IN VITRO MECHANISMS OF PROBENECID-ASSOCIATED ALTERATIONS IN ACETAMINOPHEN GLUCURONIDE HEPATIC DISPOSITION

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ABSTRACT:
The effect of probenecid (PRB) on factors regulating the hepatic disposition of acetaminophen glucuronide (AG) was investigated in vitro. Potential interactions in metabolism or binding to cytosolic proteins were examined. In the absence of PRB, AG formation in rat hepatic S9 fractions was saturable (Vmax = 2.77 ± 0.36 nmol/min/mg protein; Kmax = 18.0 ± 0.92 mM). PRB significantly decreased Vmax but not Kmax for AG formation, consistent with non-competitive inhibition. Various models were fit to the AG formation rate vs. acetaminophen (APAP) and PRB concentration data to elucidate the mechanism of inhibition by PRB. A partial noncompetitive inhibition model (K1 = 1.10 ± 0.01 mM) described the data best based on model selection criteria. AG did not bind to the cytosolic protein ligandin (glutathione S-transferase A1). These data indicate that PRB is a potent partial noncompetitive inhibitor of acetaminophen glucuronidation in vitro. PRB-associated alterations in AG hepatic disposition in vivo are not due to altered binding of AG to GSTA1 but may be attributed in part to impaired AG formation.

Phase II metabolism (i.e. glucuronidation, glutathione, and sulfate conjugation) is an important step in the hepatobiliary elimination of xenobiotics. Conjugation increases substrate polarity and negative charge, thereby facilitating removal of xenobiotics from the liver (1). Many substrates possessing hydroxy or carboxy groups undergo glucuronidation prior to elimination. APAP1 is biotransformed primarily by glucuronidation and sulfation (2, 3), processes that are capacity-limited in humans (4, 5) and rats (6, 7). N-hydroxycacetaminophen, formed via cytochrome P-450-based oxidation, is biotransformed to the reactive metabolite N-acetyl-p-benzoquinoneamine, which is conjugated with glutathione (5). Following hepatotoxic APAP doses, hepatic glutathione is depleted (8); cell death results from covalent binding of the reactive metabolite to hepatic macromolecules (2, 9, 10). Compounds that compete with APAP for glucuronidation may have therapeutic as well as toxicologic implications, especially at higher APAP doses.

PRB is a classic inhibitor of organic anion transport in the kidney (11, 12) and has been used clinically to inhibit renal secretion of penicillin and cephalosporin antibiotics (13, 14). The primary metabolic route of PRB elimination is oxidation of the N-propyl side chains, with subsequent conjugation to one ester and two other glucuronides (15). Clinical studies have suggested that PRB may impair the glucuronidation of several drugs including zomepirac (16), carprofen (17), ketoprofen (18), zidovudine (19–21), APAP (22, 23), and lorazepam (22). Recently, PRB was reported to impair the formation clearance to AG ~2-fold in the rat in vivo (24) and 3-fold in the isolated perfused rat liver (25).

Intracellular binding can influence hepatocellular translocation and storage of xenobiotics. Two classes of cytosolic proteins capable of binding nonbile acid organic anions have been identified (26, 27). The Y protein fraction (ligandin, identical to GSTA1 (28)) is the major binding protein for nonbile acid organic anions and constitutes 4–5% of cytosolic protein. Pharmacokinetic studies with DBSP (29), and multiple-indicator dilution studies with bilirubin (30) in the isolated perfused rat liver, indicated that cytosolic proteins may play an important role in regulation of hepatic egress of xenobiotics. Silberstein et al. (31) proposed that altered hepatic disposition of DBSP in rats with glycerol-induced renal failure was due to decreased binding of DBSP to ligandin. PRB binds significantly to serum proteins (~90%) (32) and may perturb the binding of organic anions to cytosolic proteins (33). Alterations in xenobiotic binding to cytosolic proteins secondary to chemical exposure or environmental toxins could influence directly hepatobiliary disposition and should be considered as a potential site of drug interactions.

