Biliary excretion of a stretched bilirubin in UGT1A1-deficient (Gunn) and Mrp2-deficient (TR$^-$) rats

Antony F. McDonagh[a,*], David A. Lightner[b], Daniel F. Nogales[b], Wilma S. Norona[a]

[a] *G.I. Unit and Liver Center, S-357, Box 0538, University of California, San Francisco, CA 94143-0538, USA*
[b] *Department of Chemistry, University of Nevada, Reno, NV 89557-0020, USA*

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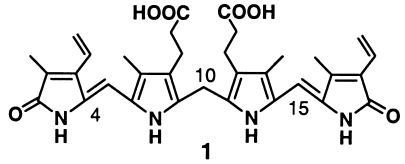
Abstract The metabolism and biliary excretion of a stretched bilirubin analog with a *p*-xylyl group replacing the central CH_2 hinge were investigated in normal rats, Gunn rats deficient in bilirubin conjugation, and TR$^-$ rats deficient in bilirubin glucuronide hepatobiliary transport. Unlike bilirubin, the analog was excreted rapidly in bile unchanged in all three rat strains after intravenous administration. In TR$^-$ rats biliary excretion of the analog was diminished, but still substantial, demonstrating that the ATP-binding cassette transporter Mrp2 is not required for its hepatic efflux. These effects are attributable to differences in the preferred conformations of bilirubin and the analog.

Key words: Bilirubin; Glucuronide; UGT1A1; Multidrug resistance-associated protein 2; Gunn rat; TR$^-$ rat

1. Introduction

Although it is well known that intramolecular hydrogen bonding and molecular conformation have overwhelming effects on the biochemistry of macromolecules, their effects on the metabolism of smaller endogenous molecules and drugs are frequently ignored. The natural dicarboxylic acids bilirubin (**1**) and biliverdin (**2**) are paradigmatic examples of small endogenous molecules (molecular weight ~600) whose metabolism seems to be highly dependent on these factors. With almost identical constitutional structures, molecular weights, and pK_as [1,2], both compounds are anions at physiologic pH and both are cleared efficiently from blood by the liver [3]. Yet their hepatic metabolism is very different. Biliverdin does not normally occur in the blood or bile of mammals because it is reduced quantitatively to bilirubin [3]. But when injected intravenously, the fraction that escapes reduction is excreted rapidly in bile unchanged [4], and in some non-mammals endogenous biliverdin is excreted intact in bile [5]. In contrast, bilirubin taken up by the liver is not secreted in bile, despite the presence of organic anion transporters in the canalicular membrane. Rather, it is converted by the glucuronosyl enzyme uridinediphosphoglucuronosyl transferase (UGT)1A1 to monoglucuronides, which are subsequently secreted rapidly from the liver into bile [3]. The presence of the ATPase Mrp2 (multidrug resistance-associated protein 2) in the canalicular membrane of the hepatocyte is essential for their secretion since mutant rats that lack it (TR$^-$ or Eisai rats) do not excrete bilirubin mono- or diglucuronides efficiently in bile [6–8]. Thus, the apical membrane at the liver–bile interface seems to be an effective barrier for efflux of unconjugated bilirubin, but not for biliverdin.



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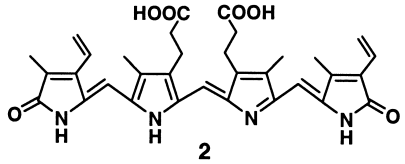

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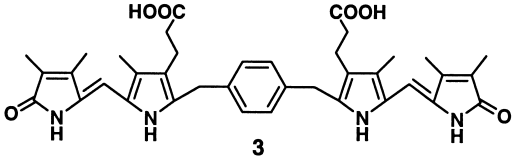

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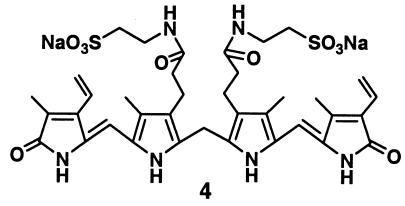

