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In vitro permeability and metabolic stability of bile pigments and the effects of hydrophilic and lipophilic modification of biliverdin

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Abstract—Bile pigments, including bilirubin and biliverdin are tetrapyrrolic, dicarboxylic acids capable of forming conjugates at their propionic acid groups via ester or amide bonds. They possess substantial antioxidant and anti-mutagenic activities and therefore their intestinal absorption might influence the development of cardiovascular disease and cancer. The aim of this study was to investigate whether altering the physico-chemical properties of bile pigments would improve their permeability in an in vitro assay of absorption. Native and synthetically modified bile pigments were tested for gastrointestinal permeability and metabolic stability using the Caco-2 cell line. In addition, a gross measure of their toxic effects was tested in a red blood cell co-incubation assay. The apparent permeability of *unconjugated bilirubin* (**1**), bilirubin ditaurate (**2**) and biliverdin (**3**) through Caco-2 cell monolayers was determined to be $10.4 \pm 1.2 \times 10^{-7}$, $35.2 \pm 3.4 \times 10^{-7}$ and $37.0 \pm 1.6 \times 10^{-7}$ cm/s (mean ± SD), respectively, while biliverdin diglucosamine (**4**), and biliverdin dioctylamine (**5**) were impermeable. Unconjugated bilirubin, biliverdin, bilirubin ditaurate and biliverdin diglucosamine did not decompose when incubated in Caco-2 cell homogenates, whereas biliverdin dioctylamine decomposed over time. Only *unconjugated bilirubin* showed toxicity towards red blood cells ($\geqslant 1000$ μM), an effect that was abolished by the addition of 40 g/L serum albumin. The data presented here suggest that bile pigments are absorbed across the Caco-2 cell monolayer and that conjugation of biliverdin to hydrophilic or lipophilic moieties decreases their absorption and can reduce their metabolic stability. In summary, exogenous bilirubin and biliverdin supplements could be absorbed across the intestinal epithelium in vivo and potentially increase circulating concentrations of these antioxidant compounds.

1. Introduction

Bile pigments are a group of compounds belonging to the *porphyrin* family of molecules. Unconjugated bilirubin (**1**) and biliverdin (**3**; Figs. 1 and 2) are examples of two endogenous bile pigments that possess tetrapyrrolic structure and two propionic acid side chains. Biliverdin (**3**) is one of many pigments excreted in the bile of reptiles, amphibians, birds, and fish and is an end product of heme catabolism.[1] The oxidation of heme to biliverdin is accomplished via the action of cytochrome P450 reductase and heme oxygenase. However, notably in mammals, biliverdin is rapidly reduced to *unconjugated bilirubin* (**1**), via the action of biliverdin reductase.[2] Unconjugated bilirubin, associated with plasma albumin in the circulation, is transported to the liver[3,4] where it is transformed via uridine glucuronosyl transferase to bilirubin mono or diglucuronide.[5] Bilirubin glucuronides are then excreted into the bile via multi-drug resistant protein-2[6] and directed into the gastrointestinal (GI) tract. The conjugation of bilirubin to glucuronic acid is a key event in the excretion of bilirubin, without which, *unconjugated bilirubin* can accumulate to toxic levels.[7]

The study of bilirubin physiology has largely involved understanding the etiology of jaundice in neonates, hereditary disorders of bilirubin excretion and how to prevent bilirubin encephalopathy.[8–11] Previous research suggested that bilirubin is absorbed from the gastrointestinal tract, undergoes entero-hepatic recirculation[12–16] and that altered bilirubin physico-chemistry (i.e., glucuronidation) affects its absorption.[14,16] However, these conclusions are based upon indirect measures of

Abbreviations: EndoH: endovinyl hydrogen; ExoH: exovinyl hydrogen; Is1: isomer 1; Is2: isomer 2.

Keywords: Unconjugated bilirubin; Bilirubin ditaurate; Amide conjugate; Caco-2 cell; Absorption; Stability; Toxicity.

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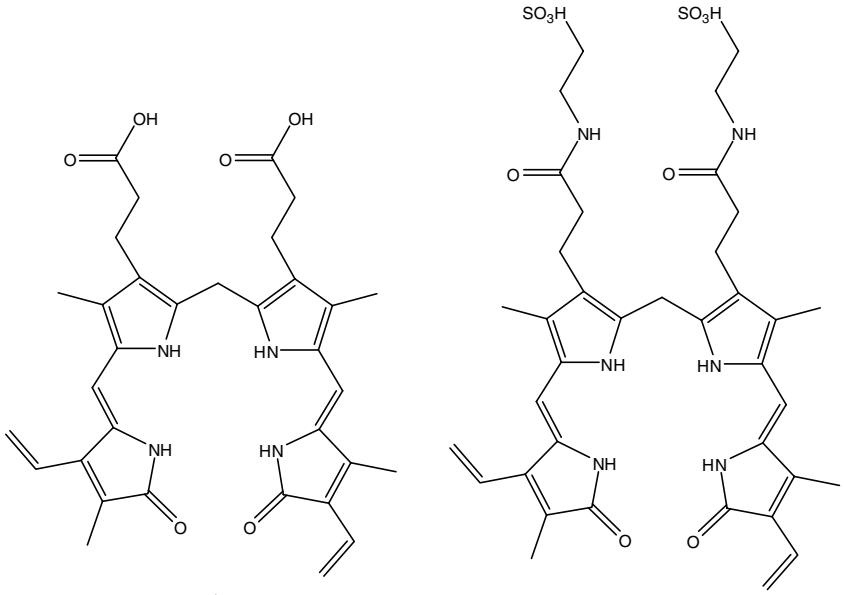

Figure 1. Structures of unconjugated bilirubin (**1**) and bilirubin ditaurate (**2**).

absorption (i.e., the quantification of radio-labeled bilirubin excretion in the bile after intraduodenal administration) and do not control for the distribution of absorbed bilirubins into the tissues of the body. In order to specifically and accurately assess the absorption of these compounds across the intestinal epithelium into the circulation, further experiments must be conducted.

