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BIODISTRIBUTION OF [^{35}S]-CYSTEINE AND CYSTEINE PRODRUGS: POTENTIAL IMPACT ON CHEMOPROTECTION STRATEGIES

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SUMMARY

Thiazolidine prodrugs of L-cysteine constructed with aldose monosaccharides as the carbonyl donor offer powerful protection against acetaminophen (APAP)-induced hepatotoxicity, but require large doses to be effective. Using disaccharides in prodrug synthesis produces a thiazolidine ring form with a cyclic sugar moiety present. This structural motif may allow the delivery of the prodrug to specific carbohydrate receptors, such as the asialoglycoprotein receptor (ASGPR) of hepatocytes, thus reducing the required dose of prodrug and enhancing the effectiveness of cytoprotection. The biodistribution of [^{35}S]-labeled prodrugs was investigated in Swiss-Webster mice, both in the presence and absence of APAP, and compared to labeled L-cysteine itself. Accumulation of radioactivity in liver appeared to be stimulated by the presence of APAP in some cases, but organ levels after prodrug administration were much lower than after the administration of L-cysteine itself. These studies identified differences in the biodistribution of L-cysteine prodrugs of different structural types, as well as effects of the hepatotoxin on localization, but the occurrence of targeted delivery to hepatocytes remains speculative.

Key Words: L-Cysteine Prodrugs, Thiazolidines, Chemoprotection, Carbohydrate Targeting.

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INTRODUCTION

Chemoprotection via elevation and/or maintenance of cellular glutathione (GSH) levels has been achieved with several types of compounds. Supplying the critical precursor of GSH, L-cysteine, is a logical approach, but cysteine can be toxic and mutagenic at protective levels (1,2). We have been working for some time on thiazolidine prodrugs of L-cysteine, prepared by the condensation of the amino acid with a variety of carbonyl containing compounds (3). Our most widely studied compound, RibCys **1** (Figure 1) is prepared from the aldose monosaccharide, D-ribose. Using naturally occurring sugars as the carbonyl donor eliminates potential problems with the aldehyde moiety that is released upon prodrug dissociation to the free L-cysteine. More recently, prodrugs based on aldose disaccharides were prepared (4). These derivatives were designed to maintain the advantage of a non-toxic carbonyl donor, but also to present a terminal sugar in cyclic form that might be recognized by carbohydrate receptors on specific cell types, increasing their organ localization and hence their protective capabilities.

In particular, we are studying protection against hepatotoxicity using acetaminophen (APAP) as a model hepatotoxin. APAP is bioactivated by the cytochromes P450 system to N-acetyl-p-quinone imine (NAPQI), a very reactive electrophile which depletes cellular GSH (5). Our hypothesis was that L-cysteine prodrugs with an appropriate cyclic sugar residue would be recognized by the asialoglycoprotein receptor (ASGPR) on hepatocytes (6), increase hepatic localization, and provide enhanced protection against APAP-induced hepatotoxicity.

In order to determine potential preferential localization, the biodistribution in mice of a [^{35}S]-labeled disaccharide-derived prodrug (β-LactCys **3**) was explored to provide information about the potential accumulation in hepatic tissue, and was compared to radiolabeled RibCys **1**, GlcCys **2**, a prodrug synthesized from the monosaccharide D-glucose, and L-cysteine itself. The effect of a sub-lethal dose (2.6 mmol/kg) of APAP on the localization properties of the amino acid derivatives was also explored.

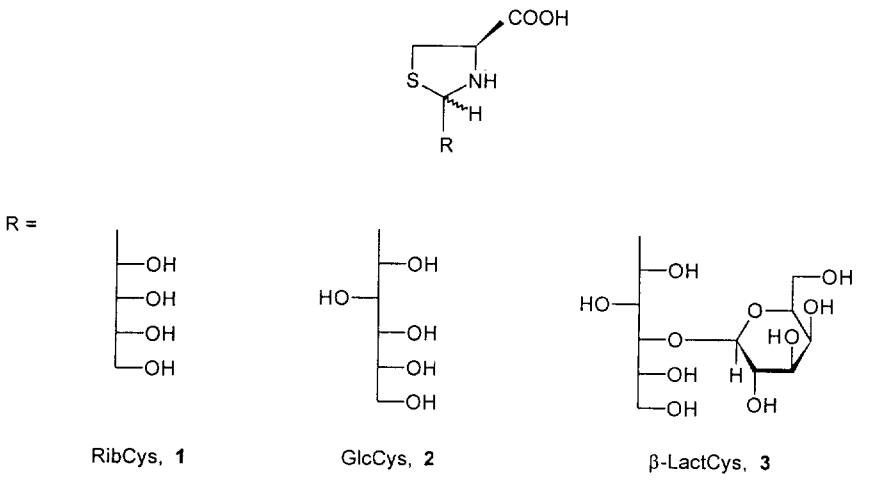

Figure 1. Structures of thiazolidine prodrugs of L-cysteine.