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The disparate biochemical behavior of **1** and **2** seems to be rooted in their very different three-dimensional structures. Whereas biliverdin adopts extended or helical lock-washer shaped conformations in solution, bilirubin is constrained by its saturated carbon atom at C10 to adopt folded, so-called ridge–tile, structures [9–12]. These are stabilized by a network of intramolecular hydrogen bonds involving the side-chain carboxyls and heterocyclic NH and C=O groups. They are more lipophilic than the conformers adopted by biliverdin in solution and are thought to be crucially important in the glucuronidation of bilirubin. For example, when intramolec-

*Corresponding author. Fax: (1)-415-476-0659.
E-mail address: tonymcd@itsa.ucsf.edu (A.F. McDonagh).

Abbreviations: HPLC, high performance liquid chromatography; MRP, multidrug resistance-associated protein; UGT, uridinediphosphoglucuronosyl transferase

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ular hydrogen bonding is prevented in one or both halves of the molecule by various chemical manipulations, glucuronidation becomes unnecessary for efflux from liver to bile [13–16].

In 1994, Nogales, Anstine and Lightner described the synthesis of the extended bilirubin analog **3**. In **3** the central C10 CH_2 group has been replaced by a *p*-xylyl group [17]. This pushes the two dipyrromethenone moieties too far apart to permit the normal type of intramolecular hydrogen bonding that prevails in bilirubin and gives a more flexible, more folded structure. In this paper we compare the hepatic excretion of **3** in normal rats, in mutant rats that, lacking UGT1A1 activity, are unable to glucuronidate bilirubin (Gunn rats) [18,19], and in mutant rats (TR^- rats) deficient in Mrp2 [6–8]. Our studies show that this stretched bilirubinoid organic anion is taken up rapidly by the liver and excreted intact in bile without undergoing glucuronidation and without the need for Mrp2. However, in the absence of Mrp2 its biliary excretion is somewhat diminished.

2. Materials and methods

2.1. Chemicals

The synthesis and properties of 2,2′-[1,4-phenylenebis(methylene)]-bis[5-[(1,5-dihydro-3,4-dimethyl-5-oxo-2*H*-pyrrol-2-ylidene)methyl]-4-methyl-(*Z*,*Z*)-1*H*-pyrrole-3-propanoic acid (**3**) have been described previously [17]. Sodium 2,2′-[(2,17-diethenyl-1,10,19,22,23,24-hexahydro-3,7,13,18-tetramethyl-1,19-dioxo-21*H*-biline-8,12-diyl)bis[(1-oxo-3,1-propanediyl)imino]]bis-ethanesulfonate (bilirubin ditaurine amide, **4**, disodium salt; sold as 'bilirubin conjugate'), obtained from Porphyrin Products (Logan, UT, USA), showed one main peak on high performance liquid chromatography (HPLC) and was used without further purification. Di-*n*-octylamine, phosphatidylcholine (Type XV-E), cholesterol, sodium cholate, taurine and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. Methanol and acetic acid were HPLC grade. Molecular stereopair drawings were made using Ball and Stick version 3.7.6 by Norbert Müller and Heinz Falk, Johannes Kepler University Linz using crystal coordinates for bilirubin IXα [9] and computed global energy minimum coordinates for rubin **3** [17].

2.2. HPLC

Isocratic HPLC analyses were run using a Beckman-Altex ultrasphere-IP 5 μm C-18 ODS column (25×0.46 cm) fitted with a similarly packed precolumn (4.5×0.46 cm) and Hewlett-Packard multiwavelength diode array detector. Compounds of interest were monitored at their absorbance maxima in the HPLC eluant and peak areas measured using HP ChemStation software. The elution solvent was 0.1 *M* di-*n*-octylamine acetate in 8% aqueous methanol, flow rate 0.75 ml/min, and column temperature ~34°C.

