Cyanidin 3-glucoside Is a Selective Inhibitor of Hepatic Bilirubin Uptake

Paola Pelizzo
University of Trieste https://orcid.org/0000-0001-7985-0582
Marco Stebel
University of Trieste https://orcid.org/0000-0002-8279-8065
Nevenka Medic
University of Trieste https://orcid.org/0000-0002-1659-1461
Paola Sist
University of Trieste https://orcid.org/0000-0003-1626-5081
Andreja Vanzo
Agricultural Institute of Slovenia https://orcid.org/0000-0002-3648-3775
Andrea Anesi
Edmund Mach Foundation https://orcid.org/0000-0002-9334-2610
Urska Vrhovsek
Edmund Mach Foundation https://orcid.org/0000-0002-7921-3249
Federica Tramer
University of Trieste https://orcid.org/0000-0003-4286-0191
Sabina Passamonti (spassamonti@units.it)
University of Trieste https://orcid.org/0000-0001-7876-4666

Research Article

Keywords: Bilirubin, cyanidin-3-O-beta-glucopyranoside, pravastatin, estradiol-17 beta-glucuronide, taurocholate, organic anion transporters, liver, rat

Posted Date: December 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1141319/v1

Version of Record: A version of this preprint was published at Biomedicine & Pharmacotherapy on January 1st, 2023. See the published version at https://doi.org/10.1016/j.biopha.2022.114044.

Abstract

Background & Aims: One of the organ-specific functions of the liver is the excretion of bilirubin into the bile. Membrane transport of bilirubin from the blood to the liver is not only an orphan function, as there is no link to the protein/gene entities that carry it out, but also a poorly characterised function. The aim of this study was to investigate the pharmacology of bilirubin uptake in the liver of the female Wistar rat to improve basic knowledge in this neglected area of liver physiology.

Methods: We treated isolated, perfused rat livers with repeated single-pass, albumin-free bilirubin boli. We monitored both bilirubin and bilirubin glucuronide in perfusion effluent with a biofluorometric assay. We tested the ability of nine molecules known to be substrates or inhibitors of sinusoidal membrane transporters to inhibit the hepatic uptake of bilirubin.

Results: We found that cyanidin 3-glucoside and malvidin 3-glucoside are the only molecules that inhibit bilirubin uptake. These dietary anthocyanins resemble bromosulfophthalein (BSP), a substrate of several sinusoidal membrane transporters. The SLCO-specific substrates estradiol-17 beta-glucuronide, pravastatin, and taurocholate inhibited only bilirubin glucuronide uptake. Cyanidin 3-glucoside and taurocholate acted at physiological concentrations. The SLC22-specific substrates indomethacin and ketoprofen were inactive. We demonstrated the existence of a bilirubin glucuronide transporter that is inhibited by bilirubin, a fact reported only once in the literature.

Conclusions: Data indicate that bilirubin and bilirubin glucuronide are transported into the liver via pharmacologically distinct membrane transport pathways. Some dietary anthocyanins may physiologically modulate the uptake of bilirubin into the liver.

Introduction

Bilirubin (BR) is the end product of cellular heme catabolism and circulates in the blood as a reversible complex with albumin. Its normal serum concentration is 5-17 µM [1], with a small amount of bilirubin diglucuronide (BRG) [2]. These values are steady-state values resulting from the balance between daily production of about 300 mg in adult humans and hepatic excretion of bilirubin diglucuronide into bile. The hepatic metabolism of BR involves transport from the blood to the liver, intracellular binding to glutathione S-transferase and conjugation with glucuronic acid by UDP-glucuronosyltransferase (UGT1A1), and transport into the bile by MRP2 (ABCC2)[2].

Elements for a molecular and pharmacological description of hepatic BR uptake are still lacking [3], although research started five decades ago. Based on kinetic data, the uptake of BR into the liver has been described as transporter-mediated, as it has been shown to be inhibited by bromosulfophthalein (BSP) and indocyanine green [4]. The concept that the sinusoidal membrane transporters OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*) are mediators of hepatic bilirubin transport has become widespread in the literature, although the evidence is not strong, as shown by a systematic review of the available data [5]. Moreover, studies on the effects of SLCO-specific drugs on bilirubinemia have consistently

shown that only conjugated bilirubin (direct bilirubin) is an endogenous biomarker for the inhibition of Oatp in rats [6] and humans [7].

Therefore, hepatic uptake of BR today is not only an orphan transport activity, since there is no link to proteins/genes that carry out this activity [8], but also a still poorly characterised function, despite being a pillar of liver physiology and clinical diagnostics.

The aim of this study was therefore to investigate the pharmacology of BR uptake in the isolated perfused rat liver model, which preserves the morphology and function of the organ. To achieve this goal, we measured BR and bilirubin glucuronides (BRG) in liver perfusion effluent using a new fluorometric method based on a recombinant protein fused to UnaG [9]. We investigated the property of nine molecules, recognised as standard substrates or inhibitors of sinusoidal membrane transporters for organic anions, to inhibit the uptake of BR into the liver.

We found that cyanidin-3-O-beta-glucopyranoside (cyanidin 3-glucoside, C3G) and its congener malvidin 3-glucoside (M3G) (but not peonidin 3-glucoside, P3G) are the only molecules that inhibit the uptake of BR. Both belong to the anthocyanin family (a subclass of flavonoids) and are found in red berries, black rice, and red wine, among others [10]. Both are competitive inhibitors [11] of bilitranslocase (TCDB 2.A.65.1.1), a genetically orphan hepatic transporter for organic anions [12]. No anthocyanin is included in the list of flavonoids targeting members of the SLCO and SLC22 transporter superfamilies expressed in the liver [13–17]. The Oatp substrates pravastatin, estradiol-17 beta-glucuronide and taurocholate inhibited only BRG uptake. Indomethacin and ketoprofen, both substrates of Oat2 (Slc22a7), were inactive in the uptake of both BR and BRG. Moreover, our experiments on inhibition of uptake of BR by C3G showed that there is a BRG transporter that is inhibited only by BR, a fact that has been reported only once in the literature [18].

