

Induction of glutathione synthesis explains pharmacodynamics of high-dose busulfan in mice and highlights putative mechanisms of drug interaction.

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List of abbreviations:

AUC, area under concentration time curve; BMT, bone marrow transplantation; Cl/F, clearance uncorrected for bioavailability; DMEM, Dulbecco's minimal essential medium; CS, γ -glutamyl-cysteine synthetase; F, bioavailability; GC, gas chromatography; GGT, γ -glutamyltranspeptidase; GLU-CYS, γ -glutamyl-cysteine; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; k_e , terminal phase elimination rate constant; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PEG-400, polyethylene glycol 400; THT, tetrahydrothiophene; THT⁺, sulfonium ion of glutathione; $T_{1/2}$, half-life.

Abstract

Busulfan is an example of a drug eliminated through glutathione S-transferase (GST)-catalyzed conjugation with glutathione (GSH). We studied the pharmacokinetics and toxicity of busulfan in C57Bl6 mice in correlation with liver GST activity and GSH synthesis by accurate determination of precursors, namely γ -glutamyl-cysteine and cysteine. A significant lower incidence of acute toxicity was observed in mice receiving busulfan 16.5 mg/kg twice a day as compared to animals receiving 33 mg/kg once a day. In both cases, a total dose of 132 mg/kg was administered over 4 days. The difference of toxicity was explained by pharmacokinetics since a strong induction of clearance was observed only in animals treated twice daily. Induction of metabolism was correlated with an increase in liver cysteine content and enhanced glutathione synthesis rate, while GST activity was unchanged. To our knowledge, this is the first time that in vivo flux of GSH synthesis is closely related to a drug plasma clearance and toxicity. These results allow hypothesizing that GSH liver synthesis may directly influence busulfan clearance in humans with possible implications in the occurrence of hepatic veno-occlusive disease.

Introduction

In mammals, reduced glutathione (GSH) is the major low-molecular-mass thiol compound involved in cellular redox reactions and thio-ether formation (Griffith, 1999). The redox reactions are catalyzed by GSH-peroxidase and GSH-reductases. Various electrophile compounds are metabolized through conjugation with GSH and thio-ether formation by glutathione S-transferases (GST). GSH is mainly used by cells for endogenous metabolism, with a complete intracellular turnover in a few hours. During treatment or intoxication with a xenobiotic which is eliminated through GSH/GSTs metabolism, sustained GSH level is a determinant for elimination kinetics and for protection against severe cell injury due to endogenous stress (DeLeve and Wang, 2000). GSH is a tripeptide synthesized from L-glutamate, L-cysteine and glycine in two steps, catalyzed by γ -glutamyl-cysteine synthetase (GCS) and glutathione synthetase (GS). Synthesis of γ -glutamyl-cysteine through GCS activity is presented as the rate limiting step of GSH synthesis (Richman and Meister, 1975). There is clear evidence that toxicity of chemicals interacting with GSH may be due either to GSH depletion (DeLeve, 1996) or to activation of different metabolic pathways enabling GSH synthesis, such as the *trans*sulfuration pathway with homocysteine accumulation (Adinolfi et al., 2005). Therefore, the simultaneous quantification of GSH and its precursors (Fig.1) can be useful to explain more precisely the role of GSH during intoxication. It is essential to define how the organism sustains glutathione synthesis during exposure to the electrophile chemical and to define how this metabolic adaptation is relevant to avoid the toxic injury. A liquid chromatography/tandem mass spectrometry assay of glutathione (GSH), glutathione disulfide (GSSG) and precursors (γ -glutamyl-cysteine, cysteinyl-glycine, cysteine, cystine, homocysteine and homocystine) was developed and validated to study glutathione synthesis in mouse liver (Bouligand et al., 2006).

Busulfan is an alkylating agent widely used in combination high-dose chemotherapy regimens followed by allogeneic (Santos et al., 1983) or autologous (Hartmann et al., 1986) bone marrow transplantation (BMT). This is a unique example of an electrophile compound administered as a drug at high-dose in humans. Busulfan is eliminated through GSH conjugation by glutathione S-transferases into the sulfonium ion of glutathione (γ -glutamyl- β -(S-tetrahydrothiophenium ion) alanyl-glycine or THT⁺)(Hassan and Ehrsson, 1987). During busulfan treatments, hepatic veno-occlusive disease (HVOD) is the most severe and frequent toxicity (McDonald et al., 1993). HVOD is defined as a concentric non-thrombotic occlusion of terminal and sublobular intra-hepatic veins by connective tissue (DeLeve et al., 2002).

Several *in vitro* laboratory experiments have shown that liver glutathione is determinant for HVOD since chemicals linked to HVOD are metabolized through the GSH/GSTs pathway (DeLeve, 1996). On the other hand, *in vivo* mechanisms of busulfan induced HVOD are still unclear. It is noteworthy that HVOD was not observed when high-dose busulfan was given as single agent in humans, *i.e.* without combination with another alkylating agent (Peters et al., 1987). Busulfan is a dose-dependent risk factor for HVOD since increased incidence was observed after augmentation of busulfan dosing in children (Meresse et al., 1992). Several clinical pharmacology studies suggested that HVOD was the consequence of a drug interaction between busulfan and the second alkylating agent of the combined regimen, *i.e.* cyclophosphamide (Hassan et al., 2000) or melphalan (Bouligand et al., 2003). We hypothesized that this drug interaction was due to the metabolism of busulfan through the GSTs/GSH pathway. Busulfan first administered may “deplete” the hepatic GSH level and thus may sensitize the liver to the toxicity of the second alkylating agent administered (Bouligand et al., 2003).

The aim of this work was thus to study the regulation of GSH synthesis *in vivo* in mice during high-dose busulfan. This information was correlated to the clearance and toxicity in order to finally highlight putative mechanisms for metabolic drug-interaction with busulfan, through the GSH/GSTs pathway.

Materials and Methods

Chemicals

Busulfan (1,4-butanediol-dimethylsulfonate) was purchased from Fluka (Steinheim, Germany) and busulfan-d4 was synthesized from [2,2,3,3-²H]-1,4-butanediol as previously reported (Vassal et al., 1988). Cyclophosphamide (Endoxan[®]) was purchased from Baxter Oncology (Maurepas, France). Tetrahydrothiophene, 2-ethylethiophene, tetraethoxypropane, sodium iodide and 4-nitrobenzylpyridine were provided by Aldrich (Steinheim, Germany), thiobarbituric acid by Sigma (Saint-Louis, USA). Polyethylene glycol 400 (PEG-400), dimethylsulfoxide (Sigma, Saint-Louis, USA) and *N,N*-dimethylacetamide (Merck, Hohenbrunn, Germany) were analytical grade. Dextrose (5%) was purchased from Macopharma (France). High-purity reduced glutathione (L- γ -glutamyl-L-cysteinyl-glycine), oxidized glutathione, γ -glutamyl-cysteine trifluoroacetate salt, cysteine hydrochloride salt, cystine, homocysteine, homocystine, cysteinyl-glycine and glutathione ethyl ester were supplied by Sigma (St Quentin Fallavier, France). HPLC grade acetonitrile, methylene chloride, heptane and hexane were provided by Carlo Erba (Rodano, Italy), formic acid by Merck (Darmstadt, Germany), potassium chloride by Prolabo (Paris, France), ethylene diamine tetracetic acid disodium salt dihydrate by Labosi (Elancourt, France). Iodoacetic acid, ammonium bicarbonate and *batho*phenanthrolinedisulfonic acid disodium salt were purchased from Aldrich (Steinheim, Germany), bovine albumin from Euromedex (Mundosheim, France). Sodium hydroxide was provided from Prolabo (Paris, France). Sterile water was provided from Fresenius (Sèvres, France) and deionized water was prepared using a Milli-QTM system (Millipore, Saint Quentin-en-Yvelines, France). DMEM was purchased from Invitrogen (Paisley, Scotland, UK).