2.3. Animal studies

The experimental procedures used have been described elsewhere [20,21]. Briefly, the femoral vein and common bile duct of adult male rats weighing >250 g were cannulated under ketamine anesthesia and a liposomal solution containing phosphatidylcholine (1.5 g), cholesterol (62 mg), sodium cholate (12.95 g) and taurine (3.75 g) in 1 l of water was infused (2 ml/h) through the femoral catheter to maintain hydration and bile flow. The total length of the biliary cannula was 7.5 cm. The animal was placed in a restraining cage under an infrared heating lamp and, once bile flow and body temperature were stable (~30–60 min), the pigment under investigation (0.25 mg), dissolved in normal rat serum (1 ml) with the aid of a small volume (0.1 ml) of DMSO, was infused via the femoral vein as a bolus over a period of 20–45 s. Bile was collected in 20-μl aliquots into micropipets from the tip of the bile duct cannula immediately before injection of pigment and at frequent intervals thereafter for 4 h. Bile samples were flash-frozen immediately in dry-ice, then kept at −70°C until analyzed by HPLC. Bile flow rates were measured gravimetrically by periodically collecting timed 3-min aliquots of bile into tared Pasteur tubes. For HPLC, frozen bile samples (20 μl) were mixed with 80 μl of ice-cold 0.1 M methanolic di-*n*-octylamine acetate, microfuged for 30 s, and the supernate (20 μl) was injected onto the column. Excretion curves were derived by plotting integrated HPLC peak areas, normalized to the maximum peak area, against time. The fraction of the injected dose excreted was estimated by comparing the area under the biliary excretion curve (HPLC peak area versus time), adjusted for total bile volume, with the HPLC peak area of the pigment in a 20-μl sample of the original serum solution injected into the rat. Areas under biliary excretion curves were determined by the trapezoidal method using Un-Scan-It software (Silk Scientific, Inc., Orem, UT, USA). Except for the animal surgery, all procedures were done under orange or red safelights in a darkroom. Although figures show excretion results from single experiments, all were reproduced in at least duplicate experiments. Homozygous Gunn rats and TR^- rats were from colonies maintained at the University of California San Francisco. Sprague–Dawley rats were from local vendors.

3. Results

On reversed phase HPLC, extended rubin **3** is considerably more polar than bilirubin (**1**), but not so polar as bilirubin

