UP-REGULATION OF GLUTATHIONE S-TRANSFERASE ACTIVITY IN ENTEROCYTES OF YOUNG CHILDREN

JOHN P. GIBBS, CHRIS A. LIACOURAS, ROBERT N. BALDISSANO, AND JOHN T. SLATTERY

Fred Hutchinson Cancer Research Center, Seattle, Washington (J.P.G., J.T.S.); Department of Pharmaceutics, University of Washington, Seattle, Washington (J.P.G., J.T.S.); and Division of Gastroenterology and Nutrition, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania (C.A.L., R.N.B.)

(Received June 16, 1999; accepted September 9, 1999)

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

ABSTRACT:

The relationship between age and busulfan apparent oral clearance (Cl/F) expressed relative to adjusted ideal body weight and body surface area (bsa) was evaluated in 135 children aged 0 to 16 years undergoing hematopoietic stem cell transplantation for various disorders. Busulfan plasma levels were measured by gas chromatography-mass spectrometry after the first daily dose of the 4-day dosing regimen. Cl/F expressed relative to adjusted ideal body weight (ml/min/kg) and bsa (ml/min/m²) was lower in 9- to 16-year-old (y.o.) compared with 0- to 4-y.o. children (49 and 30%; p < .001). We hypothesized that the greater busulfan Cl/F observed in young children was in part due to enhanced (first-pass intestinal) metabolism. Busulfan conjugation rate was compared in incubations with human small intestinal biopsy specimens from healthy young (1- to 3-y.o.) and older (9- to 17-y.o.) children. Villin content in biopsy specimens was determined by Western blot and busulfan conjugation rate was expressed relative to villin content to control for differences in epithelial cell content in pinch biopsies. Intestinal biopsy specimens from young children had a 77% higher busulfan conjugation rate (p = .037) compared with older children. We have previously shown that glutathione- S-transferase (GST) A1–1 is the major isoform involved in busulfan conjugation, and that this enzyme is expressed uniformly along the length of adult small intestine. Thus, the greater busulfan conjugation activity in intestinal biopsies of the young children was most likely due to enhanced GSTA1–1 expression. We conclude that age dependence in busulfan Cl/F appears to result at least in part from enhanced intestinal GSTA1–1 expression in young children.

Busulfan is a bifunctional alkylating agent commonly used in the preparative regimens before hematopoietic stem cell transplantation for the treatment of various malignancies and inherited disorders. In hematopoietic stem cell transplantation, outcome at a fixed milligram-per-kilogram dose has been related to the plasma concentration-time curve (AUC)² and average steady-state concentration (C_{SS}). Excessively high busulfan AUC or C_{SS} is associated with an increase in hepatic veno-occlusive disease, low levels are associated with graft rejection in allogeneic transplantation (Grochow et al., 1989; Slattery et al., 1995). AUC and C_{SS} are a function of dose and apparent oral clearance (Cl/F). Cl/F is a pharmacokinetic term defined as the ratio of clearance (a specific measure of efficiency of elimination) to the fraction of dose that transits from the site of administration (in this case, the gastrointestinal tract) to the systemic circulation (Gibaldi and Perrier, 1982).

Busulfan Cl/F is age dependent. Several studies have shown that young children [<5 years old (y.o.)] have a 1.3- to 2-fold greater Cl/F compared with adults, when Cl/F is expressed relative to actual body weight (BW) or body surface area (bsa) (Vassal et al., 1989; Grochow et al., 1990; Hassan et al., 1991; Regazzi et al., 1993; Slattery et al., 1995). However, data generated to date generally comes from studies involving small numbers of individuals. Not surprisingly, the magnitude of the differences reported varies among studies as do age groupings. Due to the limitations of these previous studies, we have reinvestigated this issue in 135 children, confirming our previous result (Slattery et al., 1995). Because Cl/F is a function of both drug absorption and metabolism, busulfan apparently either is absorbed less extensively in children or is metabolized more efficiently by them.

Busulfan is eliminated by conjugation with GSH in a glutathione S-transferase (GST)-catalyzed reaction. The product of the reaction is γ-glutamyl-β-(S-tetrahydrothiophenium ion) alanyl-glycine (THT⁺). GST is a family of enzymes that catalyze the conjugation of electrophilic substrates with GSH. The human cytosolic GSTs expressed in liver and small intestine include α, μ, and π classes (Mannervik et al., 1992). In previous studies, we have found that purified GSTA1–1 was the predominant catalyst of busulfan conjugation (Czerwinski et al., 1996). The majority of GSTA1–1 in the body is found in hepatocytes, although it is also found in enterocytes. GSTA1–1 is the major hepatic form of the enzyme.

