COMPARISON OF HUMAN LIVER AND SMALL INTESTINAL GLUTATHIONE S-TRANSFERASE-CATALYZED BUSULFAN CONJUGATION IN VITRO

JOHN P. GIBBS, JI-SUN YANG, AND JOHN T. SLATTERY

Department of Pharmaceutics (J.P.G., J.-S.Y, J.T.S.) and University of Washington and Fred Hutchinson Cancer Research Center (J.T.S.)

(Received July 24, 1997; accepted October 1, 1997)

This paper is available online at http://www.dmd.org

ABSTRACT:

The apparent oral clearance of busulfan has been observed to vary as much as 10-fold in the population of children and adults receiving high-dose busulfan. The only identified elimination pathway for busulfan involves glutathione conjugation. The reaction is predominantly catalyzed by glutathione S-transferase (GST) A1-1, which is present in both liver and intestine. The purpose of this study was to compare busulfan $V_{\text{max}}/K_m$ in cytosol prepared from adult human liver and small intestine. Tetrahydrothiophenium ion formation rate per milligram of cytosolic protein was constant along the length (assessed in 30-cm segments) of three individual small intestines. A 30-cm-long intestinal segment 90–180 cm from the pylorus was chosen to be representative of intestinal cytosolic busulfan conjugating activity. Busulfan $V_{\text{max}}/K_m$ (mean ± SD) in cytosol prepared from 23 livers and 12 small intestines was 0.166 ± 0.066 and 0.176 ± 0.086 μM/min/mg cytosolic protein, respectively, in incubations with 5 μM busulfan, 1 mM glutathione, and 2 mg of cytosolic protein. The relative content of GSTα (A1-1, A1-2, and A2-2) was compared for human liver and intestinal cytosol using Western blot. The levels of GSTα in liver and intestinal cytosol were 1.12 ± 0.56 and 1.38 ± 0.32 integrated density units mg−1 cytosolic protein, respectively. Busulfan conjugation in vitro was comparable per milligram of cytosolic protein in liver and intestinal cytosol.

Busulfan is a bifunctional alkylating agent commonly used in preparative regimens before hematopoietic stem cell (e.g., bone marrow) transplantation. The apparent oral clearance ($CL/F$) of busulfan varies at least 10-fold within the population of children and adults receiving high-dose busulfan therapy (Slattery et al., 1995; Hassan et al., 1996). In hematopoietic stem cell transplantation, excessively high busulfan plasma concentrations are associated with hepatic veno-occlusive disease, whereas low levels allow relapse of chronic myeloid leukemia, and even lower levels allow rejection of grafted marrow (Slattery et al., 1995, 1997; Grochow, 1993; Dix et al., 1996). The therapeutic window is extraordinarily narrow in some indications, e.g., a mean AUC of 16 doses of 900-1350 μM/min (average steady-state concentration of 600-900 μg/ml) for human lymphocyte antigen (HLA)-matched unrelated donors for diseases other than chronic myelocytic leukemia (Slattery et al., 1995, 1997). The metabolism of busulfan is an extremely important determinant of the outcome of hematopoietic stem cell transplantation.

The primary elimination pathway for busulfan involves glutathione (GSH) conjugation. The GSH conjugate, γ-glutamyl-β-(S-tetrahydrothiophenium)-alanly-glycine (THT$^-$), has been identified in rat bile after iv infusion (Marchand and Rennell, 1988) and in the rat liver perfusion model (Hassan and Ehrsson, 1987). THT$^-$ formation is the first step in the elimination of busulfan, as all known busulfan metabolites contain sulfur. The urinary recovery of [14C]busulfan and its radiolabeled metabolites was 30% after iv and 33% after oral administration and, in another study with [3H]busulfan, 20–30% after iv and 20–40% after oral administration to patients (Nadkarni et al., 1959; Vodopick et al., 1969). These data suggest that busulfan is not poorly absorbed in adults. In addition, a single study has estimated busulfan bioavailability to be 80% in a small group of adult subjects (Hassan et al., 1995). Recently, we demonstrated that busulfan conjugation is catalyzed by human liver cytosolic GST (Gibbs et al., 1996) and that GSTA1-1 is the predominant isoform responsible for busulfan conjugation (Czerwinski et al., 1996). This enzyme is present in both liver and intestine (Hayes and Pulford, 1995).