Animal experiments

Animal experiments were carried out in compliance with the conditions established by the European Union (Directives n°86/609/CEE and n°2003/65/CE). Male mice C57BL/6 inbred strains, 2 months old with an average weight of 25 g, were used for these experiments. Since fasting may lead to a significant decrease of hepatic glutathione contents, mice were not fasted before and during experiments.

Busulfan treatment and syngeneic bone marrow transplantation

Mice were treated with intraperitoneally injected busulfan during four days at a total dose of 132 mg/kg. Intraperitoneal route was chosen based on previous experiments which have shown a bioavailability close to 100% for crystals-free busulfan solutions (Boland et al., 1999). This route of administration was preferred to intravenous route since repeated reliable injections were required. The dose was chosen according to the literature and preliminary experiments with evaluation of single injection dose ranging from 16.5 mg/kg up to 40 mg/kg and total dose ranging from 107 mg/kg up to 133 mg/kg. Two schedules were compared: injection of busulfan twice daily and once daily. Busulfan was dissolved in *N,N*-dimethylacetamide to obtain a 60 mg/mL solution. The solution was then diluted in PEG-400 to prepare a stable 6.4 mg/mL solution. This anhydrous solution of busulfan is then diluted extemporaneously in dextrose 5% prior to each injection, to a final concentration between 1.6 and 3.2 mg/mL. Mice were transplanted with $5 \cdot 10^6$ nucleated cells of fresh syngeneic bone marrow 24 hours (twice daily schedule) after the last injection of busulfan. Syngeneic bone-marrow from sacrificed inbred animals was obtained by flushing femurs and tibias with DMEM. Survival was recorded during one year after BMT.

Busulfan pharmacokinetics

Busulfan assay

Plasma busulfan concentrations were determined using a gas chromatography and mass spectrometry (GC-MS) assay with deuterated internal standard (busulfan-d4), as previously reported (Vassal et al., 1988). The GC-MS system consisted of a GC HP6890 II and MSD 5973 detector (Agilent Technologies, Massy, France). Accuracy and stability of busulfan concentration in injectable solutions were checked using a HPTLC assay (CAMAG®, Chromacim, Lyon, Fr) with derivatization by 4-nitrobenzylpyridine, as previously reported (Bouligand et al., 2004).

Pharmacokinetics

The treatment was started at 8:00 a.m. For the pharmacokinetics determination, mice were sacrificed along the treatment at predetermined time, *i.e.* before drug injection and 1h, 4h and 8h after drug injection. Four mice were sacrificed by sampling time point. A blood sample (~1 mL) was immediately collected by cardiac puncture in a heparinized vial and plasma was separated by centrifugation (10 min at 2500 g). Liver and other organs were collected and frozen immediately in liquid nitrogen and stored at -80°C until analysis. The descending segment of concentration-time curve was mono-exponential as previously reported (Boland et

al., 1999). Elimination half-lives ($T_{1/2}$) were determined from a log-linear curve with the three time points, 1, 4 and 8 hours. The areas under the curve (AUC_{0-8h}) were calculated using the linear trapezoidal rule from 0 to 8h, and extrapolated to infinity according to the elimination half-life ($AUC_{0-\infty} = AUC_{0-8h} + C_{8h}/k_e$, with $k_e = \ln 2/T_{1/2}$). The plasma clearance uncorrected to bioavailability (Cl/F) was calculated by dividing the dose by the $AUC_{0-\infty}$.

In vitro metabolism – conjugation of busulfan to glutathione

Preparation of cytosol

Mouse liver cytosol was prepared according to the previously published method (Abernathy et al., 1971). All steps were performed at +4°C. Frozen liver (150 mg) was thawed, grossly minced, washed and suspended in about 1.5 mL of buffer (Sucrose 0.25M, EDTA 1mM, Tris 1mM, pH 7.5). The suspension was homogenized using a potter at 750 to 3000 rpm, during 1 min. Crude homogenate was centrifuged at 10 000 g for 10 min. The supernatant was centrifuged at 100 000 g during 1h. The cytosol (100 000 g supernatant) used in the experiment was stored at -80°C until analysis. The protein content was determined using the micro-BCATM protein reagent assay (Pierce, Rockford, IL, USA).

Conjugation metabolism

These experiments were performed according to the previously published methods (Gibbs et al., 1997; Ritter et al., 1999). Busulfan solubility in water (~1.2 mM) is far below the K_m value. The V_{max} and K_m therefore cannot be assessed separately. Intrinsic clearance (V_{max}/K_m) was calculated according to the method of Gibbs. Briefly, incubation mixtures contained 0.25 mg cytosolic protein and 0.7 μ mol reduced glutathione in 1.0 mL of assay buffer (100 mM sodium phosphate, pH 7.4 and 1 mM EDTA). Preincubation was carried out for 5 min at 37°C followed by addition of busulfan solution (100 mM) in dimethylsulfoxide and incubation for 20 min at 37°C. Two different final concentrations of busulfan (250 μ M and 500 μ M) were prepared. The formation of the conjugate was linear for protein content from 0.2 to 1 mg/mL. The enzymatic reaction was stopped by adding 5 mL of methylene chloride. After vortexing for 20 s, the mixture was centrifuged for 15 min at 2500 g. For alkaline hydrolysis and THT extraction, 200 μ L of hexane, 100 μ L of NaOH (1M), 40 μ L of 2-ethylthiophene (5 μ g/mL in isopropanol) were added to 700 μ L of supernatant. After vortexing and centrifugation for 10 min at 10 000 g, 100 μ L of hexane phase was removed and a 2 μ L aliquot was analyzed by GC-MS.