Previous reports indicated that PRB altered the hepatobiliary disposition of AG in the rat in vivo (24) and in the isolated perfused rat liver (25). The purpose of the present investigation was to determine whether interactions in metabolism or binding to cytosolic proteins could contribute to PRB-associated alterations in AG hepatic disposition.

Materials and Methods

Chemicals

APAP, AG, BSP, chloramphenicol, GSTA1, phthalic acid, BSA, UDPGA, and PRB were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were purchased from standard commercial sources and were of the highest purity available.

1 Abbreviations used are: APAP, acetaminophen; PRB, probenecid; AG, acetaminophen glucuronide; GSTA1, glutathione S-transferase A1; DBSP, dibromosulphthyalein; BSP, bromosulphthyalein; BSA, bovine serum albumin; UDPGA, uridine 5’-diphosphoglucuronic acid; HPLC, high-performance liquid chromatography; AIC, Akaike’s Information Criterion; UDP, uridine diphosphates.

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Male Sprague-Dawley rats (205–245 g) were obtained from Hilltop Laboratory Animals (Scottsdale, PA). Rats were housed individually in hanging wire cages and maintained on a 12-hr light/dark cycle with free access to rat chow and water at all times. Rats were allowed to acclimate for at least 5 days prior to experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

In Vitro Hepatic APAP Glucuronidation

Hepatic S9 fractions were prepared by a modification of a published method (34). The rate of in vitro APAP glucuronidation was determined by quantitating AG formation. Preliminary experiments were conducted to optimize detergent (Brij 58) concentration, cofactor (UDPGA) concentration, protein concentration, and incubation time; a single time point, at which AG formation was linear with respect to both protein concentration and incubation time, was chosen for assessing the rate of AG formation in subsequent experiments. Reaction mixtures contained final concentrations of 0.05% Brij 58, 5 mM MgCl₂, 15 mM UDPGA, 10 mg/ml S9 protein, APAP (0.5–38 mM, dissolved in 50 mM phosphate buffer), and PRB (0–10 mM; dissolved in 2.5 N NaOH, adjusted to pH 7.4 with 0.1 N HCl, and diluted to volume with 50 mM phosphate buffer) or PRB vehicle (2.5 N NaOH, 0.1 N HCl, and 50 mM phosphate buffer) in a total volume of 200 ml of 50 mM phosphate buffer (pH 7.4). Incubation mixtures were preincubated in a shaking water bath for 5 min at 37°C; APAP and either PRB or PRB vehicle were added to the preincubated mixtures to initiate the reaction. Reaction mixtures were incubated for 20 min at 37°C, and the reaction was terminated by the addition of 50 ml 6 N HCl. Samples were mixed immediately by vortex and centrifuged for 5 min; the supernatant was harvested and stored at −20°C until analysis. The concentration of AG was determined by HPLC, and AG formation rate was expressed as the total amount of AG formed/mg protein during the 20-min incubation period. Protein concentrations in the S9 fractions were determined by the method of Lowry et al. (35) with BSA as the protein standard.

In Vitro Binding to GSTA1

Binding of BSP (positive control) and AG to GSTA1 was assessed by ultrafiltration with Amicon (YMT membrane, MW cutoff 23,000 daltons) Centrifree™ micropartition systems (Amicon, Inc., Danvers, MA) over a range of substrate concentrations [0.6 μM–0.3 mM (BSP); 6.5 μM–1.3 mM (AG)] and one GSTA1 concentration (1.84 × 10⁻³ M). Preliminary experiments were conducted to assess nonspecific adsorption of substrates to ultrafiltration devices and GSTA1 membrane permeability. Since BSP bound moderately (−10%) to the ultrafiltration devices, BSP solutions (at equivalent concentrations used in binding experiments; 0.01 M phosphate buffer, pH 7.4) were filtered through ultrafiltration devices immediately prior to binding experiments. BSP or AG was mixed with GSTA1 in 0.01 M phosphate buffer (pH 7.4), transferred to prewarmed ultrafiltration devices (37°C), covered with parafilm, and incubated at 37°C for 30 min. Capping of ultrafiltration devices and gravitational force alone produced ultrafiltrate. In all cases, less than 10% of the initial volume was collected as filtrate. AG concentrations were analyzed according to a previously published HPLC method (36); BSP concentrations were determined spectrophotometrically at 580 nm following alkalination with 0.1 N NaOH (37).