Studying the intestinal absorption of bile pigments has recently become an important research topic because of a shift in the perceived importance of bilirubin and biliverdin[17] which possess antioxidant[18,19] anti-inflammatory[20], and anti-mutagenic[21] properties in vitro. Exogenous administration of these compounds have also proven efficacious in animal models of cardiovascular[22] and gastroenterological[23] pathology. These observations challenge the view that bile pigments are only toxic compounds with little or no physiological importance.[24] The strength of these findings have led researchers to call for studies investigating the degree of bile pigment absorption and their efficacy in pre-clinical trials.[25,26] To address the dearth of research in this area, we used a well-accepted in vitro model of GI absorption, the human colon adenocarcinoma (Caco-2) cell permeability assay, to estimate the in vivo bioavailability of bile pigments. In addition, we studied the effects of altering bile pigment physico-chemical structure to establish whether doing so could improve bile pigment in vitro permeability.

Cultured Caco-2 cells form polarized monolayers, which mimic the epithelium of the human small intestine.[27] When fully differentiated, these cells express all the major digestive enzymes and membrane transporters present in the small intestine and form the tight junctions and a brush border structure characteristic of the small intestine epithelia.[28,29] The apparent permeability coefficients (P_{app}) determined using the Caco-2 cell permeability assay show an excellent correlation to in vivo bioavailability for a wide variety of compounds.[30,31] Furthermore, by adding solubility enhancers (i.e., dimethyl sulfoxide; <2%) in the apical chamber and bovine serum albumin (BSA; 4% w/v) in the basolateral chamber of the assay, the data can predict in vivo absorption of lipophilic compounds that do not dissolve in the assay buffer alone.[32–34] In addition to testing the potential absorption of therapeutics, homogenizing fully differentiated Caco-2 cells provides a complex cocktail of digestive and metabolic enzymes for the assessment of compounds biological metabolic stability.

The aim of this study was to synthesize two novel biliverdin conjugates, biliverdin diglucosamine (**4**), and dioctylamine (**5**); to estimate the in vitro permeability and metabolic stability of these compounds and the bile pigments (**1**–**3**) in Caco-2 cells; and to study whether *unconjugated bilirubin* (**1**), bilirubin ditaurate (**2**), and biliverdin (**3**) were toxic to red blood cells. We could not test the toxic effects of **4** and **5** because of the limited amounts of synthesized product.

2. Results

2.1. Caco-2 cell permeability

All P_{app} data for the tested compounds can be found in Figure 3. When BSA (4% w/v) was included in the basolateral chamber, the permeability (P_{app} ($\times 10^{-7}$ cm/s) ± SD) of unconjugated bilirubin (**1**: 10.4 ± 1.2 cm/s), bilirubin ditaurate (**2**: 35.2 ± 3.4 cm/s), and biliverdin (**3**: 37.0 ± 1.6 cm/s) was significantly greater than in the no BSA condition (**1**: 0.2 ± 0.3 cm/s, $P = 0.016$, **2**: 3.9 ± 6.5 cm/s, $P = 0.010$; **3**: 10.1 ± 4.7 cm/s, $P = 0.011$) although the permeability of mannitol was not different (no BSA: 8.6 ± 0.8; BSA: 14.2 ± 4.8 cm/s; $P = 0.200$;

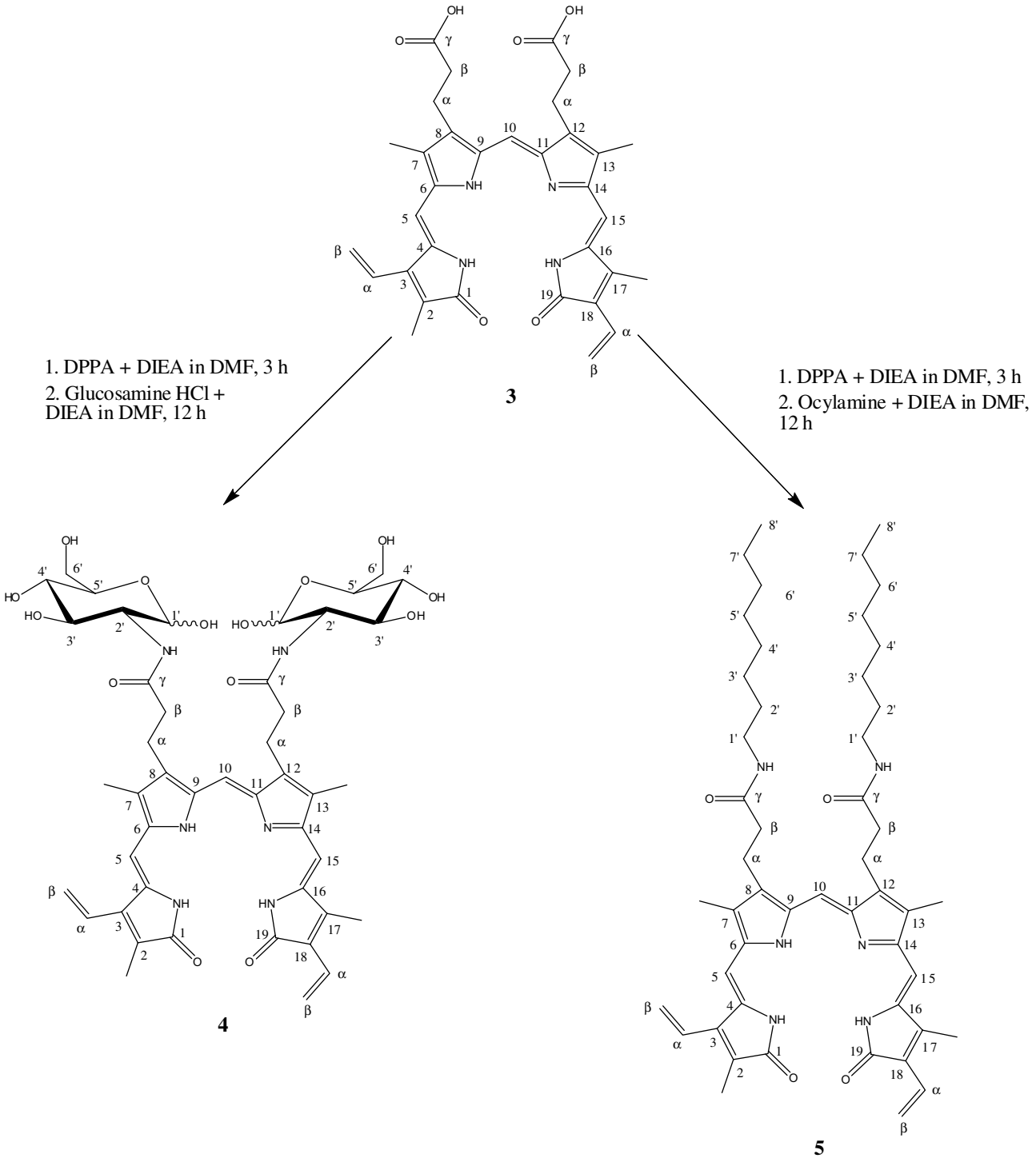

Figure 2. Reaction scheme for the conjugation of biliverdin (**3**) with glucosamine (**4**), and octylamine (**5**).