Based on the busulfan $CL/F$ observed in children and adults and a single report on busulfan iv clearance (Hassan et al., 1995), it seems that the extraction ratio for busulfan across the intestine and liver after oral administration could be in the range of 10–50%. Because the liver and small intestinal epithelium represent potential sites of busulfan first-pass metabolism that may contribute to variability in busulfan pharmacokinetics, we assessed busulfan intrinsic clearance ($V_{\text{max}}/K_m$) in vitro with livers and intestines collected as part of organ harvest for transplantation. The purpose of this study was to compare busulfan conjugation in human liver and intestinal cytosol in vitro.

$1$ Abbreviations used are: GSH, glutathione; THT, tetrahydrothiophenium; THT$^-$, tetrahydrothiophenium ion; GST, glutathione S-transferase; CL/F, apparent oral clearance; AUC, area under the plasma concentration-time curve; CDNB, 1-chloro-2,4-dinitrobenzene.

This work was supported in part by National Institutes of Health Grants CA18029, GM32165, GM07750, and ES07033.

$2$ The extraction ratio was estimated with the highest and lowest busulfan CL/F reported for a child and an adult, 430 and 80 ml/min/m², respectively, and dividing by liver blood flow, 876 ml/min/m². Because the liver and intestine are anatomically arranged in sequence, the extraction ratio represents the contributions from these two organs. We also assume that the liver is the main eliminating organ for busulfan.

Send reprint requests to John T. Slattery, Ph.D., Department of Pharmaceutics, University of Washington, Box 357610, Seattle, WA 98195-7610.
Materials and Methods

Preparation of Liver and Intestinal Cytosol. Human liver and small intestine were obtained with other organs to be used for transplantation. Intestinal cytosol was prepared by the same method as liver cytosol (Gibbs et al., 1996) except that all buffers were supplemented with 0.1 mM phenylmethylsulfonyl fluoride to inhibit intestinal peptidases (Thummel et al., 1996). Cytosol was stored at −80°C until use. Protein concentrations were determined with the Bio-Rad protein assay with bovine serum albumin as the standard (Bradford, 1976).

Incubations. All incubations were performed according to the previously described method (Gibbs et al., 1996). Linearity of product formation with time and cytosolic protein concentration were verified with cytosol prepared from a representative human liver and small intestine. The concentrations of busulfan and GSH were 5 μM and 1 mM, respectively. There was a 5-fold increase in the enzymatic formation of THT+ relative to the spontaneous formation with either liver or intestinal cytosolic protein in a 60-min incubation with 2 mg of cytosolic protein, 5 μM busulfan, and 1 mM GSH (data not shown). Thus, subsequent experiments (with one exception) were performed under these conditions. The amount of THT+ formed spontaneously was characterized in the absence of cytosolic protein. The amount of THT+ formed spontaneously was subtracted from the amount of THT+ formed in cytosolic incubations to yield the enzymatic contribution to product formation. Interday assay variability in the incubation procedure was measured by incubating liver cytosol with 5 μM busulfan and 1 mM GSH. The liver cytosolic and spontaneous rates of THT+ formation were 79.0 ± 5.5 and 17.1 ± 3.6 pmol/min (mean ± SD), respectively, determined on five consecutive days.

Analysis of THT+. Samples were prepared and analyzed according to the previously described method (Gibbs et al., 1996). Intraday assay variability was determined at 5 and 50 pmol of THT. The coefficient of variation for six determinations was 8 and 7%, respectively.