HPLC Analysis of APAP and AG

AG concentrations in S9 samples and APAP dosing solutions (50-μl aliquots) were quantitated by a modification of a previously published method (36) by using phthalic acid (60 μg/ml) as the internal standard. The mobile phase consisted of 0.75% acetic acid in 0.025 M potassium phosphate buffer (pH 3.10) and 3.55% acetonitrile delivered at a flow rate of 0.9 ml/min. The retention times for AG, APAP, and phthalic acid were 8.7, 21, and 31 min, respectively. Standard curves for AG and APAP (5–500 μg/ml) were linear (r > 0.998) and were prepared daily. The interday coefficient of variation was <9%, and the lower limit of detection for AG was ~2.5 μg/ml.

Data Analysis

The Michaelis-Menten equation (38) was fit to AG formation rate vs. APAP concentration data for control and PRB-treated conditions. To determine the mechanism of inhibition by PRB and the enzyme-inhibitor dissociation constant (Kᵢ), eight models (partial and complete competitive, partial and complete noncompetitive, partial and complete uncompetitive, and partial and complete mixed) were fit to the AG formation rate vs. APAP concentration data at each inhibitor concentration (39). Models were fit to all data sets simultaneously. A saturable binding model was fit to bound and unbound substrate concentrations to describe substrate binding to GSTA1. The number of independent binding sites (N) per molecule of protein, and Kᵢ, the association constant (μM⁻¹), were determined.

All data were analyzed by nonlinear least-squares regression analysis (PC-NONLIN; Statistical Consultants, Inc., Apex, NC). The goodness of fit for each model was assessed by visual examination of the distribution of the residuals, rank and condition number of the matrix of partial derivatives (a rank < number of parameters indicates that there are insufficient data to estimate precisely all the parameters; condition number > 10⁶ indicates a high degree of colinearity between parameters in the model), AIC (the model with the minimum AIC is regarded as the best representation of the data; 40), and visual inspection of the generated curves relative to the data.

Statistical Analysis

One-way ANOVA was used to assess differences in V_max and K_M for AG formation between different PRB treatment groups. A multiple comparison test with Bonferroni’s correction was used to determine differences between PRB treatment groups and controls. In all cases, the criterion for statistical significance was p < 0.05.

Results and Discussion

In Vitro Hepatic APAP Glucuronidation

Rat hepatic S9 fractions were used as a model system in the present study to investigate inhibition of APAP glucuronidation by PRB. AG formation rate was linear with respect to incubation time through 45 min and with respect to protein concentration through 10 mg/ml (fig. 1). Subsequent experiments were conducted at 20 min and at a protein concentration of 10 mg/ml. The kinetics of APAP glucuronidation in the absence of PRB were investigated over a range of APAP concentrations (0.5–38 mM) that were limited by APAP solubility in phosphate buffer. The rate of AG formation increased with increasing APAP concentrations and evidenced saturation at higher APAP concentrations to describe substrate binding to GSTA1. The number of independent binding sites (N) per molecule of protein, and Kᵢ, the association constant (μM⁻¹), were determined.