Fig. 3). When the basolateral compartment contained no BSA, there were no statistically significant differences in permeability between mannitol and any of the tested compounds ($P > 0.05$), however, biliverdin (**3**) was more permeable than unconjugated bilirubin (**1**: $P = 0.016$). When the basolateral compartment contained BSA, the permeability of bilirubin ditaurate (**2**: $P = 0.018$) and biliverdin (**3**: $P = 0.018$), but not unconjugated bilirubin (**1**: $P > 0.05$) was greater than that for mannitol. Bilirubin ditaurate (**2**) was no more permeable than biliverdin (**3**: $P > 0.05$; Fig. 3). The permeability of **4** and **5** could not be detected in either the BSA or the no BSA condition.

2.2. Caco-2 cell homogenate metabolic stability

The resistance of **1**–**5** to enzymatic degradation was tested under two conditions, either in the presence of a Caco-2 homogenate or in the presence of BSA, at the same total protein concentration as in the Caco-2 cell

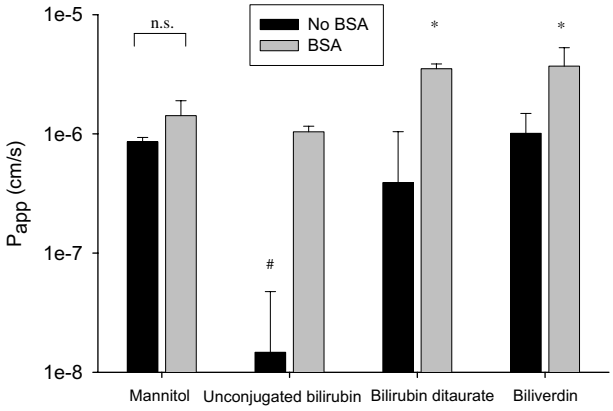

Figure 3. The apparent permeability coefficients (P_{app}) of mannitol, *unconjugated bilirubin*, bilirubin ditaurate and biliverdin in the absence and presence of 4% BSA (w/v) in the basolateral chamber (n = 3–6 per condition; mean ± SD). $^{\#}P < 0.05$ versus biliverdin no BSA; $^{*}P < 0.05$ versus mannitol BSA; n.s.: not significantly different.

homogenate (~0.6 mg/mL). Bile pigments (**1**–**5**) were incubated in BSA alone, as a negative control, to establish whether they degraded as a consequence of non-enzymatic oxidation or their disappearance was due simply to protein binding.

Unconjugated bilirubin (**1**) was not significantly metabolized in the Caco-2 cell homogenate, but was significantly ($P < 0.05$) degraded in the BSA condition (0.33%/min; see Fig. 4). Bilirubin ditaurate (**2**) was not degraded in cells or BSA over time, however, the extraction efficiency of the mobile phase for bilirubin ditaurate appeared different between the conditions ($P < 0.05$; Fig. 1; online supplemental data). Although the biliverdin (**3**) concentration decreased at certain time points during the assay ($P < 0.05$), no time dependant degradation of the compound was obvious (see Fig. 2; online supplemental data) in either the cell homogenate or BSA condition. There was no time dependent digestion of **4** over the experimental period in either the cell homogenate or BSA control condition (see Fig. 3; online supplemental data). Biliverdin

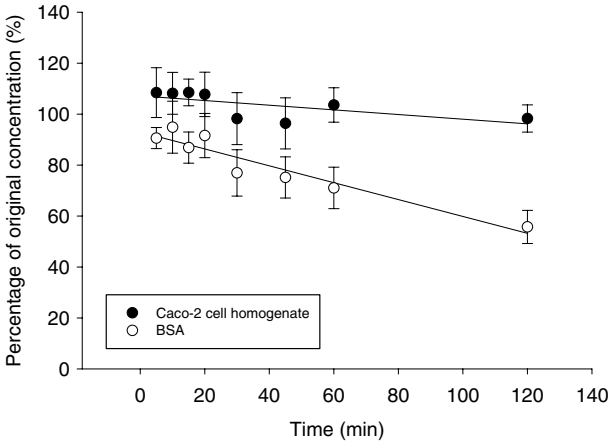

Figure 4. The stability of unconjugated bilirubin (**1**) in BSA and Caco-2 cell homogenates (n = 6 per condition; mean ± SD).

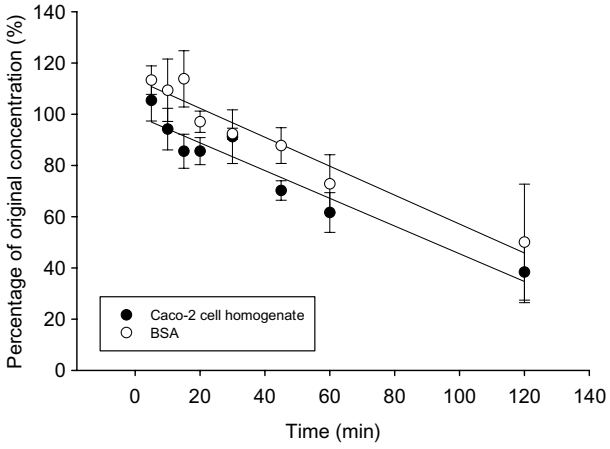

Figure 5. The stability of biliverdin dioctylamine (**5**) in BSA and Caco-2 cell homogenates (n = 6 per condition; mean ± SD).

dioctylamine (**5**) was degraded over the experimental period ($P < 0.05$). The degradation of this compound was not statistically different ($P > 0.05$) in either the cell homogenate (0.54%/min) or BSA condition (0.56%/min; see Fig. 5).