Western Blot Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under denaturing conditions with 12% polyacrylamide gels as previously described (Laemmli, 1970). Cytosolic protein (5 μg) was loaded in each lane, and each sample was run in duplicate. The proteins were transferred to nitrocellulose membrane for immunoblotting with a polyclonal antibody at a 1:1000 dilution. Human GST was detected using a goat anti-rabbit alkaline phosphatase substrate kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The relative content of GST was assessed with a scanning densitometer (Howtech Scannamaster 3+) and the program Visage (Millipore). GSTμ phenotype in the small intestine of 12 donors was determined by Western blot using an anti-human GSTμ antibody at a 1:1000 dilution. Human liver cytosols (N = 6) had been genotyped (3, GSTμ positive; 3, GSTμ deficient). There was perfect agreement between the genotype and phenotype.

Results and Discussion

To determine the appropriate segment of the small intestine for use in characterizing busulfan V_{max/K_m}, the rate of busulfan conjugation was measured in segments 0–30, 90–120, 180–210, 270–300, 360–390, and 450–480 cm from the pylorus. We chose to characterize V_{max/K_m} because peak plasma busulfan concentrations in patients receiving high-dose busulfan are around 5 μM, a concentration well below the K_m for busulfan conjugation (Gibbs et al., 1996). For this experiment, the incubation conditions were modified to 0.8 mg/ml cytosolic protein and 8 μM busulfan in a 50-min incubation. This change was necessary due to the low concentration of cytosolic protein (<2 mg/ml) in certain segments of intestinal cytosol. THT+ formation rate per milligram of cytosolic protein was constant along the length of individual intestines (fig. 1). The busulfan conjugating activity measured in the first segment (0–30 cm from the pylorus) ranged from 1.16 to 2.82 pmol/min/mg cytosolic protein and, in the last segment (450–480 cm from the pylorus), 0.92 to 3.07 pmol/min/mg cytosolic protein. The coefficient of variation of the mean value of busulfan conjugating activity in each of the 30-cm segments (N = 6) along the length of the small intestine was 17.6, 22.6, and 16.6%, for each of the three intestines, respectively. Based on these findings, an intestinal segment 90–180 cm from the pylorus (segments 3–6) was chosen to be representative of intestinal cytosolic busulfan conjugating activity. A relatively constant distribution of GST activity along the length of the small intestine has also been observed with 1-chloro-2,4-dinitrobenzene (CDNB), an isofor non-specific GST substrate (DeWaziers et al., 1989; Peters et al., 1991).

Busulfan V_{max/K_m} was measured in incubations with liver (N = 5) and intestinal (N = 3) cytosol at 3, 5, 9, and 12 μM busulfan and 1 mM GSH. In all cases, the formation of THT+ increased in proportion to busulfan concentration (data not shown). We previously found the K_m for busulfan conjugation exceeds its solubility in water, which greatly exceeds clinical concentrations (~5 μM) (Gibbs et al., 1996). To determine the validity of a single point busulfan V_{max/K_m} measurement, we examined the relationship between THT+ formation rate expressed relative to busulfan concentration (data not shown). We previously found the K_m of busulfan for GST conjugation exceeds its solubility in water, which greatly exceeds clinical concentrations (~5 μM) (Gibbs et al., 1996). For this experiment, the incubation conditions were modified to 0.8 mg/ml cytosolic protein and 8 μM busulfan in a 50-min incubation. This change was necessary due to the low concentration of cytosolic protein (<2 mg/ml) in certain segments of intestinal cytosol. THT+ formation rate per milligram of cytosolic protein was constant along the length of individual intestines (fig. 1). The busulfan conjugating activity measured in the first segment (0–30 cm from the pylorus) ranged from 1.16 to 2.82 pmol/min/mg cytosolic protein and, in the last segment (450–480 cm from the pylorus), 0.92 to 3.07 pmol/min/mg cytosolic protein. The coefficient of variation of the mean

3 F. Farin, personal communication.

FIG. 1. Busulfan conjugating activity measured in intestinal cytosols in vitro along the length of three human small intestines.