Tetrahydrothiophene assay

Sulfonium ion of glutathione was determined after alkaline conversion into tetrahydrothiophene using a GC-MS assay with a slight modification of the method (Ritter et al., 1999). Briefly, the GC-MS system consisted of a GC HP5890 II and MSD 5971A (Agilent Technologies, Massy, France). GC was performed in the splitless mode on a 30 m x 0.25 mm HP-5 capillary column (Agilent Technologies, Massy, France). Helium (99.99%) was used as carrier gas using the constant flow mode with an inlet pressure of 75 kPa. An aliquot (2 μ L) of hexane was injected at 280°C in splitless mode. The initial oven temperature was set at 45°C and held for 1.5 min, increased by 20°C/min to 165°C and maintained for 0.5 min. In these conditions, retention times were 3.5 and 4.1 min for THT and 2-ethylthiophene respectively. MS detection was performed in the electron impact mode with the following conditions of temperature and energy: transfer-line at 280°C, detector at 170°C and an electron-energy of 70 eV. THT calibrations were performed from 5 to 500 ng/mL using 2-ethylthiophene as an internal standard at a 270 ng/mL concentration. Initial THT stock solutions were prepared in isopropanol and diluted into cytosol or plasma.

In vivo synthesis of glutathione in mice liver

Assay of glutathione and precursors

Reduced and oxidized glutathione and precursors (γ -glutamyl-cysteine, cysteinyl-glycine, cysteine, cystine, homocysteine and homocystine) were quantified in mice liver using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay after iodoacetic acid derivatization, as previously reported (Bouligand et al., 2006). The LC-MS/MS system consisted of a 1100 series HPLC system (Agilent Technologies, Massy, France) and a Quattro-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Micromass, Manchester, UK).

Kinetics of glutathione and precursors

The area under the curve from 0 to 8h (AUC_{0-8h}) was calculated using linear trapezoidal rule. The mean concentration was then calculated.

Various assays

γ -glutamyltranspeptidase was analyzed in plasma sample or in liver homogenate using the Synchron LX[®] assay reagent (Bekman-CoulterTM). Thiobarbituric acid reactive species

(TBARS) were determined in order to appreciate lipoperoxidation according to the previously published method (Wallin et al., 1993).

Western blot

Frozen liver (50 mg) was thawed at +4°C, grossly minced, suspended in 100 µL of lysis buffer (containing 0.15 M NaCl, 1 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EDTA pH 6.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM benzamide, 1 µg/mL aprotinine, 10 µg/mL soybean trypsin inhibitor) and homogenized with an homogenizer (PowerGen125). A volume of 200 µL of NaCl (0.55 M) was added and incubated for 1 hour on ice for whole cell extract. After centrifugation at 13 000 g for 30 min, the protein content was determined by the micro-BCATM method (Pierce). Proteins (30 µg) were separated electrophoretically in 7.5% to 15% SDS-polyacrylamide gels and then transferred to a PVDF membrane (Hybond P Amersham Life Science, Les Ulis, France). Blots were incubated with rabbit polyclonal antibodies anti C-terminus human Nrf2 at 1:1000 dilution (anti-Nrf2 (C-20), Santa Cruz Biotechnology) during 1 hour, and followed by the anti-rabbit Ig horseradish-peroxidase-linked whole antibody from donkey (Amersham Pharmacia Biotech) at 1:2000 dilution. Detection was performed using a chemiluminescence (ECL) enzyme immunoassay (Amersham Pharmacia Biotech).

Histology

Liver, lung, intestine and femur were fixed in FinefixTM (Milestone Medical) and embedded in paraffin. For morphology analysis, 4-µm-thick paraffin sections were stained with hematoxylin-eosin-safranin. Liver was frozen with liquid nitrogen, embedded in CryomatrixTM (Thermo). For neutral lipid revelation, 4-µm frozen sections were stained with Sudan dye, a specific staining for fatty acids.

Statistical analysis

Groups of values were always reported as mean ± SD. Proportions were compared using the χ^2 test and values were compared using the Mann-Whitney and Wilcoxon tests and, for more than 2 groups, the Kruskal-Wallis nonparametric and Dunn's multiple comparison tests.

Results

Toxicology

Survival

Survival of mice treated with busulfan at a total dose of 132 mg/kg given once or twice daily is shown in figure 2. There is a strong effect of administration schedule on mortality since all mice receiving busulfan once daily died before those receiving busulfan twice daily ($p < 0.0001$). Acute toxicity occurred during the four days of once daily busulfan treatment. More than 50% of animals were dead before the day 0. The median survival was 3.5 days after the first day of busulfan treatment. All the deaths occurred more than 10 hours after the 3rd busulfan injection and this toxicity did not biased the metabolic study. However, BMT was not practicable in this group.

On the other hand, the treatment given twice daily was better tolerated and the animals died between day 5 and day 30, with a median survival of 13 days after the start of busulfan treatment. Animals treated twice daily are thus still alive for BMT at day 0. The BMT procedure successfully rescued all animals treated according to the twice daily schedule. This demonstrated that acute death of animals treated twice daily without BMT was related to haematopoietic stem cell depletion.

Body weight

Body weight is a significant marker of toxicity. Animals treated with busulfan 33 mg/kg once daily during four days presented a severe body weight loss with a mean \pm SD of $16 \pm 5\%$ after two days of treatment, and continued to decrease thereafter. This effect was reduced in animals treated according to the 16.5 mg/kg twice daily schedule, with a body weight loss of $9 \pm 3\%$ after two days of treatment. The difference was statistically significant with $p < 0.01$. Moreover, in the twice daily group, the animals presented a progressive adaptation to the treatment with a recovery of the weight, up to $-4 \pm 1\%$ of baseline after four days of treatment.

Histology

Toxicity was studied by systematic histological analysis of femur, lung, intestine and liver. At onset of toxicity, animals treated twice daily presented a dramatic drop of the bone marrow cellularity. This confirmed that animals died of bone marrow failure. During this twice daily treatment, and after bone marrow transplantation, no significant morphologic changes were identified in lung, intestine and liver. On the other hand, animals treated once daily presented

an acute liver toxicity during busulfan treatment, while no significant morphologic changes were identified in lung and intestine. After two days of treatment, 50% of animals presented a characteristic liver histology features with a diffuse microvesicular steatosis. This liver toxicity was dose-dependent since the incidence of mice with steatosis increased with the dose: 50% at 33 mg/kg/day and 80% at 40 mg/kg/day. This toxicity was associated with lethargy and severe body weight loss.

Long-time follow-up post bone marrow transplantation

BMT were performed only on animals treated with busulfan administered twice daily. All transplanted animals survived more than one year after a syngeneic BMT. The experiment was censored after one year. No significant morphological changes were identified in liver, lung and intestine. However, all busulfan-treated mice presented a hair-greying and a significant growth-delay with an average body-weight of 28 ± 1 g (-28%) one year post-BMT in the busulfan treated group as compared to 39 ± 4 g in control group ($p < 0.05$).

Busulfan plasma pharmacokinetics

We studied busulfan plasma pharmacokinetics to explain the difference of toxicity between the two schedules of administration (once and twice daily) with a same total dose of 132 mg/kg. All the animals were followed on the same period (1st day of busulfan treatment, 8 am, to 3rd day, 4pm). No deaths occurred during the study period. The plasma concentration-time curves of busulfan after intra-peritoneal administration of busulfan are shown in Figure 3. For each point, there was a low inter-individual variability (about 20%) due to the use of inbred animals and standardized conditions of experimentation. It allowed estimating the $AUC_{0-\infty}$ of busulfan after administration of busulfan at 16.5 mg/kg (1st dose and 5th dose) and at 33 mg/kg (1st dose and 3rd dose).