MATERIALS AND METHODS

General. [^{35}S]-L-Cysteine was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, MO). Radioactivity was determined using a 1900TR Packard Tricarb liquid scintillation counter.

[^{35}S]-2(R,S)-D-*ribo*-(1',2',3',4'-tetrahydroxybutyl)-thiazolidine-4(R)-carboxylic acid (RibCys, 1) and [^{35}S]-2(R,S)-D-*gluco*-(1',2',3',4',5'-pentahydroxypentyl)-thiazolidine-4(R)-carboxylic acid (GlcCys, 2). D-Ribose (0.90 g, 6.0 mmol) or D-glucose (1.08 g, 6.0 mmol) was dissolved in a minimum volume of degassed water and L-cysteine (0.64 g, 5.3 mmol), along with 1 mCi of [^{35}S]-L-cysteine, was added all at once under a flow of nitrogen. The reaction vessel was sealed, and the mixture was stirred at room temperature overnight. Ethanol was added until a slight cloudiness persisted. The solution was clarified by gentle heating, and the solution was cooled to room temperature, then to 4 °C. The resulting white precipitate was isolated by filtration and vacuum dried (3,7). Final specific activity of the radiolabeled prodrugs was 0.09-0.12 mCi/mmol.

[^{35}S]-2(R,S)-D-*gluco*-(1',2',4',5'-Tetrahydroxypentyl-3'-O-β-D-galactopyranosyl)-thiazolidine-4(R)-carboxylic acid (β-LactCys, 3). β-Lactose (2.05 g, 6.0 mmol) was dissolved in water (15 mL) and β-mercaptoethanol (2 mL) and pyridine (6 mL) were added. L-Cysteine (0.64 g, 5.3 mmol) was added to the reaction mixture, along with 1 mCi of [^{35}S]-L-cysteine, and the mixture was stirred overnight at room temperature. Ethanol was added to generate a white precipitate, then the solvent was removed under vacuum. The residue was triturated with ethanol and vacuum dried. The white powder that resulted was redissolved in water and cooled to 0 °C. Cold ethanol was added, which produced a voluminous white precipitate. The mixture was centrifuged at 10,000 rpm for 30 min at 5 °C. The supernatant was decanted and the white pellet was subjected to the trituration process four times (4). Final specific activity of this prodrug was 0.08-0.10 mCi/mmol.

Animal Care. Male Swiss-Webster mice (17 to 26 g) were purchased from Charles River Laboratories (Wilmington, MA) and were housed on cedar shavings in a 12-hour light/dark cycle in a temperature- and humidity-controlled facility accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were allowed free access to standard laboratory chow and water throughout the studies. The recommendations in the "Guide For the Care and Use of Laboratory Animals" published by the U.S. Department of Health and Human Services were followed. Mice were sacrificed by rapid CO_2 asphyxiation followed by bilateral thoracotomy in accordance with American Veterinary Medical Association guidelines. The research procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Biodistribution Studies. Separate treatment groups of five mice each received radiolabeled L-cysteine, β-LactCys, RibCys, or GlcCys, both in the absence and presence of APAP (2.6 mmol/kg) 30 minutes prior. Prodrugs were administered at a dose determined to provide 10 μCi per mouse, which represented a dose generally used in

protection studies (3,4). Specifically, animals received, on average, 2.8 mmol/kg of L-cysteine, 2.5 mmol/kg of RibCys, 2.6 mmol/kg of GlcCys, and 2.9 mmol/kg of β-LactCys. Mice were lightly anesthetized using ethyl ether prior to injection of the radiolabeled compounds; all injections were made ip. APAP was dissolved in water and maintained at 40 °C throughout the injections; [^{35}S]-L-cysteine and prodrugs were dissolved in saline, and the pH was adjusted to 7.1.