Methods

The isolated perfused rat liver was chosen as the best experimental model for BR sinusoidal uptake because of the preservation of the hepatic architecture that supports vectorial hepatobiliary transport processes. The livers of female Wistar rats were isolated and perfused with an albumin-free solution, without the addition of oxygen, to prevent the formation of radical species and redox reactions with bilirubin and anthocyanins. The solution contained glucose and the pyruvate/lactate pair to support substrate-level phosphorylation of ADP. This formula ensured organ viability and functional integrity for the duration of the uptake assays (1 hour after isolation). Up to 12 repeated boli containing BR or other compounds targeting sinusoidal membrane transporters were injected into the portal vein of a single preparation. BR, BRG, anthocyanins, and taurocholate were analysed in 120 fractions of liver perfusion effluent as described in the Supplementary Data. This study has received bioethical approval from the Italian government (project code 3379PAS18, N. 65/2019- PR, by the Ministry of Health). The methods and results comply with the ARRIVE guidelines [19].

Data Analysis

GraphPad Prism 9.1.2 software (225) was used for graphical representation of data, calculation of peak parameters (AUC, t_{max}, C_{max}), boxplot analysis of data sets (median, mean, and SD), and evaluation of differences between means by unpaired Student's t test.

Results

Hepatic uptake of bilirubin: the effect of cyanidin 3-glucoside and pravastatin

The initial tests were performed with two different inhibitors (molecular targets and kinetics in Suppl. Table 1). One was C3G (chemical structure in Suppl. Fig. 3), which inhibits the transport activity of bilitranslocase (K_i = 5.8 µM). The other was pravastatin, a specific substrate of human OATP1B1, Oatp2 (*Slco1a4*) and Oat (*Slc22a7*) from rat liver, but not of bilitranslocase.
Fig. 1a shows that when BR was injected alone or with pravastatin, some BR (10.9 pmol; 0.11%) was recovered in the effluent, whereas when it was co-injected with C3G, a sharp and high BR peak occurred, indicating inhibition of the uptake of BR. This effect was transient, as the next BR bolus was again almost completely absorbed.

In this study, inhibition of uptake of BR by C3G was performed at least once in all experiments testing pravastatin and other inhibitors as a functional control of liver preparations. No differences were observed between the BR peaks elicited by C3G that might depend on the order of administration (Suppl. Fig. 3). Therefore, we performed a comprehensive analysis of all C3G-induced peaks compared to all controls. Figure 1b shows peak analysis of BR boli alone or with either C3G or pravastatin. In contrast to pravastatin, which was inactive, C3G caused a 3-fold increase in AUC. The effect of C3G on C_{max} was even more pronounced, increasing 6-fold. We found that the t_{max} of the BR peak was decreased by C3G and increased by pravastatin (peak parameters in Suppl. Fig. 4).

Fig. 1C shows the frequency of C3G effect size from case to case. The data show that the most common observation (21%) was a 2-fold increase in AUC, although 61% of observations were above this value and notable effects (> 5-fold increase) were obtained in 16% of cases. Negative effects (AUC ratio = 1) were in the minority (7.5 %) and occurred randomly (and never serially) with some preparations.

The dose-response relationship was analysed at either constant (Suppl. Fig. 5A) or variable BR (Suppl. Fig. 5B) dose. It was found that small changes in bolus concentrations around the value of 0.05 mM resulted in large changes in the peak areas of BR, which might explain the variable effect size of C3G, shown above in Fig. 1C.

Another anthocyanin, such as malvidin 3-glucoside but not peonidin 3-glucoside (chemical structures in Suppl. Fig. 3), inhibited the uptake of BR similarly to C3G (Suppl. Fig. 6).

Hepatic uptake of cyanidin 3-glucoside: the effect of bilirubin

If C3G inhibits a BR transporter in the liver, one might think that the opposite should be true. Therefore, we repeatedly injected C3G in increasing concentrations, alone or with 0.05 mM BR (Fig. 2). C3G in the effluent peaked with C_{max} in the μM range. AUC analysis showed no net uptake of C3G into the liver. However, P3G in effluent increased linearly and was not affected by bolus administration. The recovery of P3G in the 200 fractions analyzed (10 boli) was 31.12 nmol, or approximately 9% of the total C3G injected (344 nmol). If approximately 1% of each C3G bolus is retained during its passage through the sinusoids, this tiny fraction cannot be estimated by the AUC calculation. Accordingly, inhibition of uptake by BR would not be detectable because an overwhelming proportion of C3G (approximately 99%) passed through the sinusoids. These data are consistent with the results of in vivo distribution, where only 0.64 % of the injected dose was recovered in the liver [20].

Hepatic uptake of bilirubin and bilirubin glucuronide: the effects of cyanidin 3-glucoside, pravastatin and estradiol-17 beta-glucuronide

To investigate the reason for the ineffectiveness of pravastatin in relation to the uptake of BR, we improved the analysis of liver effluent by measuring both BR and bilirubin glucuronide (BRG), the presence of which in the effluent of perfused rat liver was reported long ago [21]. The efflux of BRG through sinusoidal Mrp3 and its downstream reuptake by sinusoidal Oatp is referred to as BRG hopping [22]. Assuming that hopping should also be observed in the intact and viable isolated perfused liver, we analysed BRG in perfusion effluent and tested whether its reuptake could be inhibited by Oatp-specific substrates (Suppl. Table 1).