2.3. Red blood cell toxicity

In the absence of BSA, **1** induced hemolysis of red blood cells at concentrations equal to or above 1000 μM ($P < 0.05$ vs 0 μM; Fig. 6). Adding BSA (4% w/v) to the RBC suspension attenuated the hemolytic effect of **1**. Bilirubin ditaurate (**2**) and **3** did not induce hemolysis within the concentration range tested (0–1000 μM), either in the presence or absence of BSA (data not shown). The hemolytic effects of **4** and **5** were not tested due to difficulty in synthesizing sufficient quantities of the compounds (yield 64% and 24%, respectively).

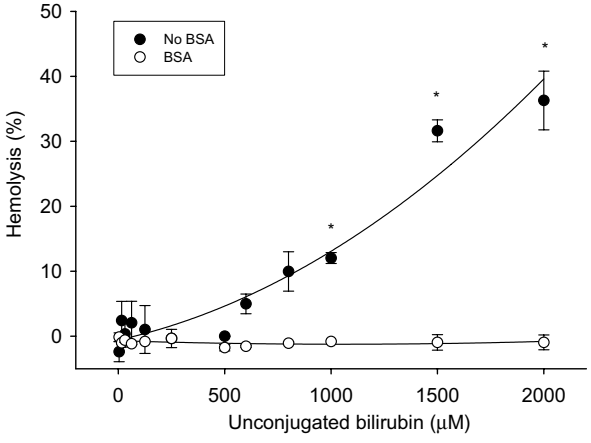

Figure 6. The hemolysis of rat red blood cells in response to increasing concentrations of unconjugated bilirubin in the absence (solid circles) and presence (hollow circles) of 4% BSA (w/v) (n = 5 per concentration; mean ± SD; $^{*}P < 0.05$ vs 0 μM *unconjugated bilirubin*).

3. Discussion

This study aimed to synthesize two novel biliverdin conjugates, biliverdin diglucosamine (**4**) and biliverdin dioctylamine (**5**); to estimate the in vitro permeability and metabolic stability of bile pigments (**1**–**5**) in Caco-2 cells; and to study whether *unconjugated bilirubin* (**1**), bilirubin ditaurate (**2**), and biliverdin (**3**) were toxic to red blood cells. The main findings of this study were the successful conjugation of biliverdin to glucosamine and octylamine, the apparent permeability of **1**, **2** and **3** across Caco-2 cell monolayers and their metabolic stability in homogenates of the same cells.

3.1. Synthesis

The synthesis of bile pigment conjugates presented many difficulties, not least because of the tetrapyrrolic moieties susceptibility to disproportionation and autoxidation in acidic (pH 1–3), or basic (pH 7–10) solution,[35–37] and on exposure to light or excessive heat.[37,38] Previous attempts at bile pigment conjugation using the coupling reagents, carbonyldiimidizole[39,40] and diphenylphosphoryl azide,[41] have been published. While Hancock et al.[41] reported success in conjugating primary amines to bilirubin few details of the reaction conditions (volume, temperature, reaction time), purification and final yields were given. The activation of biliverdin and its conjugation with glucosamine and octylamine proceeded smoothly, over 12 h, as detected by the $HPLC_2$ method. Problems in the purification of **4** and **5** were overcome by loading the crude reaction, diluted in mobile phase, onto a preparative RP-HPLC column and by adjusting the organic content of the loading solvent and gradient accordingly. Due to the poor solubility of the biliverdin conjugates, approximately 20 mg of biliverdin could be conjugated to octylamine/glucosamine and subsequently purified in one attempt. These methods resulted in yields for the two compounds, **4** and **5**, of 64% and 24%, respectively.

For NMR analysis we dissolved **4** and **5** in DMSO-d_6 to maximize their solubility. The use of high powered NMR maximized the quality of proton and carbon spectra, however, with the exception of the aliphatic peaks, most of the biliverdin signals remained broad. Poor resolution of the biliverdin NMR signal has been reported previously[42–44] and could be related to the viscosity of DMSO-d_6 or because biliverdins tend to form aggregates in solution.[42] We used our own and previously published biliverdin spectra[42–44] and spectra of glucosamine and octylamine to assign the NMR signals of the synthesized compounds. Key changes in the chemical shifts of the biliverdin conjugates included the 1.0–1.2 ppm and 2.2–1.1 ppm upfield shift of ^{13}C-resonance for C8α, C12α, and C8β, C12β pairs, respectively, compared to biliverdin (see Fig. 2 for carbon assignment). In addition, an upfield shift of the carboxylic carbonyl groups (1.4–1.5 ppm) occurred upon formation of the amide coupled conjugates. Significant changes in the C1′ and C2′ shifts of octylamine occurred from 41.8 and 33.5 (data not shown) upfield to 38.6 and 29.1, respectively, when conjugated to biliverdin. Shifts were less pronounced for the conjugation of glucosamine from 88.9, 54.5 (C1′, C2′) to 90.5, 54.3, respectively, in the biliverdin diglucosamine conjugate. In addition, the ^1H spectra exhibited new amide hydrogens at 7.5 and 7.7 ppm in the dioctylamine and diglucosamine conjugates, respectively. These data support the analytical HPLC and accurate mass analysis suggesting the successful conjugation of biliverdin to two equivalents of octylamine and glucosamine. (see 5. Experimental).

In the ^1H and ^{13}C spectra of **5** we observed doubling of peaks suggesting the formation of at least two very closely related isomers during the synthesis and/or purification process. Bile pigments are susceptible to disproportionation in basic solution where the asymmetric dipyrrone halves of the tetrapyrrole moiety undergo rearrangement and the formation of IIIα, IXα, and XIIIα isomers.[36] In addition, the rearrangement of the C5, C10 and C15 methine bridges from *syn*-Z to *anti*-E conformations could explain the splitting of carbon signals.[42,44] Finally, the possibility of aggregate formation including the formation of dimers via π–π orbital interaction and hydrogen bonding,[42] could have affected the ^1H and ^{13}C spectra.