Influence of the dose at the 1st injection

Determination of the pharmacokinetics at the 1st dose allowed checking the linearity of the pharmacokinetics with the two different doses. $AUC_{0-\infty}$ was 220 ± 34 h.nmol.mL⁻¹ after 16.5 mg/kg and 604 ± 87 h.nmol.mL⁻¹ after 33 mg/kg. According to these results, an injection of 33 mg/kg lead to an overexposure of +37% compared to two injections of 16.5 mg/kg. The pharmacokinetics of busulfan in mice are thus not linear between 16.5 mg/kg and 33 mg/kg.

Induction of plasma clearance after several busulfan injections

The influence of repeated busulfan injections on plasma clearance was studied through the determination of pharmacokinetics after the 1st dose and after two days of treatment, *i.e.* at the 3rd or the 5th dose (Fig. 3a and 3b). We observed a strong increase of plasma clearance when animals were treated according to the schedule 16.5 mg/kg twice daily. The exposure determined after the 5th dose was 91 ± 27 h.nmol.mL⁻¹ compared to 220 ± 34 h.nmol.mL⁻¹ after the 1st injection. This induction of clearance (mean \pm SD) from 7.4 ± 2.2 to 17.9 ± 2.8 mL/h was statistically significant (CI/F x 2.4, $p < 0.0001$). On the other hand, in animals treated according to the schedule 33 mg/kg once daily, the exposure determined after the 3rd dose was 490 ± 114 h.nmol.mL⁻¹ compared to 604 h.nmol.mL⁻¹ after the 1st dose. The induction of the clearance from 5.4 ± 0.8 to 6.7 ± 1.6 mL/h was less intensive (CI/F x 1.2) as compared to the twice daily schedule ($p < 0.0001$). We used a model of continuous mono-exponential decay of the exposure fit to the experimental data in order to simulate the induction. According to this model, total exposure of mice receiving busulfan once daily (4 injections, total AUC = 2081 h.nmol.mL⁻¹) was 2.4 greater fold than the exposure of mice receiving the twice daily scheme (8 injections, total AUC = 917 h.nmol.mL⁻¹). This strong and significant difference in plasma exposure ($p < 0.0001$) may explain the difference of toxicity.

Study of the mechanisms of induction of clearance

Busulfan rate of conjugation to glutathione in vivo

Plasma concentrations of sulfonium ion (THT⁺) were determined and the molar ratios of THT⁺/busulfan were calculated to estimate the rate of conjugation of GSH to busulfan. The mean molar ratio was very low ($5.6 \pm 3.8\%$) whatever the experimental conditions. Moreover, plasma concentration-time curves of busulfan and THT⁺ seem to be parallel at dose 1 (Fig. 4). In this mice strain, the elimination rate of THT⁺ was thus quite similar to the elimination rate of busulfan. For this reason, the molar ratio THT⁺/busulfan is not an accurate parameter to estimate the induction of conjugation-rate. An *in vitro* approach to measure the specific busulfan GST activity was thus proposed.

Busulfan rate of conjugation to glutathione in vitro

In vitro rate of conjugation to GSH was determined in the different experimental groups., The rate of conjugation was unchanged after two days of treatment. The intrinsic-clearance (Vmax/Km) was 0.61 ± 0.02 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in untreated mice, 0.62 ± 0.01 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in adjuvants treated mice, 0.58 ± 0.03 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in once daily treated mice and 0.62 ± 0.04

$\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in twice daily treated mice, respectively. Furthermore, the expression of Nrf2, the percent of oxidized glutathione ($5.1 \pm 2.7\%$) and lipoperoxidation determined through TBARS levels (112 ± 57 pmol/mg) were not enhanced in the liver under to the various conditions. In our experiment, induction of clearance was thus not due to an increase of busulfan specific glutathione S-transferase activity through oxidative stress and Nrf2 activation.

In vivo glutathione synthesis

GSH and its precursors were simultaneously quantified in the mouse liver samples in order to have an overview of the flux of precursors for GSH synthesis (Fig. 5). In mice treated with busulfan 16.5 mg/kg twice daily, the level of GSH was sustained throughout the activation of the metabolic pathway. The mean concentration of GSH (83.6 ± 18.4 nmol/mg) during 8 hours after the fifth busulfan injection (Fig. 5a) was not different from the mean concentration (74.8 ± 21.1 nmol/mg) after the first dose. However, GSH concentrations measured just before the 5th injection of busulfan (93 ± 26 nmol/mg) were significantly higher ($p < 0.05$) than the concentrations measured just before the 1st injection (53 ± 14 nmol/mg). Activation of the metabolic pathway was observed through a significant enhancement of the concentrations of GSH precursors, namely γ -glutamyl-cysteine and cysteine. The mean concentration of γ -glutamyl-cysteine (Fig. 5b) after the fifth busulfan injection was 2.43 ± 0.62 nmol/mg compared to the mean concentration of 1.26 ± 0.39 nmol/mg after the first dose ($p < 0.001$). The mean concentration of cysteine (Fig. 5c) after the fifth busulfan injection was 6.06 ± 1.30 nmol/mg compared to the mean concentration of 3.93 ± 0.86 nmol/mg after the first dose ($p < 0.001$). In mice treated with busulfan twice daily, activation of glutathione synthesis, with increased liver content of both γ -glutamyl-cysteine and cysteine, might explain induction of clearance. In order to explain the enhanced level of cysteine, we determined the concentration of homocysteine and cysteinyl-glycine before and after the first and fifth busulfan injections. The mean concentration of homocysteine after the fifth busulfan injection was 0.21 ± 0.04 nmol/mg compared to a mean concentration of 0.27 ± 0.04 nmol/mg after the first dose. The mean concentration of cysteinyl-glycine after the fifth busulfan injection was 1.12 ± 0.24 nmol/mg compared to a mean concentration of 0.92 ± 0.27 nmol/mg after the first dose. No statistically significant increase in mean cysteinyl-glycine levels was observed with the significant increase of liver GGT activity from 6.5 ± 1.0 mU/mg to 13.0 ± 3.3 mU/mg ($p < 0.01$).