In this series of experiments, we first tested the effect of the high-affinity Oatp substrate ß-estradiol-17 beta-glucuronide (E17G). Fig. 3A shows one of these experiments, which began with two consecutive BR boli to induce intrahepatic BRG synthesis. The boli sequence continued with pairs of boli containing BR and inhibitors (C3G or E17G), interspersed with pairs of BR-only boli. Both BR and BRG could already be detected in the first fractions, suggesting constitutive BRG efflux from the intracellular stores. Intra-portal injection of BR did not alter the apparent steady-state level of sinusoidal BRG. In contrast, when BR was co-injected with C3G or E17G, sharp spikes of BRG occurred in the effluent. However, there was a difference between C3G and E17G, as only C3G elicited simultaneous spikes of BR and BRG (boli 3-4), whereas E17G elicited only BRG spikes (boli 7-8), even when injected without BR (boli 11-12). The different pattern of C3G and E17G on sinusoidal uptake of BR and BRG was observed several times (Fig. 3B-C-D; Suppl. Fig. 7A). Pravastatin acted like E17G in that it inhibited only the uptake of BRG (Suppl. Fig. 7B).

From these data, we conclude that BR was transported to the liver (99.9% of the cumulative dose, in this experiment), conjugated by UDP-glucuronosyltransferase (UGT1A1) (to an undetermined extent), and a minute amount of BRG (0.13% of the estimated absorbed BR fraction, in this experiment) was transported

back to the sinusoid. It should be noted that under all circumstances, i.e. both before and after multiple administrations of BR, either alone or with inhibitors, the baseline BRG was constant.

Understanding the inhibition of bilirubin glucuronide uptake by cyanidin 3-glucoside

The dual action of C3G required further investigation to evaluate its ability to inhibit sinusoidal uptake of either BR or BRG. If C3G is an inhibitor of Oatp, it might be expected to inhibit reuptake of BRG even when injected in the absence of BR, similar to what has been observed with E17G and pravastatin.

In the experiment shown in Fig. 4A, pairs of BR boli were administered to induce intrahepatic BRG synthesis. When BR was co-injected with C3G, two overlapping peaks of BR and BRG (boli 3-4) appeared in the effluent, but when it was administered without BR (boli 7-8), no peak of BR or BRG appeared. Further boli of E17G, with or without BR, elicited only large peaks of BRG (boli 11-12-13). Thus, while E17G acted independently of BR on a BRG transporter (presumably Oatp), C3G acted only in its presence, suggesting that BR is the actual and necessary inhibitor of another sinusoidal BRG transporter. However, we note that C3G elicited spikes of BR only when it was co-injected with BR, whereas it had no effect on basal levels of BR. This is generally the case when a transporter has allosteric properties, such as bilitranslocase [23]. In these cases, the inhibitors are inactive at very low substrate concentrations, at the base of the sigmoid curve.

The mutual independence of these transport pathways was further challenged in an experiment in which we tested the simultaneous injection of C3G and E17G instead of C3G with BR on the sinusoidal reuptake of BRG. We found that the resulting BRG peak was never higher than that elicited by E17G alone (Fig. 4B, boli 6 vs 7 and 11 vs 12).

To complete the characterization of the differential effects of C3G and E17G, we tested their effects on basal levels of BR and BRG detectable in perfusion effluent from livers never exposed to BR boli. As shown in Fig. 4C, basal levels of BR or BRG were not affected by C3G. Only E17G triggered spikes of BRG. Simultaneous administration of C3G and E17G elicited peak values that were not different from those elicited by E17G alone. The same result was obtained with pravastatin (Suppl. Fig. 8).

Overall, these data indicate that C3G does not directly inhibit BRG uptake. In contrast, the C3G-induced BR concentration peak was the agent that inhibited an unknown BRG transporter that was not directly targeted by either C3G or E17G.

Hepatic uptake of bilirubin and bilirubin glucuronide: the effect of non-steroidal anti-inflammatory drugs

Early functional studies had demonstrated the existence of a sinusoidal BRG membrane transporter that was competitively inhibited by BR and indocyanine green (ICG) but not by glycocholate [18][24]. We

speculated that a possible candidate for this function might be rOat2 (Slc22a7), since ICG is a good inhibitor of its transport activity, unlike taurocholate and pravastatin [25]. We found that the Oat2 substrates ketoprofen or indomethacin (Suppl. Table 1B) did not affect the uptake of BR and BRG (Suppl. Fig. 9).

Hepatic uptake of bilirubin and bilirubin glucuronide: the effect of taurocholate

Finally, we studied the effect of taurocholate (TC) on the hepatic uptake of BR and BRG. Taurocholate is transported in the liver by at least three different sinusoidal transporters (Suppl. Table 1). By using taurocholate, we aimed primarily to inhibit Ntcp, which is not targeted by E17G or pravastatin (Suppl. Table 1) but transports indocyanine green [26]. In human subjects carrying mutant forms of Ntcp [27], hypercholanemia may be associated with elevated blood levels of indirect bilirubin (BR) [28] and direct bilirubin (BRG) [29][30].

We observed that serial boli of increasing concentrations of TC triggered significant BRG spikes only when the concentration of TC was 2 mM. Remarkably, they had no effect on the uptake of BR (Fig. 5). We observed a concentration-dependent inhibition of BRG uptake by TC (Suppl Fig. 10). Overall, these data are consistent with previous reports that TC does not inhibit hepatic uptake of BR in isolated perfused rat liver [31].