Animals were housed in metabolism cages until sacrifice at 1, 2, 6, 16, 24, or 48 hours after injection. Blood, liver, kidney, spleen, lung, heart, intestine, pancreas, and bladder samples from each mouse were collected and weighed. Urine from each group of five mice was pooled and weighed. Samples were placed in scintillation vials containing tissue solubilizer (2.5 mL, "Solvable", Packard Corporation, Meriden, CT) and heated to 50 °C overnight. Hydrogen peroxide (30%, 200 μL) was then added to decolorize the samples, followed by scintillation fluid (10 mL, "Ultima Flo", Packard Corporation, Meriden, CT). The average percent injected dose per gram of tissue (%ID/g) ± SEM for each organ from each group was calculated. Statistical significance between means in a particular organ at the same time point was assessed by ANOVA followed by Tukey-Kramer's Multiple Comparison Test. A P value of less than 0.05 was considered significant.

RESULTS

After injection of [^{35}S]-L-cysteine, the highest levels of radioactivity were observed in intestine, kidney, liver, and pancreas, with especially large amounts found in bladder and urine; blood levels were very low (<0.5 %ID/g) throughout the experimental time course. Figure 2A illustrates a subset of the biodistribution data, focusing on liver, kidney, and intestine. Over the first 6 hours, L-cysteine provided levels of radioactivity in the liver and intestine significantly greater ($P < 0.001$) than after the administration of β-LactCys (Figure 3A), RibCys (Figure 4A), or GlcCys (data not shown). Kidney localization at 1 hour for all four compounds was not statistically distinguishable.

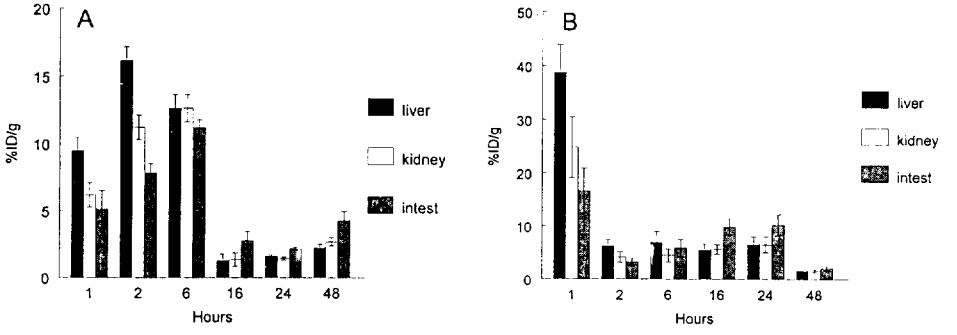

Figure 2. Biodistribution of [^{35}S]-L-cysteine in mice. (A) L-Cysteine alone; (B) L-Cysteine plus APAP (2.6 mmol/kg) given 30 minutes prior. Results are expressed as the mean %ID/g of [^{35}S] radioactivity over time ± SEM; N=5. Note the difference in the Y axes.

In the presence of 2.6 mmol/kg APAP, cysteine's biodistribution dramatically changed (Figure 2B). Large amounts of the radiolabel (~40 %ID/g) were detected in the liver at 1 hour ($P < 0.001$ compared to cysteine alone). Kidney and intestine showed a pattern similar to the liver, with large increases in radiolabel accumulation at the 1-hour time point ($P < 0.001$ compared to cysteine alone). At 2 and 6 hours, however, radioactivity levels in all three organs were statistically significantly lower in the group receiving APAP in addition to L-cysteine ($P < 0.001$). At 16 and 24 hours, the pattern shifted such that the group receiving APAP in addition to L-cysteine again exhibited significantly higher amounts of radiolabel in liver, kidney, and intestine than the L-cysteine only group ($P < 0.001$).

Following [^{35}S]-β-LactCys injection, radiolabel appeared in the liver to a far less extent than after L-cysteine administration ($P < 0.001$), and remained low throughout the 48-hour duration of the experiment (Figure 3A). For the most part, kidney and intestine followed a similar pattern. After 2.6 mmol/kg APAP, however, radiolabel accumulated to a greater extent in the liver at 1 hour ($P < 0.05$ compared to β-LactCys alone) (Figure 3B). Liver activity was increased by 6-fold over non-APAP treated animals, compared to the 4-fold increase in the L-cysteine case at this time point (Figures 2A and 2B). At 2 hours, levels in liver, kidney, and intestine when APAP was present were significantly higher than