The effects of PRB on APAP glucuronidation in rat hepatic S9 fractions were investigated over a wide range of PRB (0.5–10 mM) and APAP (0.5–38 mM) concentrations. PRB decreased significantly V_max but not the K_M for APAP glucuronidation, consistent with noncompetitive inhibition (table 1). A mathematical modeling approach was employed to determine the mechanism of inhibition by PRB and to estimate Kᵢ. A partial noncompetitive model (eq. 1) described the data best based on rank, condition number, AIC, and inspection of the generated curves relative to the data (table 2; fig. 3):

\[ u = \frac{V_{max}}{K_{C} + (I)} + \frac{K_{2}}{K_{2}} \left( \frac{(1)}{(S)K_{C} + (1)} \right) \]

where I represents the inhibitor (PRB) concentration, S represents substrate (APAP) concentration, and β is a number between 0 and 1 (39). Parameter estimates (mean ± SD; N = 3) generated from partial
noncompetitive modeling of the data were: $V_{\text{max}} = 2.89 \pm 0.58$ nmol/min/mg; $K_i = 1.10 \pm 0.01$ mM; $K_M = 17.7 \pm 4.01$ mM; and $\beta = 0.12 \pm 0.01$.

PRB is a substrate for UDP-glucuronosyltransferase (15, 45) and has been reported to inhibit the glucuronidation of other xenobiotics in vitro, including codeine (46), lorazepam (44), 4-methylumbelliferone (47), and zidovudine (48–52). Recently, PRB was demonstrated to inhibit APAP glucuronidation both competitively ($K_i = 0.46$ mM) (44) and uncompetitively ($K_i = 0.60$ mM) (23) in rat liver microsomal preparations. The results of the present study indicated a similar $K_i$ value ($1.10 \pm 0.01$ mM) but a different mechanism of inhibition by PRB. Different enzyme preparations (microsomes vs. S9 fraction), experimental design, and/or methods of data analysis could contribute to these differences. The use of only one PRB concentration (5 mM) and a narrow APAP concentration range (0.25–5 mM) in the study by Kamali (23) may have precluded full characterization of the mechanism of inhibition by PRB.

In Vitro Binding to GSTA1

The hypothesis that PRB-associated alterations in AG binding to GSTA1 may influence the hepatocellular disposition of AG was investigated in this study. BSP was used as a positive control to determine whether substrate binding to GSTA1 could be examined via ultrafiltration. BSP exhibited saturable binding to a single high-affinity binding site ($N = 0.40 \pm 0.02; K_a = 3.97 \pm 0.93$ μM$^{-1}$), consistent with previous reports of BSP binding to ligandin that used other experimental methods for determining the extent of protein binding (e.g. equilibrium dialysis, circular dichroism, and fluorescence spectroscopy).

Table 1: Influence of PRB on AG formation in rat hepatic S9 fraction

<table>
<thead>
<tr>
<th>PRB (mM)</th>
<th>$V_{\text{max}}$ (nmol/min/mg)</th>
<th>$K_M$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.77 ± 0.36</td>
<td>18.0 ± 0.92</td>
</tr>
<tr>
<td>0.05</td>
<td>2.75 ± 0.54</td>
<td>17.3 ± 3.69</td>
</tr>
<tr>
<td>0.25</td>
<td>2.44 ± 0.46</td>
<td>18.6 ± 2.96</td>
</tr>
<tr>
<td>1.25</td>
<td>1.64 ± 0.33$^a$</td>
<td>20.6 ± 4.65</td>
</tr>
<tr>
<td>6.25</td>
<td>0.80 ± 0.19$^b$</td>
<td>19.2 ± 6.55</td>
</tr>
<tr>
<td>10.0</td>
<td>0.64 ± 0.18$^b$</td>
<td>23.0 ± 7.91</td>
</tr>
</tbody>
</table>

Mean ± SD; $N = 3$; $^a p < 0.005$; $^b p < 0.0005$, control vs. PRB.