The effect of cyanidin 3-glucoside and taurocholate on hepatic uptake of bilirubin and bilirubin glucuronide at physiological concentrations

To assess the effect of C3G and TC on the uptake of BR and BRG at physiological portal concentrations, we injected boli of 0.01 mM C3G (2 nmol) alone or with either 0.05 mM BR (10 nmol) or 2 mM TC (400 nmol). The effluent was analyzed for all relevant compounds, i.e. BR and BRG, C3G and P3G, and TC, to evaluate their concentrations in the effluent (data and statistics of basal concentrations and peak parameters in Suppl. Fig. 11).

Under these conditions, C3G reached 0.44 µM, which corresponds to a physiological value in rat portal blood after intestinal absorption [32] and in human systemic circulation [33]. It elicited peak levels up to 10 nM BR, which were higher than the basal levels BR of 0.9 nM (10-fold increase). TC , which was injected together with C3G, reached 24 µM, which is within the physiological range observed in fasting humans [34]. It triggered BRG peaks that reached 8.1 nM, which was above the basal value of 1.1 nM (7-fold increase), with no effect on C3G peaks.

Discussion

Hopping of bilirubin glucuronide in isolated perfused rat liver

After several decades, this is the first study in the isolated perfused rat liver model to examine the pharmacology of hepatic BR uptake using compounds targeting specific sinusoidal membrane transporters. The liver preparations had high BR uptake efficacy. We observed a basal net release of BRG that essentially did not change with repeated administration of BR. Sinusoidal BRG resulted from efflux via an uncharacterized efflux pathway that appeared to be independent of Mrp3 (as it was not inhibited by E17G) or other sinusoidal membrane transporters targeted by the inhibitors used. Importantly, the sinusoidal concentration of BRG was kept constant by its transporter-mediated uptake in the liver, as evidenced by the specific transient inhibition by E17G, pravastatin, and taurocholate. Overall, we consider this to be evidence of BRG hopping, which in turn demonstrates the viability and functional performance of the isolated liver preparations used in this study.

Identification of a bilirubin transporter targeted by some anthocyanins

In this study, we have shown that C3G selectively inhibits the uptake of BR without direct effects on the uptake of BRG (Scheme 1). The natural pigment C3G (and all anthocyanins) is chemically similar to the synthetic dye bromosulfophthalein (BSP), an inhibitor of hepatic uptake of BR in vivo [4].

In an effort to identify the hepatic BR transporter, BSP has been widely used as a substrate for membrane transport experiments in vitro. We have performed the assay of electrogenic BSP transport in rat liver plasma membrane vesicles [35][36], for the functional evaluation of bilitranslocase transporter [37]. We had shown that C3G and 19 other anthocyanins competitively inhibit this assay [11]. Thus, this study confirms that the electrogenic BSP transport assay in plasma membrane vesicles occupies a niche among other BSP membrane transport assays because its assay conditions are such that only one of the two BSP tautomers is transported [37], and it appears that this is the unique specificity of bilitranslocase compared to all other BSP transporters such as Oatp [38], Ntcp [39], Oat [40], and Ostα-Ostβ [41]. Moreover, to our knowledge, no BSP transporter, except bilitranslocase, has the property of binding BR with high affinity (K_i = 2 nM), as we have demonstrated by experiments on the time-dependent inhibition kinetics of electrogenic BSP transport in rat liver plasma membrane vesicles by a serine-specific reagent [42] and two anti-sequence antibodies [12][43]. However, we hypothesise that several BR transporters may be involved in the highly efficient BR uptake into the liver.

Conversely, in this study we show that 6 compounds (pravastatin, E17G, indomethacin, ketoprofen, *trans*-resveratrol, and taurocholate) that are inactive in the electrogenic BSP transport assay [44], but are known substrates or inhibitors of sinusoidal membrane transporters, belonging to SLCO (Oatp), SLC10 (sodium-bile acid co-transporter), SLC22 (Oat), and SLC51 (steroid-derived molecule transporter, Ost), did not inhibit the uptake of BR into the liver. Rather, three of them (pravastatin, E17G and taurocholate) inhibited the reuptake of BRG, probably due to their specific inhibition of Oatp.

Identification of a bilirubin glucuronide transporter targeted by bilirubin

Only when injected together with BR did C3G elicit simultaneous peaks of BR and BRG. This suggests that the increase in sinusoidal BR concentration was the effector for inhibition of a BR -sensitive BRG transporter. BRG uptake in isolated perfused rat liver was previously reported to be inhibited by BR as well as by BSP and indocyanine green, but not by glycocholate [18]. Therefore, we suspected that this BRG transporter might be Oat2, which is indeed inhibited by BSP and indocyanine green, but not by taurocholate (Suppl. Table 1). However, the Oat2 substrates indomethacin and ketoprofen were inactive toward BR or BRG uptake in our model. Thus, hepatic BRG transport appears to occur via two major pathways that are inhibited by some common substrates of bile acid transporters (BSP, indocyanine green, and tauro/glycocholate) or BR, respectively. There are no data in the literature on the nature of this putative sinusoidal BRG transporter. A dual system for sinusoidal BRG uptake suggests a high physiological importance of this process. In conditions of (acute) hyperbilirubinemia, inhibition of BRG reuptake could be crucial to limit intracellular BRG concentrations, leading to product inhibition of UGT1A1 and ultimately limiting the BR uptake capacity of the liver. This system may play an important role in the neonatal period when bilirubin production is higher than hepatic excretion, with hyperbilirubinemia ranging from physiological neonatal jaundice to severe clinical forms with risk of kernicterus.

