Fluorescent Bile Acid Derivatives: Relationship Between Chemical Structure and Hepatic and Intestinal Transport in the Rat

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Studies were performed to characterize hepatic and intestinal transport, as well as biotransformation during transport, of a spectrum of fluorescent bile acids containing a fluorophore attached to the side chain. The following two classes of compounds were studied: 1) aminofluorescein (amF) coupled directly to the carboxylic group of a bile acid which was cholic, ursodeoxycholic, or cholylglycine; and 2) nitrobenzoxadiazolyl (NBD) coupled to the ε-amino group of a lysine conjugated bile acid, which was cholic or ursodeoxycholic. Fluorescein, a cholephilic organic anion, was studied as a control. Fluorescent bile acids were synthesized and their structures confirmed by nuclear magnetic resonance and mass spectrometry. Using the biliary fistula rat, hepatic transport, biotransformation, and choleretic activity were defined; intestinal absorption was assessed by jejunal or ileal perfusion studies. All fluorescent bile acids had hepatic transport maxima about one-sixth that reported for cholyltaurine, but derivatives of cholylglycine were transported best. Bile acids underwent little (<5%) biotransformation during hepatocyte transport. Only the amF conjugate of cholylglycine had normal choleretic activity; other compounds were hypocholeretic or cholestatic. In contrast, fluorescein was well transported, was partly glucuronidated, and had normal choleretic activity. NBD-tagged, but not amF-tagged, bile acids were actively transported by the intestine (ileum > jejunum), and no fluorescent bile acid had passive intestinal permeability; NBD-tagged bile acids were biotransformed during intestinal transport (jejunum > ileum). We conclude that the structure of the fluorophore as well as that of the bile acid influences transport by the hepatocyte and enterocyte. These fluorescent bile acids differ from fluorescein in being impermeable to cell membranes and undergoing little biotransformation during hepatocyte transport. Of these fluorescent bile acids, cholylglycylamF has hepatocyte transport and choleretic properties most closely resembling those of a natural bile acid. (HEPATOLOGY 1997;26:1263-1271.)

Bile acids tagged with a fluorophore have proved useful for characterizing aspects of hepatocyte,[1-13] enterocyte,[14,15] and cholangiocyte[16] transport. Several types of fluorescent bile acids have been prepared. The laboratory of G. Kurz[4,5,17] described a number of bile acid derivatives in which a hydroxy group on the steroid nucleus was replaced by nitrobenzoxadiazolyl (NBD), a well-known fluorophore. Earlier, Sherman and Fisher[1] reported the coupling of aminofluorescein (amF) to cholylglycine (glycocholate) and proposed that the amF was coupled to the oxygen atom at C-3. Subsequently, Schteingart et al.[18] showed that the assigned structure was not correct, and that in the compound prepared by Sherman and Fisher[1] the amF was coupled to the carboxyl group at the end of the side chain. Mills and his colleagues[6] coupled fluorescein to the ε-amino group of cholyllysine, thus preparing compounds in which fluorescein was coupled by an amino-*n*-butyl tether to the α-carbon of cholylglycine. More recently, our laboratory has prepared analogous derivatives by coupling NBD to the ε-amino group of cholyllysine or chenodeoxycholyllysine.[10]

If fluorescent bile acids are to be used as surrogate molecules for bile acids or other hepatophilic organic anions, detailed characterization of their hepatic handling is needed. It is also useful to know whether these molecules are transported actively or passively by the small intestine, since molecules that are well absorbed by the small intestine may undergo enterohepatic cycling.

We report here studies on the hepatic and intestinal transport of five fluorescent bile acid derivatives using the anesthetized biliary fistula rat. In the compounds that were used, the fluorophore (NBD or fluorescein) was present on the side chain. Three different bile acid moieties were used—cholic acid (C), ursodeoxycholic acid (UDC), and cholylglycine (CG)—to test whether the chemical structure of the

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Abbreviations: NBD, nitrobenzoxadiazolyl; amF, aminofluorescein; C, cholic acid; UDC, ursodeoxycholic acid; CG, cholylglycine; C-taurine, cholyltaurine; UDC-taurine, ursodeoxycholyltaurine; T_{max}, maximal hepatic transport rate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; ACA, apparent choleretic activity; CGamF, cholylglycylamidofluorescein; CamF, cholylamidofluorescein; UDCamF, ursodeoxycholylamidofluorescein; C-L-NBD, cholyl-(Nε-NBD)-lysine; UDC-L-NBD, ursodeoxycholyl-(Nε-NBD)-lysine.