In mice treated with busulfan 33 mg/kg once daily the glutathione system was saturated, which may explain the non-linearity of the pharmacokinetics. Activation of the glutathione metabolic pathway was observed with increased concentrations of glutathione, γ -glutamyl-cysteine and cysteine, 1 hour after the first busulfan injection (Fig. 5). However, the concentration of GSH (78 ± 19 nmol/mg) measured just before the 3rd injection of busulfan was not significantly higher than the concentration (53 ± 14 nmol/mg) measured just before the 1st injection. Moreover, the mean concentration of glutathione (55.9 ± 8.8 nmol/mg) during 8 hours after the third busulfan injection (Fig.5d) was lower than the mean concentration (76.3 ± 17.1 nmol/mg) after the first dose. This statistically significant ($p < 0.01$) decrease of glutathione mean concentration (-27%) was explained by the observation that the metabolic system was unable to sustain the mean concentration of glutathione precursors, γ -glutamyl-cysteine and cysteine, after the third injection. The mean concentration of γ -glutamyl-cysteine (Fig. 5e) was 1.22 ± 0.35 nmol/mg after the third busulfan injection compared to a mean concentration of 1.59 ± 0.50 nmol/mg after the first dose. This difference was not statistically significant. Furthermore, the mean concentration of cysteine (Fig. 5f) after the third busulfan injection with 4.13 ± 1.19 nmol/mg was lower than the mean concentration of 5.59 ± 1.61 nmol/mg after the first dose ($p < 0.05$). Mice treated with busulfan 33 mg/kg once daily were unable to sustain glutathione level and to adapt their metabolism through γ -glutamyl-cysteine and cysteine accumulation. The mean concentration of homocysteine after the fifth busulfan injection was 0.29 ± 0.04 nmol/mg compared to a mean concentration after the first dose of 0.35 ± 0.05 nmol/mg. The mean concentration of cysteinyl-glycine after the fifth busulfan injection was 1.05 ± 0.21 nmol/mg compared to a mean concentration of 0.97 ± 0.36 nmol/mg after the first dose and a stable GGT activity of 8.1 ± 2.0 mU/mg at third dose.

Discussion

Mice were treated with busulfan during four days at a total dose of 132 mg/kg according to two different schedules of administration: 33 mg/kg once daily versus 16.5 mg/kg twice daily. Under these conditions, busulfan 132 mg/kg given according to the once daily schedule leads to an excessive toxicity which is not compatible with the BMT procedure. No significant histological alterations in the lung and the intestine were observed at the onset of immediate toxicity. Micro-vesicular steatosis was observed in the liver which may be associated in some conditions with altered GSH synthesis at the mitochondrial level (Fernandez-Checa and Kaplowitz, 2005). Under our conditions, these histological features may be qualified of non-specific since they were associated with severe weight loss. The animals were probably not eating adequately due to lethargy. However, there was no major alteration of the metabolic capacity since GSH was not decreased at steady state.

On the other hand, animals treated twice daily tolerated properly the treatment, with adaptation phenomena assessable on the weight variations over the four days of treatment. The animals treated twice daily were efficiently rescued with a syngeneic BMT. Late tissue-specific toxicity after BMT, with growth retardation and hair-greying, was similar to previous observations (Down et al., 1989).

The difference in terms of immediate toxicity, related to the frequency of busulfan injections, may be explained through a difference of plasma clearance. A significant induction of the clearance was observed in mice treated twice daily. It is the first time that an adaptation of busulfan clearance during repeated injections is reported. Since busulfan was injected intraperitoneally, we specify that the ratio of drug-amount (A) and area under plasma concentrations-time curve (AUC) estimate the clearance “uncorrected for bioavailability” ($CL/F = A/AUC$). However, previous experiments performed in our laboratory (Boland et al., 1999) have shown that busulfan bioavailability in mice was close to 100 % when busulfan was given intraperitoneally ($CL/F \sim CL$).

Busulfan is a unique example of a drug almost exclusively eliminated through liver-metabolism with conjugation by glutathione S-transferases (Czerwinski et al., 1996; Gibbs et al., 1998). Induction of clearance may thus be due to an adaptation of busulfan metabolism through the GSH/GSTs pathway. It may concern either the conjugation rate (GST) or the substrate availability (GSH). This animal experiment allowed the study of the GSH/GSTs pathway regulation during a strong electrophile stress.

We showed that the induction of clearance is not due to an induction of the specific busulfan GST activity. GST activity was determined in vitro since the plasma ratio THT⁺/busulfan determined in vivo did not allow estimating accurately the conjugation rate. This was probably due to a high rate of elimination of THT⁺ in mice contrary to the observations done in humans with THT⁺ accumulation (Gibbs et al., 1997). On the other hand, the induction of busulfan clearance was correlated with enhanced GSH synthesis. In our conditions, GSH synthesis rate through availability of precursors is the limiting parameter for conjugation of busulfan to GSH. The induction of the clearance was very rapid and a saturation of this biochemical pathway was observed for a dose of busulfan between 16.5 and 33 mg/kg/injection. It is a relevant observation since the synthesis of GSH influenced directly the clearance and the toxicity of busulfan in C57BL6 male mice. Previous studies have shown that GSH stressors such as acetaminophen frequently lead to a rebound effect of GSH synthesis after a prior depletion (Buttar et al., 1977).

Our study is the first report of the in vivo flux of liver GSH synthesis being so closely related to the plasma clearance and toxicity of a drug. γ GCS activity has been presented for a long time as the rate limiting step of GSH synthesis, because this enzymatic activity is inhibited in vitro by high-level of GSH (Richman and Meister, 1975). Our study in mice supports the recently published results observed in yeasts (Lafaye et al., 2005). It was proposed that the enzymes of the metabolic pathway are not saturated under standard conditions and that increasing the pool of cysteine is sufficient to increase γ -glutamyl-cysteine level and GSH synthesis. Enhanced cysteine flux through modification of the intra-cellular metabolism with enhanced anabolism (Lafaye et al., 2005) or decreased catabolism (Lee et al., 2004) may thus be determinant for glutathione synthesis. Furthermore, this observation is very interesting for therapeutic purposes. In children receiving high-dose busulfan every 6 h over 4 consecutive days, busulfan plasma levels exhibited a significant circadian rhythm with a higher mean level at 6 a.m. compared to that at 12 p.m., 6 p.m. and midnight (Vassal et al., 1993). It was proposed that this circadian rhythm was due to hepatic GSH content (Vassal et al., 1993) since hepatic GSH, and not GST, exhibits circadian variations (Belanger et al., 1991). These observations are strong arguments to hypothesize that GSH synthesis in the liver may directly influence busulfan clearance, not only in mice, but also in humans.

It has been proposed a long time ago that busulfan administered in the first place in a BMT conditioning regimen might deplete the hepatic GSH content and sensitize the liver to the toxicity of the second alkylating agent, melphalan (Bouligand et al., 2003; Meresse et al., 1992) or cyclophosphamide (Hassan et al., 2000). This hypothesis was supported by clinical

observations and laboratory experiments. Shulman *et al.* showed that melphalan injected after a strong alteration of GSH synthesis can produce HVOD in dogs (Shulman et al., 1987). DeLeve demonstrated with various *in vitro* or *ex vivo* experiments that sinusoidal endothelial cells (SEC) isolated from mice liver are significantly more susceptible than hepatocytes to the toxicity of various toxins implicated in HVOD (DeLeve, 1996). During these *in vitro* experiments, the profound depletion of GSH preceded the onset of toxicity (DeLeve, 1996). This observation explains why injury is initiated on SEC and not on hepatocytes during HVOD. On the other hand, the differences observed between SEC and hepatocytes could be a consequence of cell isolation procedure or cell culture conditions. Furthermore, only high concentrations of busulfan ($\geq 250 \mu\text{g/mL}$), not achievable *in vivo*, were able to profoundly deplete GSH in murine hepatic cells *in vitro* (DeLeve and Wang, 2000).