Table 2: Model selection criteria after nonlinear least-squares regression analysis of AG formation rate vs. APAP concentration data at various PRB concentrations

<table>
<thead>
<tr>
<th>Model$^a$</th>
<th>No. of Parameters</th>
<th>Rank</th>
<th>Condition No.</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4.89E + 06</td>
<td>209 (65.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5.74E + 11)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3.66E + 11</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.23E + 11)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
<td>46.8 (8.80)</td>
<td>-59.9 (36.5)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>127 (47.3)</td>
<td>-89.9 (46.1)</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>4</td>
<td>70.2 (6.42)</td>
<td>-58.7 (36.5)</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>5</td>
<td>177 (26.8)</td>
<td>-89.5 (46.8)</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>3</td>
<td>70.7 (15.6)</td>
<td>-37.7 (39.9)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>4</td>
<td>306 (54.5)</td>
<td>-34.5 (50.8)</td>
</tr>
</tbody>
</table>

Mean (SD); $N = 3$.

$^a$ Model 1 (linear competitive); Model 2 (partial competitive); Model 3 (linear noncompetitive); Model 4 (partial noncompetitive); Model 5 (linear mixed); Model 6 (partial mixed); Model 7 (linear uncompetitive); Model 8 (partial uncompetitive).

noncompetitive modeling of the data were: $V_{\text{max}} = 2.89 \pm 0.58$ nmol/min/mg; $K_i = 1.10 \pm 0.01$ mM; $K_M = 17.7 \pm 4.01$ mM; and $\beta = 0.12 \pm 0.01$.

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Nonlinear least-squares regression. Values represent mean ± SD for three separate incubations from a representative S9 fraction.

Incubation mixtures contained in final concentrations 0.05% Brij 58, 5 mM MgCl₂, 15 mM UDPGA, 10 mg/ml S9 protein, APAP (0.3–38 mM), and PRB [0.05 mM (●), 0.25 mM (▲), 1.25 mM (●), 6.25 mM (●), and 10 mM (▼)] in 50 mM phosphate buffer (pH 7.4). Reactions were terminated at 20 min by the addition of 6 N HCl. The solid line represents the fit of equation 1 to AG formation rate vs. APAP concentration data at various PRB concentrations by nonlinear least-squares regression. Values represent mean ± SD for three separate incubations from a representative S9 fraction.

Fig. 3. AG formation rate (nmol/min/mg) vs APAP concentration (mM) at various PRB concentrations (mM).

Binding of AG to GSTA1 was assessed by ultrafiltration over a range of AG concentrations (6.5 μM–1.3 mM) and one GSTA1 concentration (1.84 × 10⁻⁵ M) in 0.01 M phosphate buffer. Values represent the average of two determinations. Linear regression analysis of the data yielded: slope = 1.03, intercept = 0, r² = 1.00.

Incubation mixtures contained in final concentrations 0.05% Brij 58, 5 mM MgCl₂, 15 mM UDPGA, 10 mg/ml S9 protein, APAP (0.3–38 mM), and PRB [0.05 mM (●), 0.25 mM (▲), 1.25 mM (●), 6.25 mM (●), and 10 mM (▼)] in 50 mM phosphate buffer (pH 7.4). Reactions were terminated at 20 min by the addition of 6 N HCl. The solid line represents the fit of equation 1 to AG formation rate vs. APAP concentration data at various PRB concentrations by nonlinear least-squares regression. Values represent mean ± SD for three separate incubations from a representative S9 fraction.

In summary, PRB inhibits AG formation in vitro by a partial noncompetitive mechanism, and the low Kᵢ value relative to the apparent Kₘ value for APAP glucuronidation indicates that PRB is a potent inhibitor of APAP glucuronidation in vitro. AG did not bind to GSTA1, suggesting that this protein does not influence AG hepatocellular translocation. These results confirm PRB-associated impairment of AG formation reported in the rat in vivo and in the isolated perfused rat liver, and indicate that PRB-associated perturbations in AG hepatic disposition can be attributed in part to impaired AG formation.

References