Conclusions

In conclusion, this study has shed light on previously unknown functional properties of the liver. Given its specific action as an inhibitor of hepatic BR uptake, C3G emerges as a probe for phenotypic studies of BR transport that should be incorporated into broader functional screenings of other organic anion membrane transporters, with both positive and negative data reported. More importantly, BR should be used as a transport substrate in programs aimed at characterizing the functional properties of SLC transporters. The lack of knowledge about the structure of this BR transporter and its encoding gene requires intensive experimental efforts.

Declarations

Financial support

Funded by the European Regional Development Fund through both Interreg V-A Italy-Slovenia 2014-2020 (Agrotur II project, ID 1473843258; WP3.2.1 Knowledge on the bioactive compounds of wine) and Interreg V-A Italy-Croatia 2014-2020 (AdriAquaNet, ID 10045161, WP4.3 Welfare monitoring); by the University of Trieste (co-funding of PhD fellowship for P.P., and internal funds); by the Slovenian Research Agency Research Programme no. P4-0133. ADP 2021 Autonomous province of Trento, Italy.

Conflicts of interests

None.

Authors' contributions

Conceptualization: Sabina Passamonti.
Data curation: Paola Pelizzo and Nevenka Medic.
Formal analysis: Paola Pelizzo, Andreja Vanzo, Andrea Anesi, and Federica Tramer.
Funding acquisition: Sabina Passamonti.
Investigation: Paola Pelizzo, Marco Stebel, Nevenka Medic, Andreja Vanzo, Andrea Anesi, and Federica Tramer.
Methodology: Marco Stebel, Paola Sist, Andreja Vanzo, Urska Vrhovsek, Federica Tramer, and Sabina Passamonti.
Project administration: Federica Tramer and Sabina Passamonti.
Resources: Paola Pelizzo, Marco Stebel, Nevenka Medic, Paola Sist, Andreja Vanzo, Andrea Anesi, Urska Vrhovsek, and Federica Tramer.
Supervision: Nevenka Medic, Paola Sist, Urska Vrhovsek, Federica Tramer, and Sabina Passamonti.
Validation: Paola Pelizzo, Marco Stebel, Nevenka Medic, Paola Sist, Federica Tramer, and Sabina Passamonti.
Visualization: Paola Pelizzo and Sabina Passamonti.
Writing - original draft: Sabina Passamonti.
Writing - review & editing: Sabina Passamonti.

Data availability

Data are included in the paper or supplementary materials. Primary data sets will be available after acceptance of this manuscript for publication.

Acknowledgments

The staff of the University of Trieste's bioethics committee (OBPA) and animal facility is gratefully acknowledged.

Supplementary data

Supplementary data are attached.

Abbreviations

BR, bilirubin; BRG, bilirubin glucuronide; C3G, cyanidin 3-glucoside; estradiol-17 beta glucuronide, E17G; M3G, malvidin 3-glucoside; P3G, peonidin 3-glucoside; PRV, pravastatin; taurocholate, TC.

References

1. Vítek L, Tiribelli C: **Bilirubin: The yellow hormone?** *J Hepatol* 2021.
2. Levitt DG, Levitt MD: **Quantitative assessment of the multiple processes responsible for bilirubin homeostasis in health and disease**. *Clin Exp Gastroenterol* 2014, **7**:307–28.
3. Lin L, Yee SW, Kim RB, Giacomini KM: **SLC transporters as therapeutic targets: emerging opportunities**. *Nat Rev Drug Discov* 2015, **14**:543–560.
4. Scharschmidt BF, Waggoner JG, Berk PD: **Hepatic organic anion uptake in the rat**. *J Clin Invest* 1975, **56**:1280–1292.
5. Čvorović J, Passamonti S: **Membrane Transporters for Bilirubin and Its Conjugates: A Systematic Review**. *Frontiers in Pharmacology* 2017:887.
6. Watanabe T, Miyake M, Shimizu T, Kamezawa M, Masutomi N, Shimura T, Ohashi R: **Utility of bilirubins and bile acids as endogenous biomarkers for the inhibition of hepatic transporters**. *Drug Metab Dispos* 2015, **43**:459–466.
7. Takehara I, Yoshikado T, Ishigame K, Mori D, Furihata K, Watanabe N, Ando O, Maeda K, Sugiyama Y, Kusuhara H: **Comparative Study of the Dose-Dependence of OATP1B Inhibition by Rifampicin Using Probe Drugs and Endogenous Substrates in Healthy Volunteers**. *Pharm Res* 2018, **35**:138.
8. Sorokina M, Stam M, Medigue C, Lespinet O, Vallenet D: **Profiling the orphan enzymes**. *Biol Direct* 2014, **9**:10.
9. Bandiera A, Corich L, Tommasi S, De Bortoli M, Pelizzo P, Stebel M, Paladin D, Passamonti S: **Human elastin-like polypeptides as a versatile platform for exploitation of ultrasensitive bilirubin detection by UnaG**. *Biotechnol Bioeng* 2020, **117**:354–361.
10. Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL: **Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption**. *J Agric Food Chem* 2006, **54**:4069–4075.
11. Passamonti S, Vrhovsek U, Mattivi F: **The interaction of anthocyanins with bilitranslocase**. *Biochem Biophys Res Commun* 2002, **296**:631–636.
12. Battiston L, Passamonti S, Macagno A, Sottocasa GL: **The bilirubin-binding motif of bilitranslocase and its relation to conserved motifs in ancient biliproteins**. *Biochem Biophys Res Commun* 1998, **247**:687–692.
13. Stieger B, Mahdi ZM, Jager W: **Intestinal and Hepatocellular Transporters: Therapeutic Effects and Drug Interactions of Herbal Supplements**. *Annu Rev Pharmacol Toxicol* 2017, **57**:399–416.
14. Lu X, Chan T, Xu C, Ng WV, Zhu L, Zhou F: **The Interactions of Herbal Compounds with Human Organic Anion/Cation Transporters**. *J Pharmacogenomics Pharmacoproteomics* 2014, **5**:645–2153.
15. Wang X, Wolkoff AW, Morris ME: **Flavonoids as a novel class of human organic anion-transporting polypeptide OATP1B1 (OATP-C) modulators**. *Drug Metab Dispos* 2005, **33**:1666–1672.
16. Riha J, Brenner S, Srovnalova A, Klameth L, Dvorak Z, Jager W, Thalhammer T: **Effects of anthocyans on the expression of organic anion transporting polypeptides (SLCOs/OATPs) in primary human hepatocytes**. *Food Funct* 2015, **6**:772–779.