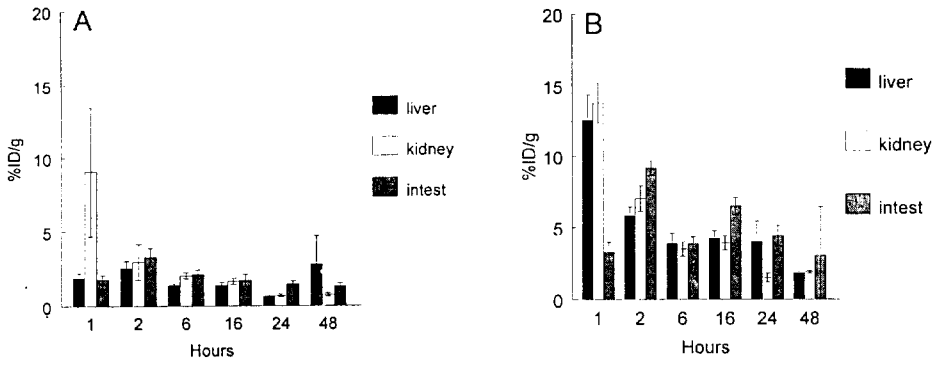

Figure 3. Biodistribution of [^{35}S]-β-LactCys in mice. (A) β-LactCys alone; (B) β-LactCys plus APAP (2.6 mmol/kg) given 30 minutes prior. Results are expressed as the mean %ID/g of [^{35}S] radioactivity over time ± SEM; N=5.

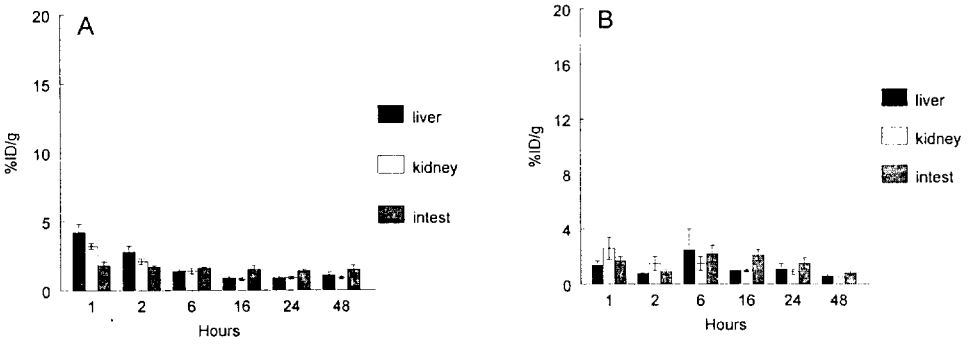

Figure 4. Biodistribution of [^{35}S]-RibCys in mice. (A) RibCys alone; (B) RibCys plus APAP (2.6 mmol/kg) given 30 minutes prior. Results are expressed as the mean %ID/g of [^{35}S] radioactivity over time ± SEM; N=5.

with β-LactCys alone ($P < 0.05$, 0.05, and 0.001, respectively). Statistical significance was not achieved at the 6-hour time point, but differences were again significant at the 16-hour time point in liver, kidney, and intestine compared to β-LactCys alone ($P < 0.01$, 0.05, and 0.001, respectively).

In the case of [^{35}S]-RibCys, radioactivity levels were again low in all organs (Figure 4A). In contrast to the previous experiments, however, the accumulation of radioactivity appeared to decrease in the presence of APAP (Figure 4B), although the differences did not achieve statistical significance.

The biodistribution profile for GlcCys, both in the absence and presence of APAP (data not shown), was virtually identical to that of RibCys.

Figure 5 illustrates the cumulative radioactivity excreted in urine over time as a percentage of the administered dose. The bolus dose of cysteine was excreted to a large extent in the urine over the first 6 hours. When APAP was present, however, urinary excretion was decreased, perhaps indicating the trapping or preferential retention of protective thiols in the body. This corresponds with the higher organ levels found at early time points (Figure 2). In the case of β-LactCys, cumulative urinary excretion was even more rapid, reaching 62% of the administered dose by 6 hours. The same "trapping" phenomenon was visible here as well, with urinary excretion of the radiolabel decreasing in the presence of APAP. RibCys showed rapid urinary excretion as well, reaching 63% by 6 hours; however, in the presence of APAP, the cumulative 6-hour excretion value increased to 70%, in contrast to either β-LactCys or L-cysteine itself.