Our study is original since it elucidates the *in vivo* role of GSH synthesis during high-dose busulfan treatment. We showed that mice treated with high-dose busulfan sustain efficiently their liver GSH content through activation of synthesis and cysteine accumulation. In clinical practice, patients receive busulfan at a total dose of 16 mg/kg in adults and up to 30 mg/kg in children. Busulfan is generally given every 6h over 4 consecutive days. According to either oral or intravenous administration, mean busulfan concentrations are far below the concentrations determined in mice receiving 132 mg/kg (Bouligand et al., 2003). On the other hand, the GSH content in human liver with 50 nmol/mg (Srivastava et al., 2004) is quite similar to the content determined in mice. It is thus unlikely that high-dose busulfan profoundly deplete GSH content in human liver in therapeutic conditions. Another mechanism of drug interaction may be proposed, such as the activation of different metabolic pathways enabling increase of GSH synthesis in response to the electrophile stress. The conception of GSH/GSTs pathway may be reviewed considering that this metabolic pathway is also a sensor of the chemical stress (Griffith, 1999). It has been demonstrated through the Nrf2/keap-1 pathway (Motohashi and Yamamoto, 2004) that several phase-2 genes are activated in response to chemical stress in parallel to the activation of GSH synthesis. Moreover, the role of many other pathways is still unknown (Tsuji, 2005). We think that the electrophile stress during busulfan treatment, detected through intensive GSH conjugation, may enhance the expression of several proteins, procoagulant, proinflammatory or profibrotic, participating to the biological events characterizing HVOD (DeLeve et al., 2002). The second alkylating agent administered in the conditioning regimen would initiate the systemic disease through alteration of the endothelium and cytokine release. This hypothesis is supported by recent observations showing that endothelial cells enhanced their production of

thromboplastin (Ritter et al., 2002) or activin-A (Dressel et al., 2003) during exposure to busulfan through an efficient GSH conjugation. The activation of the GSH pathway could be related to the elevation of homocysteine levels during HVOD (Gerecitano et al., 2003). It is noteworthy that cysteine and homocysteine are potentially toxic for the endothelium (Adinolfi et al., 2005). Moreover, cysteine was shown to be a strong reducer of iron and elevation of cysteine could thus maintain free iron in the toxic ferrous form responsible for the “Fenton Reaction” (Park and Imlay, 2003). That is very interesting since it has been proposed that iron-generated pro-oxidant state may partially explain the occurrence of HVOD during BMT (Evens et al., 2004).

In summary, we showed that activation of GSH synthesis through the increase of liver cysteine content is essential for clearance and detoxification of busulfan in mice. To our knowledge, it is the first time that the *in vivo* flux of liver GSH synthesis is so closely related to plasma clearance and toxicity of a drug.

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Footnotes

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List of figures and legends

Figure 1 – Glutathione metabolic pathways and busulfan metabolism

Figure 2 – Influence of busulfan dosing schedule on mice survival.

Mice were treated with busulfan injected intraperitoneally during four days (day -4 to day -1). There were 8 mice by experimental group. Two schedules were compared: injection of busulfan 16.5 mg/kg twice daily *versus* injection of 33 mg/kg once daily. The twice daily schedule (median survival of 13 days without BMT) was significantly better tolerated ($p < 0.0001$) than the once daily schedule (median survival 3.5 days). Only the twice daily schedule without immediate toxicity allowed to perform BMT at day 0 and to rescue all animals from the bone marrow failure.

Figure 3 – Busulfan pharmacokinetics in mice

Plasma busulfan pharmacokinetics was determined after injection of busulfan 16.5 mg/kg twice daily (a) *versus* injection of 33 mg/kg once daily (b). These determinations were performed on untreated mice (1st dose) and in “induced” mice after two days of treatment (3rd or 5th doses). For the pharmacokinetics, mice were sacrificed along the treatment at predetermined time, *i.e.* before drug injection and 1h, 4h and 8h after drug injection. Four mice were sacrificed by sampling time. Busulfan concentration was determined in blood plasma by GC/MS. Time (hours) is plotted on x-axis and busulfan concentration (nmol/mL) on y-axis.

Figure 4 – Plasma concentrations of busulfan and THT+

Plasma busulfan and THT+ concentrations were determined after injection of busulfan 16.5 mg/kg twice daily (a, b) *versus* injection of 33 mg/kg once daily (c, d). These determinations were performed on untreated mice at 1st dose (a, c) and in “induced” mice after two days of treatment at 5th (b) or 3rd (d) dose. For the study, mice were sacrificed along the treatment at predetermined time, *i.e.* before drug injection and 1h, 4h and 8h after drug injection. Four mice were sacrificed by sampling time. Busulfan concentration (◆) and THT+ concentration (■) were determined in blood plasma by two different GC/MS assay methods. Time (hours) is plotted on x-axis and concentration (nmol/mL) on a log-scale y-axis.

Figure 5 – Kinetics of liver glutathione and precursors after busulfan administration

Glutathione (GSH) and precursors were determined in mice liver after injection of busulfan 16.5 mg/kg twice daily (a, b, c) *versus* injection of 33 mg/kg once daily (d, e, f). These determinations were performed on untreated mice (1st dose) and in “induced” mice after two days of treatment (3rd or 5th doses). For the kinetics study, mice were sacrificed along the treatment at predetermined time, *i.e.* before drug injection and 1h, 4h and 8h after drug injection. Four mice were sacrificed by sampling time. GSH (a, d), γ -glutamyl-cysteine (b, e) and cysteine (c, f) were determined in liver mice by LC-MS/MS. Time (hours) is plotted on x-axis and content (nmol/mg proteins) on y-axis.