3.2. Caco-2 cell permeability

Our results showed that bilirubin ditaurate and biliverdin (**2**, **3**) were more permeable than mannitol (Fig. 3), which is known to cross Caco-2 cell monolayers via paracellular absorption only and so has a very low permeability. Unconjugated bilirubin (**1**) was also permeable, however, the results were no greater than that for mannitol. The mannitol permeability coefficients in the presence of 2% (v/v) DMSO fell within acceptable limits,[30] suggesting it did not affect membrane permeability.

That bilirubin ditaurate (**2**) was more permeable than **1** was a surprising result in this study. Bilirubin ditaurate is an artificially synthesized analogue of physiologically conjugated bilirubin diglucuronide. Studying the in vitro absorption of bilirubin glucuronide is not possible because it is labile and not commercially available.[45] Therefore, studying the permeability of **2** might reflect the absorption of physiologically conjugated bilirubin through the intestinal mucosa. Radio-labeled bilirubins, after intestinal administration in animals, are absorbed and subsequently excreted in the bile. Lester et al.[14,16] reported that the relative intestinal absorption and hepatic excretion of unconjugated bilirubin was greater than that for bilirubin diglucuronide. These data suggest that water soluble bilirubin conjugates may not be readily absorbed. We found that adding the assay buffer (HBSS, $CaCl_2$, $MgCl_2$ and $MgSO_4$ free) to a solution of unconjugated bilirubin in DMSO (2% v/v) caused the formation of visible bilirubin aggregates. Therefore, it is possible the P_{app} of unconjugated bilirubin has been underestimated in this study. Both biliverdin conjugates also aggregated in the apical chamber of the Caco-2 cell permeability assay and therefore, it is likely their permeability has also been underestimated. Increasing the apical DMSO concentration to 10%,[32] including bile acids in apical chamber[46] or slightly increasing the pH of

the assay buffers[47] might assist in determining more accurate P_{app} values for bilirubin and the biliverdin conjugates in future.

The most important finding of this study was that adding BSA to the basolateral chamber significantly improved the permeability of **1**, **2**, and **3** (Fig. 3). The inclusion of BSA did not increase mannitol permeability, suggesting the plasma protein did not disrupt the integrity of the monolayer. That unconjugated bilirubin (**1**) and bilirubin glucuronide bind to circulating albumin,[4,48] provides strong evidence that the inclusion of BSA in the basolateral chamber is essential for the accurate determination of bile pigment permeability and for other lipophilic and plasma protein bound drugs.[33,49]

3.3. Caco-2 cell homogenate metabolic stability

The metabolic stability of bile pigments (**1**–**5**) in homogenates of intestinal epithelial cells has not been studied to date. These results could provide important data on the differing permeability of the compounds based upon their metabolism in Caco-2 cell homogenates. Importantly, we acknowledge the metabolic stability data presented here probably overestimate the bile pigments true in vivo metabolic stability because the Caco-2 cell line lacks cytochrome P450 (CYP3A4) activity.[50]

In this experiment, the solubility of unconjugated bilirubin (**1**) was good and was probably associated with proteins in the Caco-2 cell homogenate.[51] The incomplete solubility of **4** and **5** was still apparent. The results show that **1** was not metabolized significantly by the cellular enzymes over the assay period. A control condition where **1** was incubated with BSA suggested it remained more stable in the cell homogenate (Fig. 4). It is possible that the antioxidant activity in Caco-2 cells spared **1** from oxidation.[52] Interestingly, some authors have suggested that **1** is conjugated to glucuronic acid in intestinal cells,[53,54] however, there was no evidence of this in our HPLC chromatograms. Bilirubin ditaurate (**2**) and biliverdin were not significantly metabolized in the cell homogenate or in the BSA control condition (Figs. 1 and 2; online supplemental data). Very little biliverdin was reduced to bilirubin (via biliverdin reductase[55]) in the cell homogenate condition (data not shown). The sparingly soluble **4** remained stable over the period the assay was run (Fig. 3; online supplemental data). Despite its poor solubility, **5** did not remain stable in either the cell homogenate or BSA, both of which gave the same linear degradation profile (Fig. 5). Clearly, auto oxidation and not enzymatic degradation was responsible for the disappearance of this compound. The metabolic stability of **1**, **2**, and **3** in Caco-2 cell homogenates suggests that enzymatic degradation did not contribute to the varying permeability of these compounds.

3.4. Red blood cell toxicity

A crude measure of red blood cell toxicity was calculated only for the bile pigments **1**, **2**, and **3** because insufficient quantities of **4** and **5** were synthesized. Unconjugated bilirubin exerts toxic effects on RBC's,[56] however, there have been no previous reports concerning the toxic effects of **2** or **3**. In our experiment only unconjugated bilirubin described a toxic effect, doing so at concentrations greater than or equal to 1000 μM. This effect was attenuated by the addition of 4% (w/v) BSA (Fig. 6). Therefore, it is unlikely that **1** would exert toxic effects on red blood cells at concentrations below the binding capacity of circulating albumin (~35 mg/dL; 600 μM),[57] in vivo.

4. Conclusion

This study describes the first in vitro permeability data for endogenous and synthetic bile pigments. The data suggest that *unconjugated bilirubin*, bilirubin ditaurate and biliverdin are absorbed through cells that share a similar morphology and function to intestinal epithelial cells. The Caco-2 cell permeability assay may be useful for investigating ways to improve the bioavailability of bile pigments, however, it should be noted that some limitations in the in vivo prediction of bile pigment absorption might exist using the model (i.e., the lack of bile salts and a mucosa, which might aid in bile pigment absorption). The metabolic stability results show that native bile pigments are relatively stable compounds, which Caco-2 cells do not metabolize. Bile pigments have been reported to possess antioxidant,[58] anti-inflammatory[20], and anti-mutagenic[21] actions and therefore their supplementation and intestinal absorption might prove beneficial.

