMI 2650 cell culture (Kürti et al., 2012; Kiss et al., 2014).

The triple co-culture model for BBB is suitable for *in vitro* permeability measurements and the drug permeability is generally comparable with *in vivo* data (Nakagawa et al., 2009). In our experiments, this culture model was applied to estimate the permeability of DMTS through the BBB. Results showed that the endothelial cell layers did not present a barrier against DMTS as indicated by similar permeability values in culture models and cell-free inserts. The P_{app} of DMTS was similar to that of the highly lipophilic and BBB penetrating drug caffeine on the same model (Hellinger et al., 2012). The recovery (mass balance) of DMTS was 67.7 % and 69.4 % in culture and in cell free permeability assays, respectively. These results indicate 32.3 % and 30.6 % loss of DMTS in these systems. Our unpublished

preliminary data show that DMTS attach with high affinity to the surface of glass and plastic. Therefore, we assume that the loss of DMTS was mainly caused because of the binding of DMTS to culture insert membranes or to the walls of plastic plates. Cellular metabolism can also eliminate DMTS as it was mentioned in our previous paper (Kiss et al., 2017), where the DMTS amount decreased by time in sheep blood. However, due to the little difference between the recovery data in the presence of cells and in cell free assays we assume that cellular metabolism can be negligible as compared to the binding of DMTS to surfaces and this phenomenon may cause the DMTS loss in the assay systems. Based on the high TEER values and the low permeability of fluorescein the cellular barrier remained intact ten minutes after the treatment. Furthermore, proteins building up the tight junctions, ZO-1 and claudin-5, and adherent junctions, β-catenin, showed a typical morphology with strong staining between the neighboring endothelial cells after the DMTS treatment (Figure 4).

Double-nuclei staining is an indicator of membrane damage and cell death (Kiss et al., 2013) which was performed after the DMTS permeability measurement. The endothelial cells were alive with intact membranes and no staining of ethidium homodimer-1 was observed. Furthermore, DMTS did not decrease the metabolic activity of the brain endothelial cells at tested concentrations, but even slightly enhanced this activity at higher concentrations as shown by the MTT assay. These cell culture data indicate that DMTS can be safely used at 0.1 mg/ml concentration within 10 minutes, while the effect of longer exposure of DMTS should be tested in the future.

The permeability of DMTS measured in the BBB culture model (158×10^{-6} cm/s) was ~13 times higher than in the BBB-PAMPA system (12×10^{-6} cm/s). This difference between the permeability values measured in PAMPA and cell culture models can also be observed for other drugs. For example, caffeine and alprazolam have permeabilities of 2.03×10^{-6} and 11.12×10^{-6} cm/s through the BBB-PAMPA, respectively, and 44.40×10^{-6} and 103.63×10^{-6} cm/s through the Caco-2 cellular barrier membranes (Mensch et al., 2010). Caffeine ($\log P_{caffeine} = -0.07$; https://pubchem.ncbi.nlm.nih.gov/compound/2519#section=LogP) can pass through the cellular barrier with passive and facilitated diffusion (McCall et al., 1982), while alprazolam ($\log P_{alprazolam} = 2.12$; https://pubchem.ncbi.nlm.nih.gov/compound/alprazolam#section=LogP) penetrates by passive diffusion and active transport using human organic cation transporter 2 (Chiba et al., 2013). The significant difference in permeability of DMTS in the BBB-PAMPA – containing only lipids – and the BBB model, which expresses transporters (Walter et al., 2015) suggests that in addition to passive diffusion DMTS may cross the BBB by an other, facilitated or active transport mechanism.

DMTS uptake into the blood and soon thereafter into the brain occurred fast enough to reach the c_{max} within 10 min following intramuscular injection. The toxic effects of CN can appear from a few minutes to an hour after exposure depending on the concentration. The rapid penetration of a CN antidote is essential to efficiently defend the body in cases of acute intoxication. In the previous paragraph we discussed a possible active transport mechanism of DMTS and the quick and significant penetration into the brain observed *in vivo,* which together do not contradict the *in vitro* experimental results. The concentration of DMTS reached the maximum of 25.2 µg/ml in the blood and 1.07 µg DMTS/g in the brain ten minutes after the injection. To prevent the biasing effects of blood in the brain experiments, the cerebral blood was flushed out of the brain by cerebral perfusion prior to extracting the brain for analysis. This ensured that the DMTS determination from the brain was not affected by the DMTS in the blood and only the DMTS that entered the brain was analyzed. The disappearance of blood from brain tissue during perfusion can easily be observed. The volume of distribution for DMTS showed higher value than the mouse total body water (approximately 14.5 ml; Davies and Morris, 1993). This high V_D is typical for lipophilic drugs, and may indicate a greater amount of tissue distribution.

Detailed studies of the mechanisms of DMTS penetration through BBB were beyond the scope of the present work. DMTS may also undergo chemical transformations in the brain, as it appears to happen in the blood (Kiss et al., 2017). Ongoing efforts in our lab are focused on the investigation of these interactions between the DMTS and the blood or the brain and will be published separately.