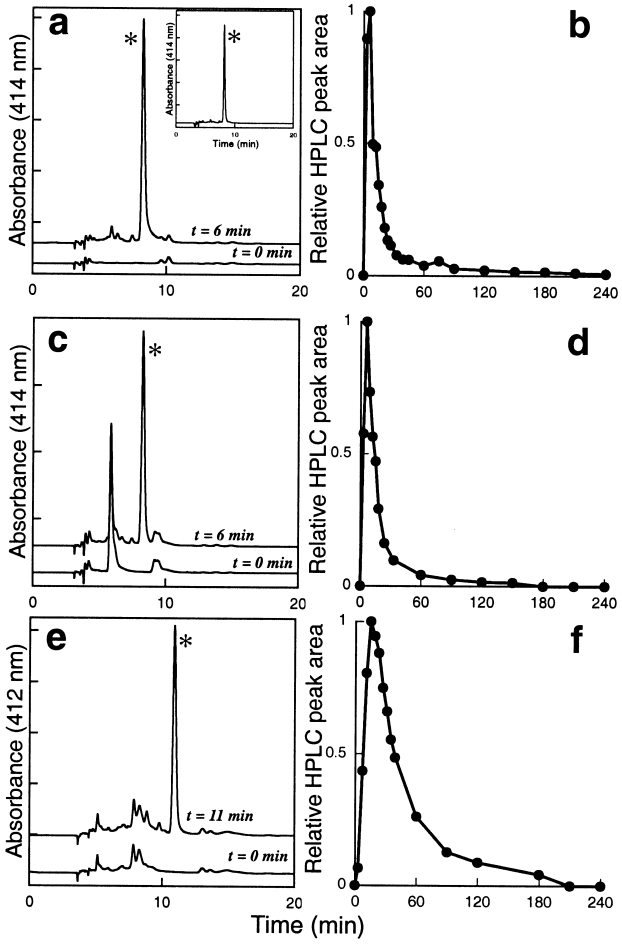


Fig. 1. The left-hand column shows HPLC chromatograms of bile collected just before ($t=0$) and shortly after intravenous injection of a 0.25-mg bolus of rubin **3** into a Gunn rat (a), a Sprague–Dawley rat (c) and a TR^- (e) rat. The right-hand column shows the biliary excretion profiles for **3** in a Gunn rat (b), a Sprague–Dawley rat (d) and TR^- (f) rat. The inset in a shows a representative HPLC chromatogram of the injectate solution used for each animal. The asterisk denotes unchanged **3** and the peaks near 6 min and 9.5 min in c correspond to endogenous bilirubin di- and monoglucuronides respectively. (Different HPLC instruments were used in c and e, resulting in different retention times for **3**.)

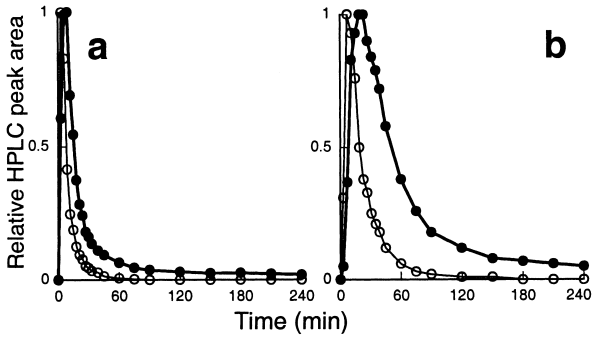


Fig. 2. Biliary excretion profiles obtained after injecting 0.25 mg each of bilirubin ditaurine amide (**4**) and extended rubin **3** simultaneously into (a) a Sprague–Dawley rat and (b) a TR$^-$ rat. Open circles represent **4**; closed circles **3**.

ditaurine amide (**4**). Relative retention factors were 1.0:2.1:9.0 for **4**, **3** and bilirubin, respectively. Rubin **3** is more polar than bilirubin monoglucuronides and less polar than bilirubin diglucuronide.

On intravenous injection into the homozygous Gunn rat, **3** was detectable in bile within 3 min in unchanged form, reaching a maximum concentration by 6 min (Fig. 1a,b). No significant quantities of yellow metabolites of **3** were detected. Similar behavior was seen in the Sprague–Dawley rat (Fig. 1c,d) and glucuronide metabolites of **3** were not detected. The fraction of the injected dose excreted in bile in 4 h was estimated to be 0.5 and 0.6 in the Gunn and Sprague–Dawley rat respectively. In four animals (two Gunn, two Sprague–Dawley) the maximum concentration of unchanged **3** measured in bile ranged from 1.1 to 1.9 times that in the injectate and the mean bile flow rate was 1.7 ml/h.

Rubin **3** was also excreted rapidly in unchanged form when administered to the TR$^-$ rat (Fig. 1e,f), but the biliary excretion curve was broader. The fraction of the injected dose excreted in bile in 4 h was estimated to be 0.4 and the maximum concentration of unchanged **3** in bile was 0.5 and 0.6 times that in the injectate in duplicate experiments. Mean bile flow was 0.9 ml/h.