5. Experimental

5.1. General

5.1.1. Materials. Peptide grade dimethyl formamide (DMF) was purchased from Lomb Scientific (Taren Point, NSW, Australia). All other reagents were of analytic reagent grade (DMSO) or better (diphenylphosphoryl azide, DPPA; di-*n*-octylamine; octylamine; glucosamine hydrochloride; trifluoroacetic acid, TFA; acetonitrile, MeCN; methanol, MeOH) and were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Transwell polycarbonate inserts were obtained from Costar (Cambridge, MA, USA) and cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). A modified Hanks buffered salt solution (HBSS; $CaCl_2$, $MgCl_2$, and $MgSO_4$ free; Invitrogen, Mount Waverley, Vic., Australia) was purchased so as to prevent the formation of a calcium bilirubinate precipitate when unconjugated bilirubin was added to HBSS solutions. Unconjugated bilirubin (**1**; >98% pure) [635-65-4] was obtained from BioPharma Pty. Ltd (Brisbane, Qld, Australia) bilirubin ditaurate disodium (**2**) [89771-93-7] and biliverdin hydrochloride (**3**) [55482-27-4] and were obtained from Frontier Scientific (Logan, UT, USA). All bile pigments were tested in their native form and were not radio-labeled. In addition all chemical reactions

were performed in solvents purged with nitrogen and in vessels protected from light.

5.1.2. Preparative high performance liquid chromatography. Purification of the synthesized biliverdin conjugates was achieved using preparative RP-HPLC on a C4 silica column (Grace/Vydac, Deerfield, IL, USA; 250 mm × 25 mm) using a HPLC pump (Waters, Milford, MA, USA; 10 mL/min). Gradient elution separated the compounds of interest using a mixture of solvent A (H_2O [0.1% TFA]) and solvent B (H_2O/MeCN 10:90 v/v [0.1% TFA]).

5.1.3. Analytical high performance liquid chromatography. The purity of the synthesized biliverdin conjugates (**4**, **5**) was assessed using two separate RP-HPLC protocols. The first protocol (RP-$HPLC_1$) used an isocratic mobile phase of di-*n*-octylamine acetate (0.1 M) in methanol/H_2O (95:5 v/v) over 20 min, at a flow rate of 1 mL/min. The mobile phase perfused an Ultrasphere IP C18 reverse phase HPLC guard (Beckman-Coulter, Fullerton, CA, USA; 5 μm, 45 mm × 4.6 mm) and analytical RP-HPLC column (Beckman-Coulter, Fullerton, CA, USA; 5 μm, 250 mm × 4.6 mm) and spectrophotometric data were collected using a photo diode array detector (SPD-M10A, Shimadzu, Japan).

The second protocol (RP-$HPLC_2$) used a mixture of solvent A (H_2O [0.1% TFA]) and solvent B (H_2O/MeCN 10:90 [0.1% TFA]), 10–100% solvent B over 30 min, followed by 100% solvent B for 5 min, at a flow rate of 1 mL/min. The mobile phase perfused a C18 RP-HPLC column (Agilent, Santa Clara, CA, USA; 3 μm, 150 mm × 4.6 mm) and the spectrophotometric detector (1100, Agilent, Santa Clara, CA, USA) was set at 375 nm.

5.1.4. Nuclear magnetic resonance spectroscopy. NMR spectra of the starting material biliverdin (**3**), and the synthesized biliverdin conjugates (**4**, **5**) were measured on either a Bruker Avance 750 MHz (5 mm broadband probe) or a Bruker Avance 900 MHz (5 mm TCI cryoprobe) instrument. The spectra were run at room temperature in DMSO-d_6. ^{13}C spectra were typically collected with a pulse width of 45–90° and a 2-min recycle time. The reported assignments are approximations based on previously published biliverdin spectra.[42,44] The quality and resolution of the spectra were insufficient to obtain 2D-spectra to confirm our assignment.

5.1.5. Mass spectrometry. HRESIMS accurate mass measurements were recorded in positive mode on a Bruker MicrOTOF-Q (quadrupole—Time of Flight) instrument with a Bruker ESI source. Accurate mass measurements were carried out with external calibration using Agilent Tunemix as reference calibrant.

5.2. Synthesis

Biliverdin conjugates were synthesized using biliverdin hydrochloride (**3**) as a starting material, purchased from Frontier Scientific (Logan, UT, USA). Reverse phase HPLC and NMR characterization of this compound were conducted so that the synthesized biliverdin conjugates (**4**, **5**) could be compared to the retention times and NMR spectra of the starting material. Biliverdin (**3**) was analyzed using both RP-HPLC protocols ($HPLC_1$; purity 92.9%, rt = 5.86 min; $HPLC_2$; purity 94.3%, rt = 17.29 min). ^1H NMR 750 MHz, (DMSO-d_6) δ 11.9 (br s, 1H, pyrrole NH); 10.2, 10.0 (2 × br s, 1H, lactam NH); 7.1 (br s, 1H, H10); 6.8 (dd, J = 11.5, 17.6 Hz; 1H, exoHx), 6.6 (dd, J = 11.5, 17.5 Hz; 1H, endoHx); 6.2 (s, 1H, H5); 6.1 (s, 1H, H15); 6.1 (d, J = 18 Hz; 1H, endoHb); 5.7 (d, J = 18 Hz; 1H, exoHb); 5.7 (d, J = 11.6 Hz; 1H, exoHa); 5.4 (d, J = 11.6 Hz; 1H, endoHa); 2.9 (m, 4H, CH_2–CH_2–COOH); 2.5 (m, 4H, CH_2–CH_2–COOH); 2.2, 2.1, 2.1 (3 × s, 3H, C7-Me, C17-Me, C13-Me); 1.9 (s, 3H, C2-Me). ^{13}C NMR 188 MHz, (DMSO-d_6) δ 173.2 (2 × COOH), 171.4 (C1), 170.6 (C19), 150.8 (C14), 147.6 (C6), 141.1 (C9,11), 140.7 (C4,16), 139.4 (C3, C17), 132.5 (C8, C12), 129.3 (C18), 128.5 (C13) 128.2 (C7), 127.8 (C2), 126.5 (C3α), 126.3 (C18α), 122.6 (C3β), 119.7 (C18β), 113.8 (C10), 96.7 (C5), 96.0 (C15), 34.8 (C8β, C12β), 19.3 (C8α, C12α), 9.2–8.9 (C2-Me, C7-Me, C13-Me, C17-Me).