Our results with DMTS showed better pharmacokinetic parameters than commercially available CN antidotes, the thiosulfate or the hydroxocobalamin. Thiosulfate, the sulfur donor component of Nithiodote®, has limited ability to cross the BBB (Pollay and Kaplan, 1971; Neuwelt et al., 1998) and has a shorter half-life (15 - 20 mins; Schulz, 1984; https://www.drugs.com/pro/sodium-thiosulfate-injection.html) in the blood than DMTS. The half-life of DMTS in blood is 36 mins (De Silva et al., 2016) and it can effectively cross the BBB as the results of this paper proved. Van den Berg et al. (2003) measured the concentration of hydroxocobalamin (component of Cyanokit®) in the cerebrospinal fluid (CSF) and plasma after intranasal or intravenous administration. The partitioning coefficient ($Kp_{CSF:plasma}$) of hydroxocobalamin was between 0.5 - 0.6 %. However, in our paper the DMTS was measured from whole brain and blood instead of CSF and plasma, but if we compare the two Kp-s DMTS ($Kp_{brain:blood}$ = 1.79 %) has 10 times higher partitioning to brain than hydroxocobalamin to CSF. Furthermore, hydroxocobalamin hardly appeared in CSF 30 min after the administration, while DMTS reached its highest concentration in brain within 10 minutes.

CONCLUSION

The permeability of DMTS through the BBB was measured in a BBB-PAMPA system and in a cell culture BBB model. The high penetrability of DMTS through the BBB-PAMPA indicates that the rate of passive diffusion is significant. The thirteen times greater penetrability of DMTS through the BBB cell culture model suggests that the possibility of an active transport mechanism for DMTS in the BBB of living systems can not be excluded. The results of the pharmacokinetics investigations in a mouse model (blood and brain) were consistent with those of the *in vitro* experiments. The DMTS reached a peak brain concentration in alive mice within 10 min following the intramuscular injection. These permeability and pharmacokinetic results highlight the promise of DMTS as a brain protecting antidote. Together with the prior observations of high *in vivo* antidotal efficacy against CN, these results strengthen the potential of DMTS for clinical studies.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest associated with this study.

DISCLOSURE

Patent, related to DMTS: Ilona Petrikovics (SHSU), Steven Baskin (ICD), and Gary Rockwood (ICD). Dimethyltrisulfide as a cyanide antidote (2015 October), Patent No.#: US20150290143 A1.

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Tables

TABLE 1. BBB-PAMPA experimental setup of the DMTS and verapamil treatments, buffer solutions and sample collection times. The donor compartments and the corresponding acceptor compartments are placed in the same row.

Donor compartment		**Acceptor compartment**	
Sample	**Sample collection times (min)**	**Sample**	**Sample collection times (min)**
0.1 mg/ml DMTS in 0.05 % (w/v) Poly80 and Prisma HT buffer	0; 90	Brain Sink Buffer 0.05 % (w/v) Poly80	30; 60; 90
200 µM verapamil in DMSO + 0.05 % (w/v) Poly80 and Prisma HT buffer	0; 90	Brain Sink Buffer 0.05 % (w/v) Poly80	90
200 µM verapamil in DMSO and Prisma HT buffer	0; 90	Brain Sink Buffer	90

TABLE 2. Apparent permeability (P_{app}), transfer and recovery of DMTS in the *in vitro* BBB model and in cell-free inserts. Values are presented as mean ± SD, n = 4. The apparent permeability calculation is described in the supplementary materials.

Samples	**P_{app} (10^{-6} cm/s)**	
	BBB model	***Cell-free insert***
DMTS permeability	158 ± 14	159 ± 7
Transfer of DMTS (%)[a]	21.2 ± 1.9	21.4 ± 1.1
Recovery of DMTS (%)[b]	67.7 ± 2.4	69.4 ± 1.3

[a] *Transfer of DMTS (%) = (µg of measured DMTS in the acceptor compartment after the experiment / µg of DMTS in the donor compartment at the beginning of the experiment) x 100*

[b] *Recovery of DMTS (%) = [(µg of measured DMTS in the acceptor compartment after the experiment + µg of measured DMTS in the donor compartment after the experiment) / µg of DMTS in the donor compartment at the beginning of the experiment] x 100*

Legends

FIG. 1. Calibration curve for BBB-PAMPA and cell culture studies. Data are presented as mean ± SD, n = 3. Error bars are not visible due to the low SDs.

FIG. 2. Clearance volume and apparent permeability for DMTS and verapamil in the BBB-PAMPA system. A) The clearance volume of DMTS was plotted against time. Data are presented as mean ± SD, n = 10. B) Apparent permeability of verapamil combined with Poly80, and verapamil alone. Data are presented as mean ± SD, n = 5. Significance analysis was performed using unpaired Student t-test: statistically significant difference was detected between verapamil + Poly80 and verapamil permeability (* $p < 0.05$).

FIG. 3. Cell viability measured by MTT dye conversion assay after a 10-min treatment with DMTS solutions in 0.05 % (w/v) Poly80. The viability is given as the percentage of the control group. Values are presented as means ± SD, n = 6 - 8. Statistical analysis: ANOVA followed by Dunnett's test, $p < 0.05$ as compared with the control groups. Control, non-treated cells; TX, Triton X-100.

FIG. 4. Effects of DMTS (10-min treatment) on junctional morphology of the brain endothelial cells. Immunostaining for zonula occludens-1 (ZO-1), claudin-5 and β-catenin junction proteins are shown in green color. Blue color: staining of cell nuclei. Bar = 25 µm.

FIG. 5. Concentration-time profile of DMTS in (a) blood and (b) brain. Values are presented as means ± SD, n = 3 - 4.