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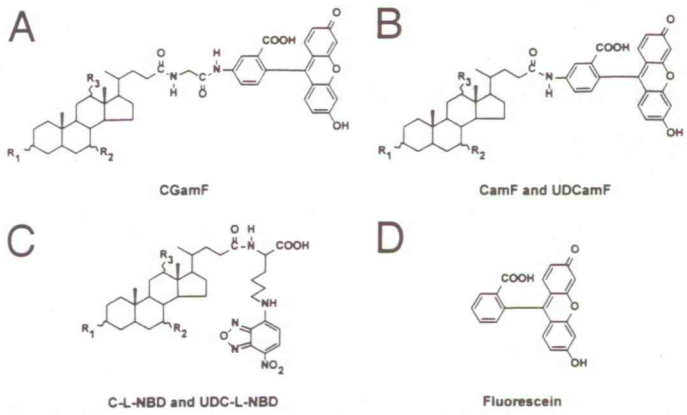

FIG. 1. Chemical structures of fluorescent bile acid derivatives and fluorescein.

steroid moiety influenced hepatic or intestinal handling. Since three of the molecules were tagged with fluorescein (as amF), we also characterized the hepatic handling of fluorescein to factor out the effect of the bile acid moiety on the amF-tagged bile acids. Results with these organic anions were compared with literature values for cholyltaurine (C-taurine) and ursodeoxycholyltaurine (UDC-taurine), common conjugated bile acids, whose transport characteristics by the rat liver[19-21] and intestine[22-25] have been well defined.

MATERIALS AND METHODS

Experimental Design

Experiments were performed using the anesthetized, biliary fistula rat because we wished to characterize the disposition of these molecules in the whole animal and because our laboratory has had considerable experience with hepatic transport[20,26,27] and intestinal transport[22] of bile acids in the biliary fistula rat. Two types of studies were performed. In the first set of studies, hepatocyte transport was assessed by measuring the maximal transport rate (T_{max}). Bile acid biotransformation [by thin-layer chromatography (TLC) and HPLC] and bile flow in relation to bile acid recovery [apparent choleretic activity (ACA)] were measured at the same time. In the second, intestinal absorption was assessed by perfusing the jejunum or ileum and equating biliary recovery with intestinal absorption.

Chemicals: Fluorescent Bile Acids and Fluorescein

Five fluorescent bile acids were used in this study, three tagged with amF [cholylglycylamidofluorescein (CGamF), cholylamidofluorescein (CamF), and ursodeoxycholylamidofluorescein (UDCamF)] and two with NBD [cholyl-(Nϵ-NBD)-lysine (C-L-NBD) and ursodeoxycholyl-(Nϵ-NBD)-lysine (UDC-L-NBD)]. Fluorescein sodium salt was purchased from Sigma Chemical Co. (St. Louis, MO); it was found by HPLC to have a purity >98% and was used as received. Chemical structures of all compounds are shown in Figure 1. Trivial names, abbreviations, orientation of nuclear substituents, molecular weights, and HPLC retention times are given in Table 1.

All amF-tagged bile acids were synthesized by conjugation of commercially available 5-aminofluorescein (Sigma Chemical Co., St. Louis, MO) with the carboxylic group of C, UDC, CG. Conjugation was performed using the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in a pyridine-hydrochloric acid buffer. This reaction couples the amino group of the fluorescein molecule to the carboxyl group of the bile acid side chain by an amide bond. Details of synthetic methods have been published elsewhere.[18] The Nϵ-NBD–tagged bile acid derivatives were prepared by coupling the relatively smaller NBD fluorophore to the ϵ amino group of cholyl- and ursodeoxycholyl-lysine using a series of reactions similar to the ones described by Schneider et al.[17]

Compounds were purified by adsorption chromatography using silica gel column chromatography as previously described.[28] The final preparations were >98% pure by HPLC.[29]

Physiological Experiments

Animal studies were approved by the Animal Subjects Committee of the University of California, San Diego.