Indirect measures have suggested that the age dependence of busulfan Cl/F in children is due to enhanced ability to form the busulfan-
GSH conjugate. The AUC for busulfan and THT\(^+\) measured in children (0–4 y.o.) is 50% greater than in adolescents and adults (12–54 y.o.), and the ratio of THT\(^+\) to busulfan AUC and busulfan C\(\text{I/F}\) were elevated to a similar extent (Gibbs et al., 1997). Children and adults have comparable busulfan elimination half-lives (Vassal et al., 1989, 1992; Grochow et al., 1990; Regazzi et al., 1993; Hassan et al., 1994), a parameter that reflects hepatic metabolism to a greater extent than metabolism in the intestine. Taken together, these results suggest that children may have a higher busulfan C\(\text{I/F}\) because they conjugate a greater fraction of an oral dose of busulfan on first pass through the intestine. Herein we compare the busulfan conjugation rates in intestinal biopsy specimens in young children (<5 y.o.) with those of older children (9–17 y.o.).

Materials and Methods

Patients. Records collected as part of routine clinical busulfan monitoring between January 1990 and January 1998 at the Fred Hutchinson Cancer Research Center were examined retrospectively. All patients from whom age, dose, disease, gender, height, weight, and two or more determinations of busulfan C\(\text{I/F}\) were available were included in the analysis. The final database included patients from the Fred Hutchinson Cancer Research Center (Seattle, WA; \(n = 87\)), University of California at San Francisco (San Francisco, CA; \(n = 24\)), Cardinal-Glennon Children’s Hospital (St. Louis, MO; \(n = 9\)), Riley Hospital for Children (Indianapolis, IN; \(n = 11\)), and University of Louisville Medical Center (Louisville, KY; \(n = 4\)). The combined data set was comprised of C\(\text{I/F}\) determinations after a test dose (21), dose 1 (115), dose 5 (94), dose 9 (99), and dose 13 (56). The mean of at least two C\(\text{I/F}\) determinations in each patient was reported. All patients were between the ages of 3 months old and 16 y.o., and received oral busulfan tablets every 6 h for 4 days as part of their transplant preparative regimen. For those unable to swallow tablets, a busulfan suspension was administered via a nasogastric tube. Phenytoin was administered for seizure prophylaxis. No other cytotoxic agent or irradiation was given immediately before or concomitantly with busulfan. To test for the effect of disease on busulfan C\(\text{I/F}\), the patients were subdivided into two categories based on disease: malignant (\(V = 78\); including the following major disease categories, acute myelogenous leukemia (37), juvenile chronic myelogenous leukemia (3), ALL (4), Ewing’s Sarcoma (4), and myelodysplastic syndrome (13)) and nonmalignant (\(V = 33\); including the following major disease categories: severe combined immunodeficiency (11), Wiskott-Aldrich Syndrome (5), and Hurler’s syndrome (2)).

Determination of C\(\text{I/F}\). Blood samples were collected just before and 60, 120, 240, and 360 min after the administration of busulfan tablets. A 30-min sample was included after the administration of busulfan suspension. Plasma busulfan concentrations were determined by gas chromatography with mass spectrometry. Busulfan C\(\text{I/F}\) was correlated with hepatic metabolism by calculating C\(\text{I/F}\) relative to body size. The ratio of C\(\text{I/F}\) to body weight (bwa) was calculated using the following equation:

\[
\text{bwa} (\text{kg}) = \frac{\text{IBW}}{0.25} \times (\text{BW} - \text{IBW})
\]

Statistical Analysis. All statistical comparisons were performed using SPSS version 7.5 (Chicago, IL). The Mann-Whitney U test was used to compare the means of separate groups.

Biopsy Specimens. Pinch biopsies of the third portion of the duodenum were obtained at the Children’s Hospital of Philadelphia from two groups of children aged 1 to 3 and 9 to 17 years, respectively, undergoing endoscopy secondary to symptoms of gastroesophageal reflux. The pinch biopsies were snap frozen in liquid nitrogen and shipped frozen on dry ice to the Fred Hutchinson Cancer Research Center by overnight courier. The Human Subjects Review Committee at the Children’s Hospital of Philadelphia approved the study protocol.

Preparation of S9. Thawed biopsy specimens were homogenized after the addition of 100 mM potassium phosphate, 1 mM EDTA, pH 7.4 in a Thompson tube with a Teflon pestle on ice. The homogenate was centrifuged at 12,000g for 10 min. An aliquot of the supernatant fraction (S9) was diluted in homogenization buffer and saved for protein and villin analysis. Protein concentrations were determined using the Micro Bio-Rad assay with BSA as the standard (Bradford, 1976).