To better characterize the biliary excretion of **3** in the TR$^-$ rat we ran experiments in which **3** was injected in admixture with an equal weight of bilirubin ditaurine amide (**4**), a compound that is excreted extensively in bile in Sprague–Dawley and Gunn rats and reported to have normal hepatic excretion in TR$^-$ rats [22,23]. Fig. 2a shows biliary excretion profiles obtained when the two compounds were injected simultaneously into a Sprague–Dawley rat. Essentially identical curves (not shown) were obtained in the Gunn rat. In both strains the excretion curves for each compound were similar to those obtained when the two compounds were injected independently, indicating that at the very small doses used ($\sim$1–2 μmol/kg) neither had an effect on the hepatic uptake or efflux of the other. In duplicate experiments, the fraction of injected **4** excreted in bile was 0.8–1.0 and the fraction of **3**, 0.5. The peak concentration in bile for **4** was 4.4–4.6 times that in the injectate, and for **3** equal to that in the injectate. These values are similar to those found when the pigments were injected individually.

Fig. 2b shows biliary excretion curves obtained when the recipient was a TR$^-$ rat, in which the mean bile flow rate was 1.0 ml/h compared to 1.4 ml/h for the animal shown in Fig. 2a. Both pigments were excreted rapidly, but more slowly than in normal rats or Gunn rats. The curve for **3** lags behind that of **4** and is considerably broader, consistent with the greater polarity of **4** compared to **3**. In duplicate experiments the fraction of injected **3** excreted in bile in 4 h was 0.4, whereas excretion of **4** was quantitative. For each compound, the ratio of the maximum concentration in bile to the concentration in the injectate was lower than in Gunn or Sprague–Dawley rats. Thus, for **3** the ratio was 0.4 and for **4** the ratio was 2.4–3.0.

4. Discussion

Our results show extended rubin **3** is cholephilic. When administered intravenously to normal rats, to rats deficient in UGT1A1 and incapable of glucuronidating bilirubin, and to rats lacking the ATP-binding cassette transporter Mrp2, a substantial fraction was excreted rapidly in bile in unchanged form, with the peak concentration in bile exceeding that in the original injectate solution. Rubin **3** differs from natural bilirubin **1** in having the two vinyl groups replaced by methyl groups and the central C10 methylene hinge group replaced by *p*-xylyl. Replacement of the vinyl groups by methyls has been shown to have little effect on the metabolism or lyophilic properties of bilirubins [21]. Replacing the C10 CH_2 group with the planar aromatic *p*-xylyl function might be expected to markedly increase the lipophilicity of the pigment and diminish its biliary excretion. In fact, it has the opposite effect, as shown by the markedly more polar behavior of **3** on HPLC compared to bilirubin and by its rapid excretion in bile in unconjugated form after intravenous administration. This is undoubtedly because insertion of the *p*-xylyl group prevents **3** from forming the preferred, hydrogen-bonded lipophilic

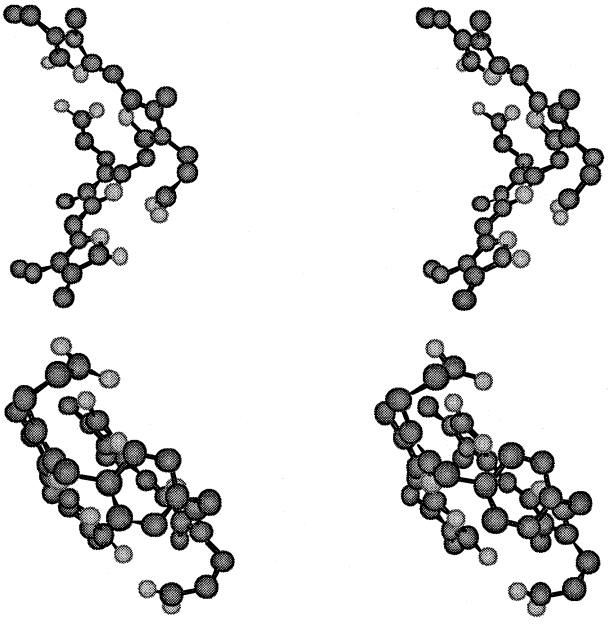
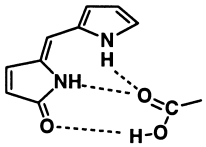