Figure 1

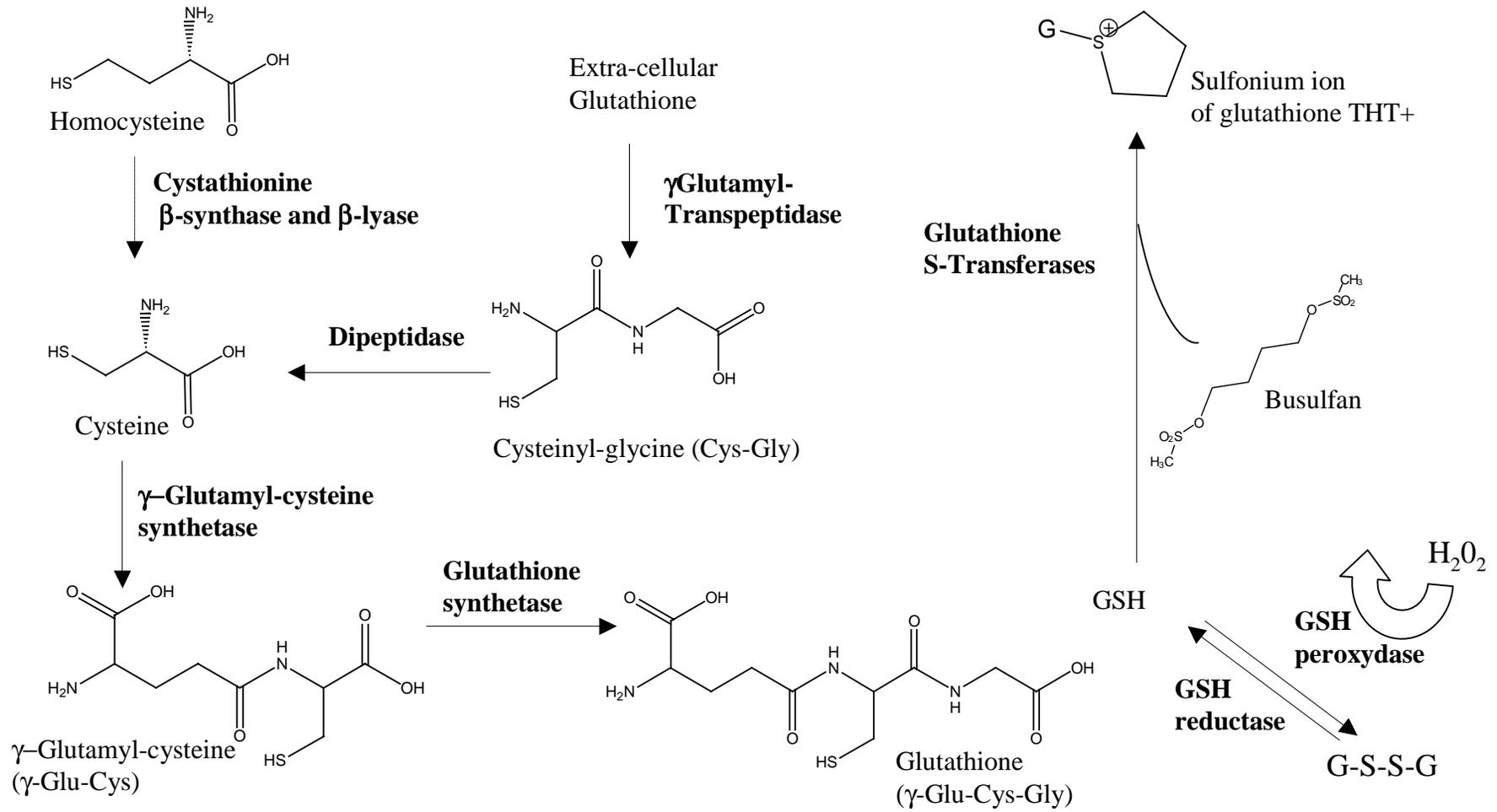


Figure 2

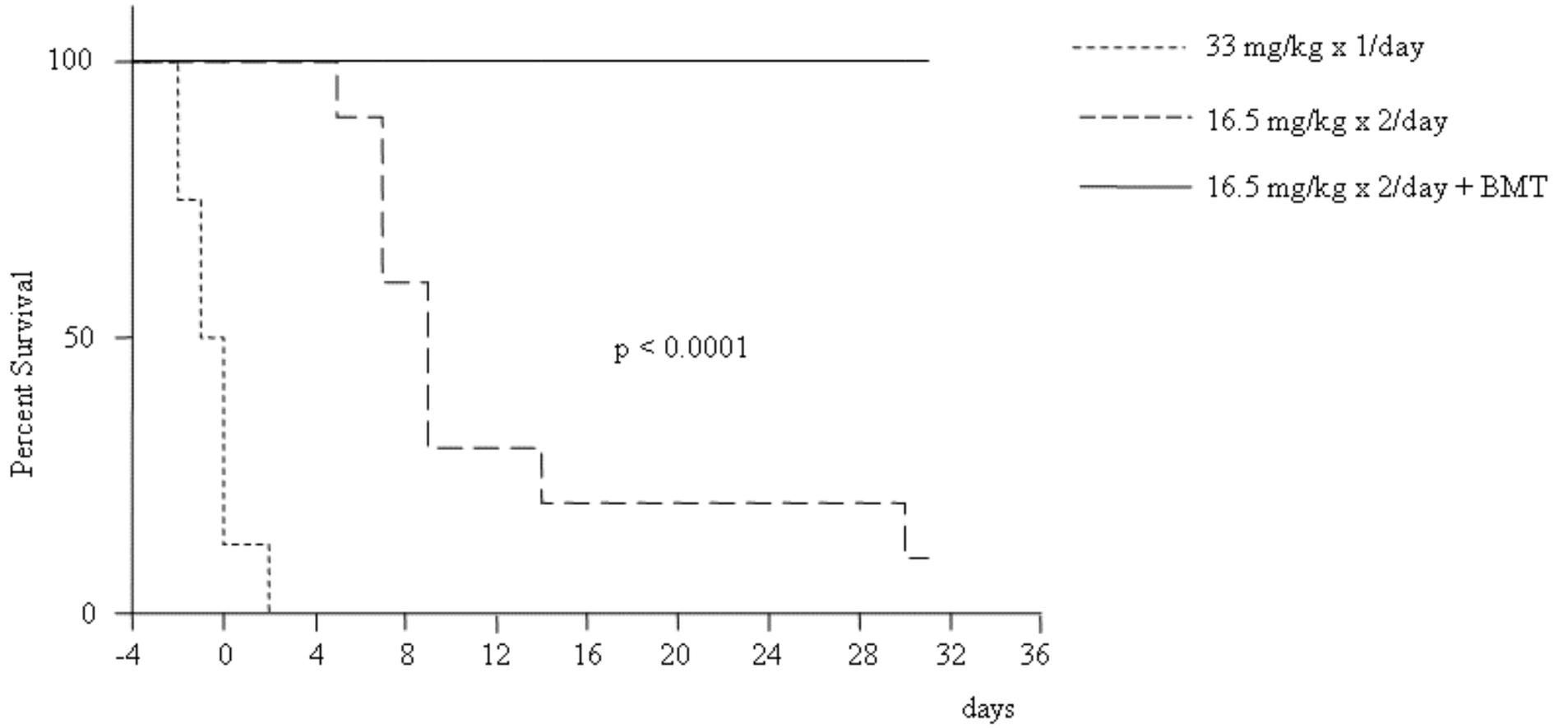


Figure 3