Incubations. THT\(^+\) formation rate for each S9 sample was measured in 30-min incubations with 1.5 mg/ml S9 protein, 500 \(\mu\)M busulfan, and 1 mM GSH at 37°C. The total volume of the incubation was 0.200 ml. The spontaneous rate of busulfan conjugation was determined in incubations without cytotoxic protein. The difference between THT\(^+\) formation rate in incubations with and without S9 protein was the enzymatic rate of busulfan conjugation.

Western Blot Analysis of Villin. The relative villin content for biopsy specimens was determined using a method similar to that of Lown et al. (1994). Protein electrophoresis was performed using the Xcell II Mini-cell (Novex, San Diego, CA) with MOPS buffer and NuPage 4 to 12% gradient gels. Each lane was loaded with 3.1 \(\mu\)g of S9 protein. Villin was detected using a mouse monoclonal antibody raised against bovine villin (Transduction Laboratories, Lexington, KY). The secondary antibody was anti-mouse IgG with horseradish peroxidase enzyme (Kirkegaard and Perry, Gaithersburg, MD). Villin bands were detected using a chemiluminescence kit (Amersham, Piscataway, NJ) and exposure to Hyperfilm (Amersham) for 15 to 30 s. The molecular mass of the bands detected with the villin monoclonal antibody were assessed using Bio-Rad molecular mass standards (Bio-Rad, Hercules, CA) as a reference.

The relative villin band was detected in a molecular weight of approximately 95 kilodaltons, the reported molecular mass of villin (West et al., 1988). This band was taken as villin (no bands were detected without the addition of the villin monoclonal antibody). The relative content of villin was assessed using a scanning densitometer (Howtek Scannmaster 3+, Hudson, NH) and the program Visage (Millipore, Bedford, MA). Each sample integrated optical density (IOD) was expressed relative to a reference sample that was run on all gels to allow comparison of IOD values across gels. All samples were run in duplicate.

Results

The relationship between age and busulfan C\(\text{I/F}\) expressed relative to AIBW and bsa is shown in Fig. 1. We have previously shown that bsa and AIBW were the best body size measures for reducing C\(\text{I/F}\) variability among adult patients (Gibbs et al., 1999). Busulfan C\(\text{I/F}\) declined with age in a manner that was consistent with previous reports. Children were categorized based on age to determine the magnitude of differences in clearance when C\(\text{I/F}\) was expressed relative to actual AIBW and bsa. The data are shown in Table 1. C\(\text{I/F}\) expressed relative to AIBW was lower in 5- to 8- (24%: \(p < 0.001\)) and 9- to 16- (49%: \(p < 0.001\)) compared with 0- to 4-y.o. children. C\(\text{I/F}\) expressed relative to AIBW was lower in 9- to 16- (33%: \(p < 0.002\)) compared with 5- to 8-y.o. children. C\(\text{I/F}\) expressed relative to bsa was not different among 0- to 4- and 5- to 8-y.o. children (\(p = .114\)) or 5- to 8- and 9- to 16-y.o. children (\(p = .341\)). C\(\text{I/F}\) relative to bsa was 30% lower in 9- to 16-y.o. children in comparison with 0- to 4-y.o. children (\(p < .001\)).

The impact of disease on busulfan C\(\text{I/F}\) expressed relative to bsa or AIBW was assessed by comparing C\(\text{I/F}\) in children within the same age group. Children were categorized as having malignant or inherited diseases. In univariable regression analysis, age was a significant predictor of busulfan C\(\text{I/F}\) expressed relative to bsa (\(r^2 = 0.327, p <\)
The relationship between age and busulfan CI/F expressed relative to AIBW (top) and bsa (bottom).

Different symbols represent patients from different medical institutions.

**TABLE 1**

*Busulfan CI/F determined in children 0 to 4, 5 to 8, and 9 to 16 y.o.*

<table>
<thead>
<tr>
<th>Age Category, years</th>
<th>0 to 4</th>
<th>5 to 8</th>
<th>9 to 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>68</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td>ml/min/kg AIBW</td>
<td>6.77 ± 2.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.14 ± 2.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.45 ± 0.60&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ml/min/m² AIBW</td>
<td>144 ± 52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131 ± 61</td>
<td>111 ± 17</td>
</tr>
</tbody>
</table>

<sup>a</sup> Children ages 0 to 4 versus 9 to 16, p < .05.

<sup>b</sup> Children ages 0 to 4 versus 5 to 8, p < .05.