5.2.1. Biliverdin diglucosamine (4). Biliverdin (**2**; 20 mg, 0.032 mmol) was added to a dry 25 mL round bottom flask containing 2 mL of dry DMF. Diphenylphosphoryl azide (DPPA; 27.8 μL, 0.129 mmol; 4 equiv) was added to the solution, followed by diisopropylethylamine (DIPEA; 5.6 μL, 0.129 mmol; 4 equiv). The reaction was stirred under N_2 for 3 h. Glucosamine hydrochloride (28.6 mg, 0.129 mmol; 4 equiv) was added to the reaction, followed by an excess of DIPEA (5.6 μL, 0.129 mmol; 4 equiv). The pH of the reaction contents was 9. The reaction was left to stir overnight (12 h), under N_2.$HPLC_2$ analysis of the reaction suggested mono and diconjugated biliverdin species were present and the disappearance of the starting material. The reaction was diluted with 50 mL of 80% solvent A/20% solvent B, filtered using a 0.45 μm syringe filter, and loaded onto a preparative C4 RP-HPLC column. The compound was purified using a linear gradient of 20–100% solvent B over 60 min. $HPLC_2$ was used to determine which fractions contained pure **4** and these were lyophilized. Analytical $HPLC_{1/2}$ confirmed the purity of the resultant powder. $HPLC_1$ analysis suggested the compound was homogenous (purity: 94.5%, rt: 3.15 min). However, $HPLC_2$ analysis showed the lyophilized powder comprised of two closely eluting compounds of equal area (purity: 94.2%, rt: 13.6, 13.7 min), yield: 64.1%. NMR analysis suggested one conformation of the biliverdin conjugate was present. ^1H NMR 750 MHz, (DMSO-d_6) δ 11.8 (br d, 1H, pyrrole NH); 10.7 10.6 (2 × br s, 1H, lactam NH); 7.7 (br s, 2H, amide); 7.5 (br s, 1H, H10); 6.9 (br t, 1H, exoHx), 6.7 (br t, 1H, endoHx); 6.3 (m, 3H, H5, H15, endoHb); 5.8 (br m, 2H, exoHa and b); 5.5 (br s, 1H, endoHa); 4.9 (s, 2H, H1′); 3.6–3.5 (m, sugar Hs); 3.5–3.4 (m, sugar Hs); 3.1 (t, J = 9.2 Hz, 2H, H5′); 3.03 (m, 4H, CH_2–CH_2–CONH); 2.5 (m, 4H, CH_2–CH_2–CONH); 2.3, 2.2, 2.1 (3 × s, 3H, C7-Me, C17-Me, C13-Me); 2.0 (s, 3H, C2-Me). ^{13}C NMR 188 MHz, (DMSO-d_6) δ 171.8 (2 × CONH), 171.4 (C1), 171.1 (C19), 152.2 (C14), 150.0 (C8, C12),

147.4, 147.0 (C6, split), 146.0 (C11), 143.0, 142.4 (C9, split), 141.4 (C4, C16), 139.7 (C3, C17), 131.7 (C8, C12), 130.1 (C18), 129.6 (C13), 129.3 (C7), 128.6 (C2), 127.1 (C3α), 126.3 (C18α), 124.0 (C3β), 121.6, (C18β), 118.7 (C10), 95.2 (C5), 94.7 (C15), 90.5 (C1′), 76.7 (unassigned), 72.0 (C3′), 71.0 (C4′), 70.4 (C6′), 61.0 (C5′), 54.3 (C2′) 36.4, 35.9 (C8β, C12β), 20.3 (C8α, C12α), 9.7–9.2 (C2-Me, C7-Me, C13-Me, C17-Me). HRESIMS: Calcd for $C_{45}H_{57}N_6O_{14}$ = 904.3855, obsd = 905.3898 [M+H$^+$].

5.2.2. Biliverdin dioctylamine (5). Essentially the same experimental activation procedure as above was followed. Octylamine (5.4 μL, 0.129 mmol; 4 equiv) and DIPEA (5.6 μL, 0.129 mmol; 4 equiv) were added to the activated biliverdin solution. After leaving the solution to stir overnight, HPLC analysis of the reaction suggested mono and diconjugated biliverdin species were present with the disappearance of the starting material. The crude reaction was then diluted in 50 mL 30% solvent A/70% solvent B, filtered through a 0.45 μm syringe filter and loaded onto a preparative C4 RP-HPLC column. Purification of the filtrate was achieved using a linear gradient of 70–100% solvent B over 30 min, followed by 100% solvent B for 15 min. The purified product was isolated as described above. Analytical $HPLC_{1/2}$ confirmed the purity of the resultant powder. $HPLC_1$ and $HPLC_2$ analysis suggested the compound was homogenous (purity: 92.3%, rt: 7.44 min, purity: 93.9%, rt: 32.7 min, respectively), yield: 24.2%. ^{1}H and ^{13}C NMR spectra showed clear evidence of two closely related isomers or conformers formed in a ratio of ~3:2 (Is1:Is2). ^{1}H NMR 900 MHz, (DMSO-d_6) δ 11.8, 11.7 (2 × br s, 1H, pyrrole NH, Is1&2); 10.7, 10.6 (2 × br s, 1H, lactam NH, Is1); 10.3, 10.0 (2 × br s, 1H, lactam NH, Is2); 7.9 (br s, 1H, amide, Is2); 7.8 (br s, 1H, amide, Is1); 7.5 (br s, 1H, H10 Is2); 6.9 (m, 2H, exoHx Is2, H10 Is1), 6.8 (br t, J = 13.86 Hz; 1H, exoHx Is1); 6.7 (br t, J = 12.1, 1H endoHx Is2); 6.6 (br t, J = 13.1, 1H endoHx Is1); 6.3 (m, 3H, C5, C15, endoHb Is2); 6.1–6.0 (m, 3H, H5, H15, endoHb Is1); 5.8, 5.7 (2 × m, 2H each, exoHa, exoHb Is1&2); 5.6 (br d, J = 7.0 Hz; 1H, endoHa Is2); 5.4 (br d, J = 9.3 Hz; 1H, endoHa Is1); 3.0 (m, 4H, CH_2–CH$_2$–CONH Is2); 2.9 (br m, 10H, CH_2–CH$_2$–CONH Is1 + octyl chain Hs); 2.8 (br s, 4H, CH$_2$–CH_2–CONH Is1); 2.4 (br s, 4H, CH$_2$–CH_2–CONH Is2); 2.3 (br s, 6H, 2 × Me) 2.2 (s, 3H, Me); 2.1 (br s, 6H, 2 × Me); 2.0, 2.0, 1.8 (3 × s, 3H each, 3 × Me); 1.20 (m, 8H, octyl Hs); 1.2–1.1 (m, 40H, octyl Hs); 0.82 (t, J = 7.3 Hz, 3H, Me Is1); 0.81 (t, J = 7.3 Hz, 3H, Me Is2). ^{13}C NMR 226 MHz, (DMSO-d_6) δ 171.7 (2 × CONH), 171.1 (C1), 170.9 (C19), 151.0 (C14), 149.9 (C8, C12), 147.7, 147.0 (C6; Is$_1$, Is$_2$), 146.0 (C11), 143.0, 142.4 (C9; Is1, Is2), 141.4, 139.7 (C4, C16), 140.0, 139.2 (C17; Is1, Is2), 139.0, 138.0 (C3; Is1, Is2), 131.7, 131.6 (C8, C12), 129.6 (C18) 128.9, 128.7 (C13), 128.1, 128.0 (C7) 127.3, 127.2 (C2) 126.8 (C3α), 126.2 (C18α), 124.0 (C3β), 121.6 (C18β), 118.6 (C10), 97.8, 97.1 (C5; Is1, Is2), 95.0, 94.5 (C15; Is1, Is2), 38.6 (C1′), 37.0, 36.2 (C8β, C12β), 31.3 (C6′), 29.1 (C2′), 28.7–28.6 (C4′–C5′), 26.4 (C3′), 22.1 (C7′), 20.5, 20.3 (C8α, C12α), 13.9 (C8′) 9.7–9.1 (C2-Me, C7-Me, C13-Me, C17-Me). HRESIMS: Calcd for $C_{49}H_{69}N_6O_4$ = 804.5302, obsd = 805.5352 [M+H$^+$].