Segments were 30 cm in length, and the data from the 90–120-cm segment are reported as 120 cm from the pylorus, etc. Incubations were performed in 100 mM potassium phosphate buffer, pH 7.4, with 8 μM busulfan, 1 mM GSH, and 0.8 mg/ml cytosolic protein concentration for 50 min at 37°C. Each data point represents the mean ± SD of three determinations.
Incubations were performed in 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.4, with 1 mM GSH and 5 μM busulfan for 60 min at 37°C. Each data point represents the mean of three determinations.

The relative content of GSTα (A1-1, A1-2, and A2-2) was compared for human liver and intestinal cytosol using Western blot (data not shown). The levels of GSTα in liver and intestinal cytosol were 1.12 ± 0.56 and 1.36 ± 0.32 integrated optimal density units/5 μg cytosolic protein, respectively. This result supports our findings that the intestine and liver have comparable busulfan conjugating activity. As judged by the coefficient of variation, GSTα expression was less variable in the intestine (23%) relative to the liver (48%). Based on the level of GST expression and busulfan \( V_{\text{max}}/K_{\text{m}} \), GSTA-1 seems to be the major isoform catalyzing busulfan conjugation in both the liver and intestine. However, the lack of specific GST substrates and inhibitors for use in cytosolic enzyme preparations prevent direct proof of the specific isoform responsible for busulfan conjugation in the intestine.

Although CL/F and urinary recovery data suggest that there may be a first-pass effect on busulfan, one has not been definitively proved. We recently measured the AUC for busulfan and THT in children and adults and found that the ratio of THT* to busulfan AUC and busulfan CL/F expressed relative to body surface area were elevated (to a similar extent) in children relative to adults (Gibbs et al., in press). This finding suggests that children apparently eliminate busulfan through GSH conjugation more efficiently than adults. However, children and adults have comparable busulfan elimination half-lives, which reflects hepatic activity to a greater extent than the intestine because of blood flow differences. Thus, children may differ from adults in that they may conjugate a greater fraction of an oral dose of busulfan on first pass through the intestinal epithelium than do adults. Although the liver and intestine have comparable busulfan \( V_{\text{max}}/K_{\text{m}} \) and GSTα content per milligram of cytosolic protein, the liver as a whole organ has a total busulfan \( V_{\text{max}}/K_{\text{m}} \) approximately 67 times greater than that of the intestine. It might therefore be questioned as to whether intestinal first-pass metabolism would be significant relative to a hepatic first-pass effect. A similar calculation has been made for CYP3A in the intestine vs. liver. Although midazolam \( V_{\text{max}}/K_{\text{m}} \) is comparable per milligram of microsomal protein, total organ \( V_{\text{max}}/K_{\text{m}} \) of the liver is 70 times the intestinal \( V_{\text{max}}/K_{\text{m}} \) (Paine et al., in press). Pharmacokinetic studies designed to directly quantitate midazolam extraction by human intestinal epithelium in patients undergoing liver transplant surgery found that the contribution of the intestine was equal to that of the liver (both with extraction ratios of 0.4) (Thummel et al., 1996; Paine et al., 1996). Although the total mass of enzyme present in the intestinal epithelium is much less than that of the liver, the midazolam data suggest that the intestine nevertheless can exert an appreciable first-pass effect relative to the liver.

The major finding of this study was that busulfan conjugation \textit{in vitro} was comparable per milligram of cytosolic protein in liver and intestinal cytosol. Both organs may contribute to the first-pass metabolism of busulfan. That busulfan does indeed undergo an intestinal or hepatic first-pass effect after oral administration has not been proved.

**References**