<sup>c</sup> Children ages 5 to 8 versus 9 to 16, p < .05.

ranges of 1 to 3 (n = 9) and 9 to 17 (n = 16) y.o., which corresponds to the age ranges between which differences in busulfan CI/F have consistently been observed. Because we were comparing GSTA1–1 activity in pinch biopsy specimens, and GSTA1–1 is predominantly expressed in the intestinal epithelial cells (Hayes et al., 1989), we used the relative content of villin to control for variability in the epithelial villi of biopsy specimens. Figure 2 shows a representative Western blot of villin. We found a major band at 95 kD, the molecular mass of villin (West et al., 1988), in all samples, with the exception of two specimens that showed no band at this molecular mass. The overall content of villin in biopsy samples was similar in young (1- to 3-y.o.) and older (9- to 17-y.o.) children (0.97 ± 0.16 versus 1.31 ± 0.63 IOD, p = .15, respectively).

Single determinations of busulfan conjugation activity were performed in incubations with 1.5 mg/ml human intestinal S9, 500 μM busulfan, and 1 mM GSH. We have previously shown that the K<sub>m</sub> for busulfan conjugation by GSTA1–1 is greater than 1.2 mM (Czerwinski et al., 1996). Figure 3 shows the busulfan conjugation rate expressed relative to villin in relation to age. There was a 77% greater THT<sup>a</sup> formation rate in biopsy samples from young children in comparison with older children (34.1 ± 16.6 versus 19.3 ± 10.7 pmol/min/U villin, p = .037, respectively).

**Discussion**

The major findings of this study were: 1) that busulfan CI/F expressed relative to bsa was 30% greater in children aged 0 to 4 years than in children aged 9 to 16 years; and 2) that busulfan-GSH conjugase activity was 77% greater in intestinal biopsies obtained from children in the younger age group. The difference in CI/F among age groups was increased to 95% when CI/F was expressed relative to AIBW, most likely due to the greater liver weight to BW ratio in young children (Murray et al., 1995; Gibbs et al., 1997). Liver weight expressed relative to bsa is not different between the age groups between which the difference in busulfan CI/F was observed.

---

*Fig. 1. Western blot analysis of villin.*

A reference sample was run on all gels, and the IOD value of each sample was expressed relative to the reference sample IOD to allow comparison across gels. All samples were run in duplicate.
have higher busulfan Cl/F than those with malignancies \( n = 12 \) (Hassan et al., 1996). However, in that study, the differences in disease category corresponded with differences in ages; the children with inherited disorders were 0.8 to 7.75 y.o. and those with leukemias were 0.5 to 14 y.o. A higher portion of the children were 0 to 4 y.o. in the group with inherited disorders, 7 of 8, versus 7 of 12 in the malignancy group. Results herein suggest that the claimed difference attributed to disease (Hassan et al., 1996) may have been due to differences in age.

We conclude that busulfan Cl/F is elevated in young children more than can be explained by a difference in the relationship of liver size to body size. The enhanced Cl/F is at least in part due to up-regulated metabolism of busulfan, apparently due to enhanced GSTA1–1 activity. The results suggest that it is likely that GSTA1–1 is up-regulated in young children.

References


Grochow LB, Jones RJ, Brundrett RB, Braine HG, Chen T-L, Sural R, Santos GW and Colvin GM (1989) Pharmacokinetics of busulfan. Correlation with veno-occlusive disease attributed to disease (Hassan et al., 1996) may have been due to differences in age.

We conclude that busulfan Cl/F is elevated in young children more than can be explained by a difference in the relationship of liver size to body size. The enhanced Cl/F is at least in part due to up-regulated metabolism of busulfan, apparently due to enhanced GSTA1–1 activity. The results suggest that it is likely that GSTA1–1 is up-regulated in young children.

References


Grochow LB, Jones RJ, Brundrett RB, Braine HG, Chen T-L, Sural R, Santos GW and Colvin GM (1989) Pharmacokinetics of busulfan. Correlation with veno-occlusive disease attributed to disease (Hassan et al., 1996) may have been due to differences in age.

We conclude that busulfan Cl/F is elevated in young children more than can be explained by a difference in the relationship of liver size to body size. The enhanced Cl/F is at least in part due to up-regulated metabolism of busulfan, apparently due to enhanced GSTA1–1 activity. The results suggest that it is likely that GSTA1–1 is up-regulated in young children.

References


Grochow LB, Jones RJ, Brundrett RB, Braine HG, Chen T-L, Sural R, Santos GW and Colvin GM (1989) Pharmacokinetics of busulfan. Correlation with veno-occlusive disease attributed to disease (Hassan et al., 1996) may have been due to differences in age.

We conclude that busulfan Cl/F is elevated in young children more than can be explained by a difference in the relationship of liver size to body size. The enhanced Cl/F is at least in part due to up-regulated metabolism of busulfan, apparently due to enhanced GSTA1–1 activity. The results suggest that it is likely that GSTA1–1 is up-regulated in young children.