17. Li Y, Revalde J, Paxton JW: **The effects of dietary and herbal phytochemicals on drug transporters**. *Adv Drug Deliv Rev* 2017, **116**:45–62.
18. Shupeck M, Wolkoff AW, Scharschmidt BF, Waggoner JG, Berk PD: **Studies of the kinetics of purified conjugated bilirubin-3H in the rat**. *Am J Gastroenterol* 1978, **70**:259–264.
19. du Sert NP, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M: **Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0**. *PLoS Biol* 2020, **18**.
20. Fornasaro S, Ziberna L, Gasperotti M, Tramer F, Vrhovsek U, Mattivi F, Passamonti S: **Determination of cyanidin 3-glucoside in rat brain, liver and kidneys by UPLC/MS-MS and its application to a short-term pharmacokinetic study**. *Sci Rep* 2016, **6**:22815.
21. Barnhart JL, Clarenburg R: **Factors determining clearance of bilirubin in perfused rat liver**. *Am J Physiol Content* 1973, **225**:497–507.
22. Iusuf D, Van De Steeg E, Schinkel AH: **Hepatocyte hopping of OATP1B substrates contributes to efficient hepatic detoxification**. *Clin Pharmacol Ther* 2012, **92**:559–562.
23. Passamonti S, Battiston L, Sottocasa GL: **Bilitranslocase can exist in two metastable forms with different affinities for the substrates--evidence from cysteine and arginine modification**. *Eur J Biochem* 1998, **253**:84–90.
24. Adachi Y, Roy-Chowdhury J, Roy-Chowdhury N, Kinne R, Tran T, Kobayashi H, Arias IM: **Hepatic uptake of bilirubin diglucuronide: analysis by using sinusoidal plasma membrane vesicles**. *J Biochem* 1990, **107**:749–754.
25. Morita N, Kusuhara H, Nozaki Y, Endou H, Sugiyama Y: **Functional involvement of rat organic anion transporter 2 (Slc22a7) in the hepatic uptake of the nonsteroidal anti-inflammatory drug ketoprofen**. *Drug Metab Dispos* 2005, **33**:1151–1157.
26. de Graaf W, Häusler S, Heger M, van Ginhoven TM, van Cappellen G, Bennink RJ, Kullak-Ublick GA, Hesselmann R, van Gulik TM, Stieger B: **Transporters involved in the hepatic uptake of 99mTc-mebrofenin and indocyanine green**. *J Hepatol* 2011, **54**:738–745.
27. Boyer JL, Soroka CJ: **Bile formation and Secretion: An update**. *J Hepatol* 2021.
28. Qiu J-W, Deng M, Cheng Y, Atif R-M, Lin W-X, Guo L, Li H, Song Y-Z: **Sodium taurocholate cotransporting polypeptide (NTCP) deficiency: Identification of a novel SLC10A1 mutation in two unrelated infants presenting with neonatal indirect hyperbilirubinemia and remarkable hypercholanemia**. *Oncotarget* 2017, **8**:106598–106607.
29. Vaz FM, Paulusma CC, Huidekoper H, de Ru M, Lim C, Koster J, Ho-Mok K, Bootsma AH, Groen AK, Schaap FG, Oude Elferink RPJ, Waterham HR, Wanders RJA: **Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype**. *Hepatology* 2015, **61**:260–267.
30. Yan YY, Wang MX, Gong JY, Liu LL, Setchell KDR, Xie XB, Wang NL, Li W, Wang J-S: **Abnormal Bilirubin Metabolism in Patients With Sodium Taurocholate Cotransporting Polypeptide Deficiency**. *J Pediatr Gastroenterol Nutr* 2020, **71**:e138–e141.