Anesthetized Biliary Fistula Rat. Unfasted adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 330 g were anesthetized by intraperitoneal injection of pentobarbital, 7.5 mg/100 g body wt (Nembutal®, Abbott Laboratories, North Chicago, IL). Anesthesia was maintained by repeated intramuscular doses (10 mg) of pentobarbital given every 2 to 2.5 hours. Abdominal temperature was maintained throughout the experiment at 38 ± 0.5°C with a temperature-controlled heating lamp connected to a rectal temperature probe (Yellow Springs Instrument Co., Yellow Springs, OH). The jugular vein was cannulated using PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ; inner diameter, 0.28 mm; outer diameter, 0.61 mm). 0.9% sodium chloride (Baxter, Deerfield, IL) was infused at a rate of 2.2 mL/h using a Harvard syringe pump (Harvard Apparatus Co., Millis, MA). The abdomen was then opened by a midline incision and an external biliary fistula was constructed using PE-10 polyethylene tubing. Bile was collected every 15 minutes in preweighed plastic vials. After a 2 hour control period, the fluorescent bile acids or fluorescein (dissolved in 0.9% sodium chloride and 0.05 N NaOH, pH ≈ 8.0) were infused. Fluorescent bile acids were infused over a dose range from 0.3 to 8 μmol/ kg · min, and usually one to three doses were studied per animal. Each dose was infused for 90 minutes before proceeding to the next dose. (This procedure gives a value for T_{max}, but not for recovery, because of the confounding effect of toxicity at higher doses.)

Higher doses of fluorescent bile acids were not administered either because of toxic effects (see "Results") or lack of compound (an infusion of a fluorescent bile acid at a rate of 8 μmol/ kg · min requires ≈ 150 mg of the compound). However, an infusion rate of 8 μmol/ kg · min exceeds the usual flux of bile acids to the liver in the healthy rat.[30] Fluorescein was infused over a dose range from 0.5 to 14 μmol/ kg · min. At the end of the infusion period of the tested compound, saline was infused for an additional 2 hours.

To test for renal excretion of infused compounds and their metabolites, the urinary bladder was aspirated at the end of each experiment, and urine was analyzed by TLC.

Intestinal Perfusion. Adult male Sprague-Dawley rats were fasted overnight before the experiment. After completion of the biliary fistula as described above, a jejunal segment distal to the ligament of Treitz (≈20 cm in length) or a segment of the terminal ileum (≈20 cm in length) was surgically isolated. A PE-90 polyethylene tube (inner diameter, 0.86 mm; outer diameter, 1.27 mm) was introduced into the proximal lumen and tied in place. A large-bore polyethylene tube (Tygon R-3603, Fisher Scientific Co., Pittsburgh, PA; inner diameter, 1.60 mm; outer diameter, 3.20 mm) was inserted into the distal part of the isolated segment. (In the case of the ileum the tube was carefully pushed through the ileocecal valve for about 5 mm and tied in place.) The isolated segment was then rinsed with 20 to 40 mL 0.9% sodium chloride at 38°C using a syringe in order to remove intestinal contents. After the tube placements, the abdominal temperature was maintained at 38° ± 0.5°C by means of a temperature-controlled heating lamp. The perfusate, which was kept at 38°C in a water bath, consisted of isotonic TRIS buffer (TRIS, 25 mmol/L; NaCl, 130 mmol/L, pH 8.0). Fluorescent bile acids or fluorescein were dissolved in the perfusate to a concentration of 1 mmol/L.

A 60-minute control period, during which buffer was perfused, was followed by a 60 minute perfusion of the test compound. A peristaltic pump (Harvard Apparatus, Millis, MA) was used to per-