5.3. Caco-2 cell permeability assay

The cell culture and experimental conditions were similar to those in our previously published work,[59] however, we used a modified ($CaCl_2$, $MgCl_2$, and $MgSO_4$ free) Hank's buffered salt solution. Permeability studies for **1**–**5** were carried out both in the absence and presence of 4% (w/v) bovine serum albumin in the basolateral chamber and the presence of 2% DMSO in the apical chamber. All experiments were conducted at least in triplicate.

The permeability of ^{14}C-mannitol was measured in order to assess the viability of the monolayers in the absence and presence of BSA in the basolateral compartment. All samples from the basolateral layer were frozen immediately on dry ice after collection. The permeability coefficient (P_{app}) of each compound was calculated using a previously published formula.[60]

5.4. Caco-2 cell homogenate metabolic stability assay

A detailed description of the Caco-2 cell homogenate metabolic stability assay can be found elsewhere.[61] HBSS ($CaCl_2$, $MgCl_2$, and $MgSO_4$ free) was used in place of regular HBSS and the total protein content of the homogenated Caco-2 cell supernatant or BSA (control) was adjusted with HBSS to ~0.6 mg/mL in a clean 96-well plate.

The compounds to be tested were first dissolved 2% DMSO (of final volume) and then HBSS-25 mM Hepes buffer, to a final concentration of 200 μM. Hundred microliters of this solution was added to each well containing 100 μL of cell homogenate. The assay was performed at 37 °C with shaking at 400 rpm in the dark. Samples (20 μL) were taken at selected time points (0, 5, 10, 15, 20, 30, 45, 60, and 120 min) and immediately frozen on dry ice. The concentration of the test compounds in each sample was determined by RP-HPLC (see below). Results are presented relative to the concentration of the compound at time 0 (100% = 100 μM).

5.5. Sample analysis

Samples from the Caco-2 cell permeability and homogenate stability assay were analyzed using the two previously described RP-HPLC analytical techniques. Unconjugated bilirubin (**1**), **2** and **3** were analyzed using the $HPLC_1$ method and compounds **4** and **5** were analyzed on the $HPLC_2$ method. Samples from the Caco-2 cell permeability (100 μL) and homogenate stability assay (20 μL) were combined with 200 μL and 80 μL of mobile phase, respectively. For compounds **1**–**3** methanolic di-*n*-octylamine acetate mobile phase was added, whereas 100% solvent B was added for **4** and **5**. The samples were combined with these mobile phases so as to precipitate any protein within the sample, vortexed briefly, centrifuged at 10,000*g* for 5 min and 50 μL of the supernatant was injected onto the respective col-

umns. Correction for the dilution of each sample was made prior to data analysis.

The retention times for **1** (λ_{max} 450 nm), **2** (λ_{max} 450 nm), and **3** (λ_{max} 375 nm) were 15.5, 4.4 and 5.9 min, respectively ($HPLC_1$). The retention times of **4** and **5** were 13.6 and 32.7 min, respectively ($HPLC_2$). In both HPLC assays, the sample concentrations of the compounds were quantitated using solutions of known standard concentrations (0.2–200 μM).

5.6. Red blood cell toxicity experiment

Permission from the University of Queensland Animal Ethics Committee was obtained prior to conducting the red blood cell toxicity study. Blood from three male Wistar rats was collected and placed into sodium heparin vacutainers. A detailed description of the study method can be found in previously published work.[59] The experimental conditions were identical except that the bile pigments (**1**–**3**) were solubilized as their sodium salts pH ~ 8 before being added to RBC suspensions. Each compound was tested at concentrations spanning 0–1000 μM ($n = 5$). The concentration range tested for unconjugated bilirubin was extended beyond 1000 μM because toxic effects were observed at this concentration (see Section 2). Bovine serum albumin was also added at a concentration of 4% (w/v) to assess the effects of protein binding on bilirubin toxicity.

5.7. Statistical analysis

Statistical analysis was conducted using SigmaStat Ver. 3.0. The change in the permeability coefficient for each compound was compared to that for mannitol using one-way analysis of variance followed by Tukey's post hoc test. The time dependant change in bile pigment and hemoglobin concentrations (Caco-2 cell homogenate stability, hemolysis assays) was tested using repeated measures analysis of variance and Bonferroni post hoc tests. If the data were not normally distributed a repeated measures analysis of variance on Ranks followed by Tukey's post hoc tests evaluated the significance of the results. A P-value of <0.05 was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.008.

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