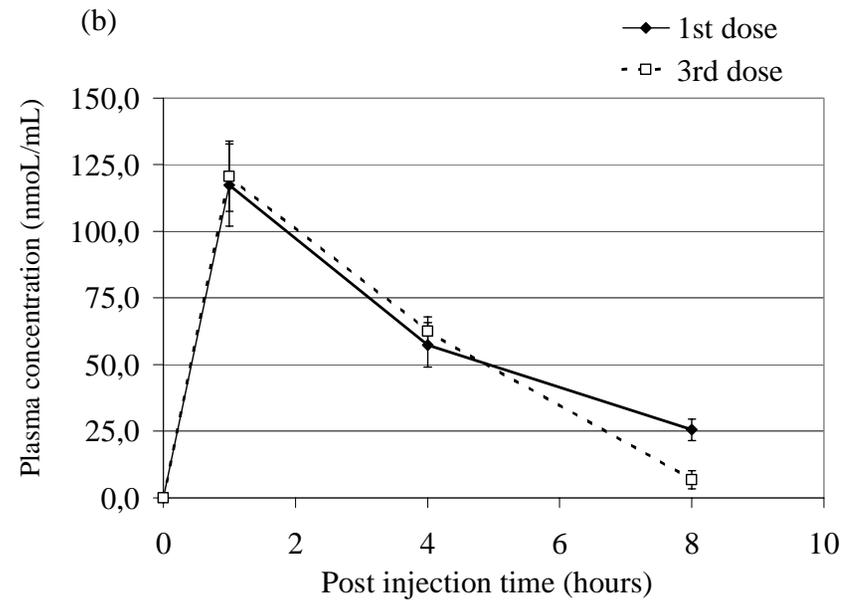
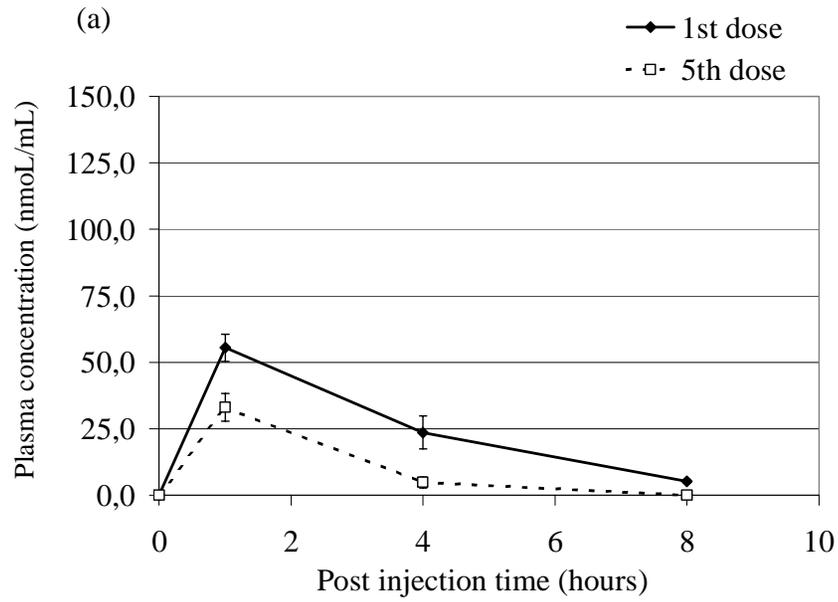


Figure 4

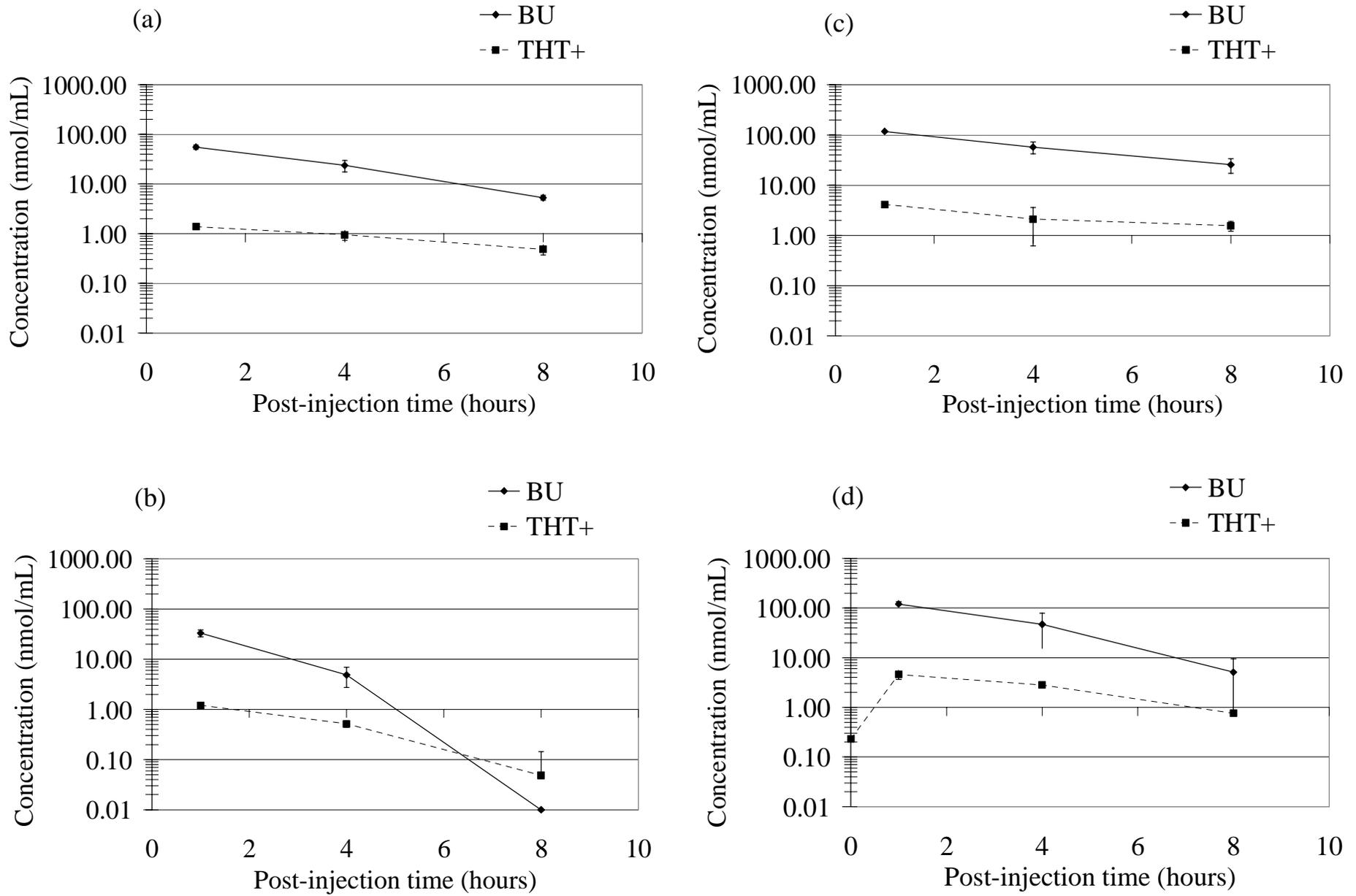


Figure 5