31. Paumgartner G, Reichen J: **Kinetics of hepatic uptake of unconjugated bilirubin**. *Clin Sci Mol Med* 1976, **51**:169–176.
32. Passamonti S, Vrhovsek U, Vanzo A, Mattivi F: **The stomach as a site for anthocyanins absorption from food**. *FEBS Lett* 2003, **544**:210–213.
33. de Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, Kay CD: **The pharmacokinetics of anthocyanins and their metabolites in humans**. *Br J Pharmacol* 2014, **171**:3268–3282.
34. Ahlberg J, Angelin B, Björkhem I, Einarsson K: **Individual bile acids in portal venous and systemic blood serum of fasting man**. *Gastroenterology* 1977, **73**:1377–1382.
35. Baldini G, Passamonti S, Lunazzi GC, Tiribelli C, Sottocasa GL: **Cellular localization of sulfobromophthalein transport activity in rat liver**. *Biochim Biophys Acta* 1986, **856**:1–10.
36. Passamonti S, Tramer F, Petrussa E, Braidot E, Vianello A: **Electrogenic bromosulfalein transport in isolated membrane vesicles: implementation in both animal and plant preparations for the study of flavonoid transporters**. *Methods Mol Biol* 2010, **643**:307–335.
37. Sottocasa GL, Passamonti S, Battiston L, Pascolo L, Tiribelli C: **Molecular aspects of organic anion uptake in liver**. *J Hepatol* 1996, **24 Suppl 1**:36–41.
38. Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ: **Expression cloning of a rat liver Na(+)-independent organic anion transporter**. *Proc Natl Acad Sci U S A* 1994, **91**:133–137.
39. Hata S, Wang P, Eftychiou N, Ananthanarayanan M, Batta A, Salen G, Pang KS, Wolkoff AW: **Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake**. *Am J Physiol Liver Physiol* 2003, **285**:G829–G839.
40. Saito H, Masuda S, Inui K: **Cloning and functional characterization of a novel rat organic anion transporter mediating basolateral uptake of methotrexate in the kidney**. *J Biol Chem* 1996, **271**:20719–20725.
41. Seward DJ, Koh AS, Boyer JL, Ballatori N: **Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta**. *J Biol Chem* 2003, **278**:27473–27482.
42. Passamonti S, Battiston L, Sottocasa GL: **Arylsulfonylation of bilitranslocase in plasma membranes from rat liver enables to discriminate between natural and artificial substrates**. *Biochim Biophys Acta - Biomembr* 1997, **1323**:130–136.
43. Passamonti S, Cocolo A, Braidot E, Petrussa E, Peresson C, Medic N, Macri F, Vianello A: **Characterization of electrogenic bromosulfophthalein transport in carnation petal microsomes and its inhibition by antibodies against bilitranslocase**. *FEBS J* 2005, **272**:3282–3296.
44. Zuperl S, Fornasaro S, Novic M, Passamonti S: **Experimental determination and prediction of bilitranslocase transport activity**. *Anal Chim Acta* 2011, **705**:322–333.