Fig. 3. Stereopair representations of the preferred conformations of (upper) bilirubin IXα (**1**) and (lower) extended bilirubin (**3**). Hydrogen atoms are omitted. Light shading denotes O and N atoms, dark shading C atoms.

ridge–tile conformations that are characteristic of bilirubin in solution and in the crystalline state [9–11]. Instead, as shown by molecular mechanics calculations, **3** preferentially adopts a sandwich type of structure (Fig. 3) in which some intramolecular hydrogen bonding of the propionic acid groups to pyrromethenone lactam groups can still occur, but is weaker and less complete than in bilirubin [17]. This has little apparent effect on hepatic uptake, since both bilirubin and **3** are taken up rapidly by the liver from the circulation. But it has a marked effect on transhepatic transport and efflux into bile. Whereas bilirubin is secreted rapidly into bile only after glucuronidation by UGT1A1, the extended rubin **3** was secreted unchanged and at similar rates in both normal and Gunn rats. This does not necessarily mean that **3** is not a substrate for UGT1A1; merely that glucuronidation is not required for its biliary excretion. However, we suspect that it is unlikely to be a good substrate because it is unable to adopt the structural motif **5** that seems to be preferred by the enzyme for efficient glucuronidation of bilirubins [14].



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Stretched rubin **3** has many structural features present in bilirubin glucuronides. Like them it is undoubtedly a dianion at physiologic pH and on reversed phase HPLC it is even more polar than bilirubin monoglucuronides. In addition it has a hydrophobic aromatic benzene ring at the hinge region of the molecule. It would thus appear to be an ideal candidate for canalicular transport by Mrp2 [8,24]. Yet, it was excreted promptly in bile after administration to TR^- rats that lack Mrp2. Thus, it does not require the presence of Mrp2 for efflux from the liver. In this respect it is similar to the ditaurine amide of bilirubin (**4**) [23], to the disulfonic acid indocyanin green [25,26], to the aliphatic hydroxylated carboxylic acid privastatin [27], and to the glucuronide of the aromatic carboxylic acid telmisaltan [28], which do not require Mrp2 either. However, its biliary excretion was impaired in TR^- rats, which have a much slower bile flow than either Gunn rats or wild type animals. The biliary excretion curve was less sharp and more prolonged than in rats with Mrp2, and the peak concentration and proportion of the dose excreted within 4 h was somewhat lower. This effect was seen most clearly in experiments in which **3** was injected along with ditaurobilirubin (**4**) as an internal standard. Ditaurobilirubin has been shown to be excreted as efficiently in TR^- rats as in normal Mrp2-containing controls [23]. Although either compound could potentially influence the excretion of the other, at the trace doses used in our experiments we found no evidence for this. In normal rats or Gunn rats, stretched rubin **3** showed biliary excretion curves that were slightly delayed with respect to those of ditaurobilirubin (Fig. 2a). This is consistent with the greater HPLC polarity of ditaurobilirubin compared to **3**. In contrast, in the TR^- rat the difference in the biliary excretion curves for the two compounds is much more pronounced (Fig. 2b).

The slower excretion of **3** in TR^- rats compared to normal rats would be consistent with the hypothesis that the compound is, at least in part, actively transported into bile by Mrp2. However, it is also possible that the effect of Mrp2 is a less direct one. Our data offer no insight into this question, nor into the question of how **3** or ditaurobilirubin are secreted into bile in the absence of Mrp2. It might be argued that they are actively transported by some other canalicular organic anion transporter. However, that argument merely raises the alternative question of why the other transporter does not facilitate the efflux of bilirubin glucuronides or even unconjugated bilirubin.

These studies show the crucial role that intramolecular hydrogen bonding and three-dimensional structure can play in the Phase II metabolism and hepatic transport of drugs and, along with other studies [23,26–28], they suggest that Mrp2 might not be essential for the rapid efflux from liver to bile of many non-bile salt organic anions.

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