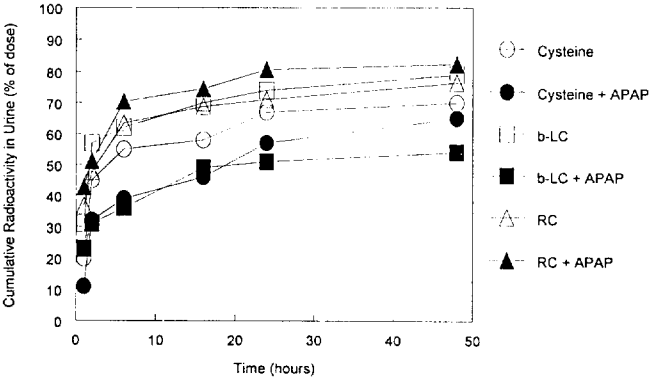

Figure 5. Cumulative urinary excretion of [^{35}S] radioactivity over time. Each data point represents the combined output of groups of five mice.

DISCUSSION

The ASGPR is present on hepatocytes and, to a much smaller extent, in kidney and other tissue (8), and normally functions in the removal of senescent glycoproteins from the blood. The ASGPR recognizes and internalizes molecules containing cyclic galactose into hepatocytes, and this system has been capitalized upon to target drugs to these cells (9-12).

Because of the requirement by the ASGPR for galactose in its cyclic pyranosyl form, the linear polyhydroxy chains present on monosaccharide-based thiazolidines such as RibCys are not appropriate for recognition by such receptors. However, when a galactose-terminal disaccharide such as lactose is condensed with L-cysteine, the thiazolidine presents a terminal galactose in cyclic form which may be recognized by the ASGPR. While the ASGPR prefers more than one galactose moiety for optimal binding ("the cluster effect"), only a single cyclic sugar residue is required for recognition (6).

No information on the extent or the location of dissociation can be inferred from these experiments, and no distinction in the physical form of the labeled sulfur atom can be made after administration of the radiolabeled compounds. Besides remaining in the prodrug form, or being released as L-cysteine, the sulfur atom can be converted into various metabolites such as sulfate, cystine, and taurine, in addition to being incorporated into GSH. In studies with rat hepatocytes, the pattern of cysteine metabolism varied with availability of the amino acid (13). At high cysteine availability, the production of taurine and sulfate were favored. At low availability, the cysteine was preferentially converted to GSH. In rats receiving a cysteine adequate diet, oxidation of intraperitoneally administered cysteine to sulfate accounted for 6% of the dose, oxidation to taurine for 35% (14).

In the present study, the animals not receiving APAP were not fasted and can be considered cysteine adequate, in contrast to the animals receiving 2.6 mmol/kg APAP. While this is well under a lethal dose of APAP, biological effects were observed. Untreated mice showed an average of 7.2 umol GSH/g liver tissue (4). Those receiving 2.6 mmol/kg

APAP showed a dramatic decrease in hepatic GSH to 3.1 umol GSH/g liver at 4 hours after the toxin; GSH levels rebounded to control values by 24 hours (4). The GSH status of these mice was closely correlated with the hepatotoxic index, as measured by histological rating of APAP-induced hepatic necrosis. In mice receiving 2.6 mmol/kg APAP, two out of nine animals showed minor necrosis at the 4-hour time point. That value increased to eight out of nine at 24 hours, with a corresponding increase in the severity of damage (4). β-LactCys completely protected against hepatic damage, and RibCys partially protected; GlcCys has shown reduced hepatoprotective capacity (3).

Very low levels of radioactivity were observed in most organs after prodrug administration, compared to L-cysteine; urinary excretion was high and rapid in all cases. This may simply confirm that no cysteine deficiency existed in these animals. Pretreatment with APAP resulted in a very rapid elevation in radiolabel in the liver and kidney with cysteine and β-LactCys, possibly indicating the redirection of critical components for detoxication; urinary excretion was correspondingly decreased in the presence of the hepatotoxin. These phenomena were reversed in the case of either RibCys or GlcCys.

CONCLUSIONS

The disaccharide-derived prodrug, β-LactCys, appeared to offer a slight advantage over the monosaccharide-derived analogs, RibCys and GlcCys, in that hepatic localization was greatly increased after β-LactCys in the presence of the hepatotoxin, APAP. This observation may be due to the preferential hepatic localization of the disaccharide-derived prodrug over those without a "targeting" moiety. However, since β-LactCys and RibCys, but not GlcCys, are effective hepatoprotectors (3,4), the importance of these biodistribution observations, either in the profile or the magnitude, is unclear.

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