Figures

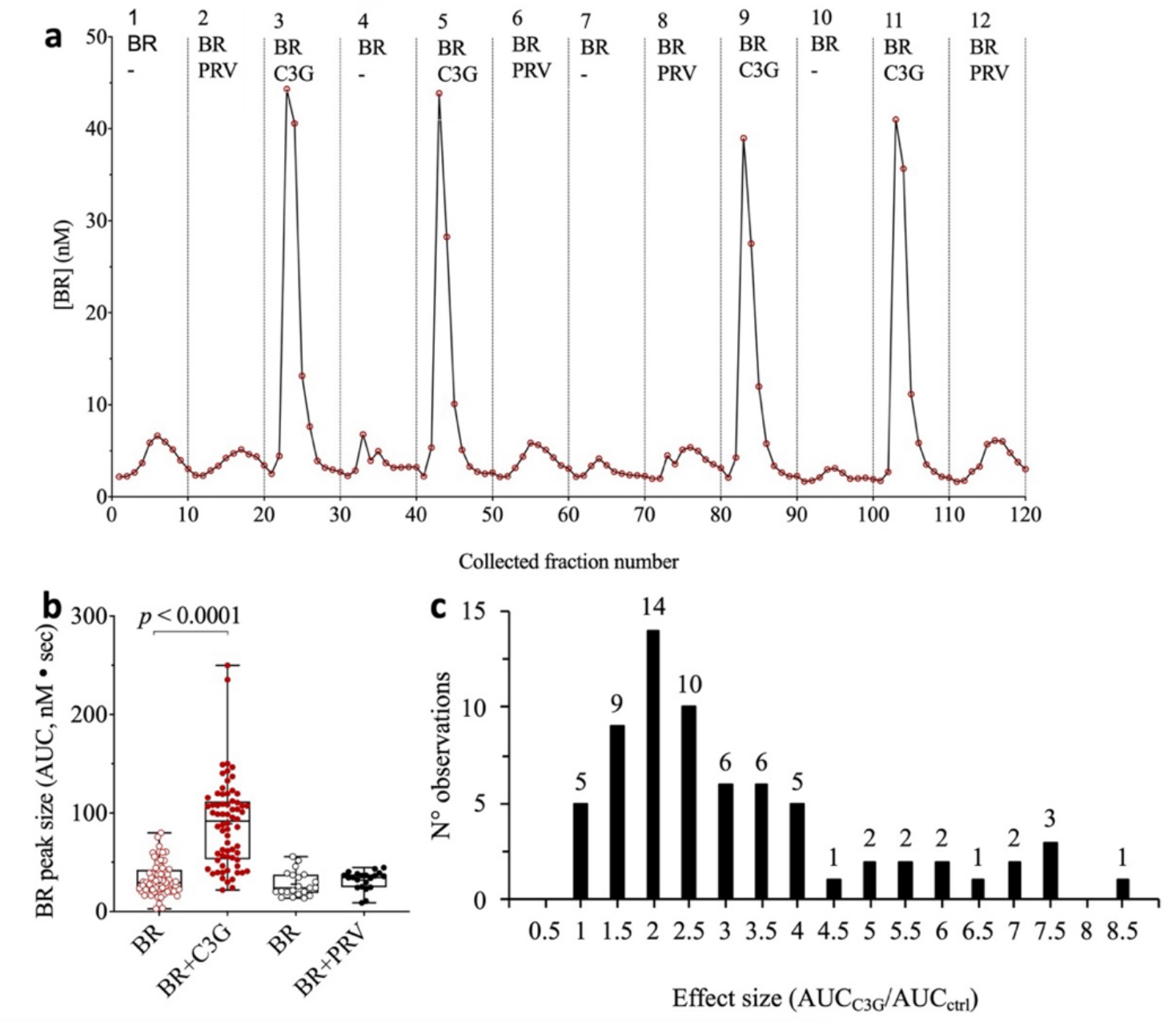

Figure 1

The effects of cyanidin 3-glucoside and pravastatin on the hepatic uptake of bilirubin. a Concentrations of bilirubin (BR) in the venous effluent of a representative isolated perfused rat liver, following sequential intra-portal boli (0.2 mL) of 0.05 mM BR without and with either 2 mM cyanidin 3-glucoside (C3G) or 6 mM pravastatin (PRV). b Box plots of BR peak areas, resulting from co-injection of 0.05 mM BR without (open symbols) or with either 2 mM C3G (filled red circles) (livers, n = 21; peak areas, n = 68) or 6 mM PRV (filled black circles) (livers, n = 6; peak areas, n = 21). c Frequency analysis of the effect size of C3G inhibition of hepatic BR uptake. The effect size was calculated as ratio of each peak area resulting from co-injection of BR with C3G (AUCC3G) vs each respective control BR peak area (AUCCtrl) (livers, n = 21; peak pairs, n = 68).

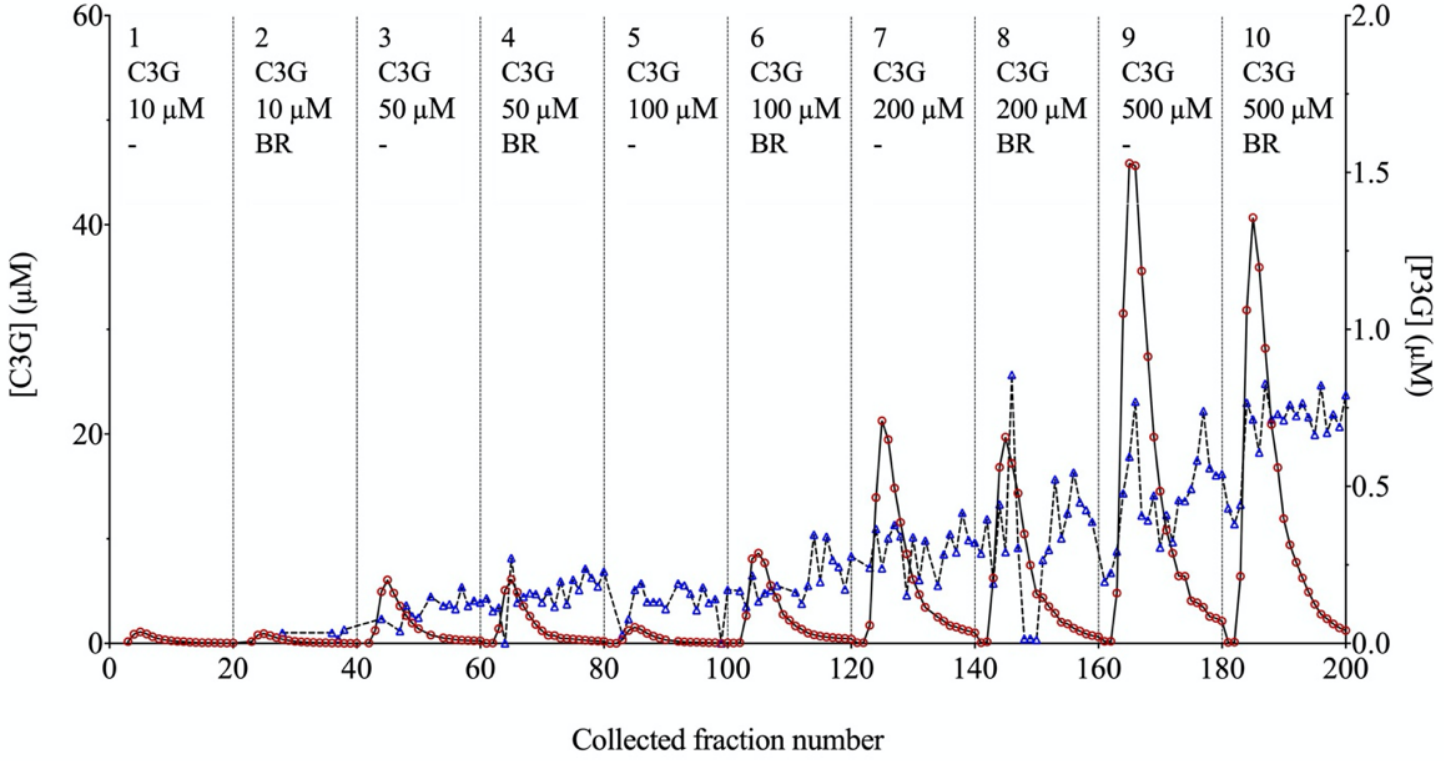

Figure 2

The effect of bilirubin on the hepatic uptake of cyanidin 3-glucoside and efflux of peonidin 3-glucoside. Concentration of C3G (in red) and P3G (in blue) in the venous effluent of an isolated perfused rat liver, following sequential boli (0.2 mL) with increasing concentrations of C3G (0.01, 0.05, 0.1, 0.2, and 0.5 mM) without or with 0.05 mM BR.

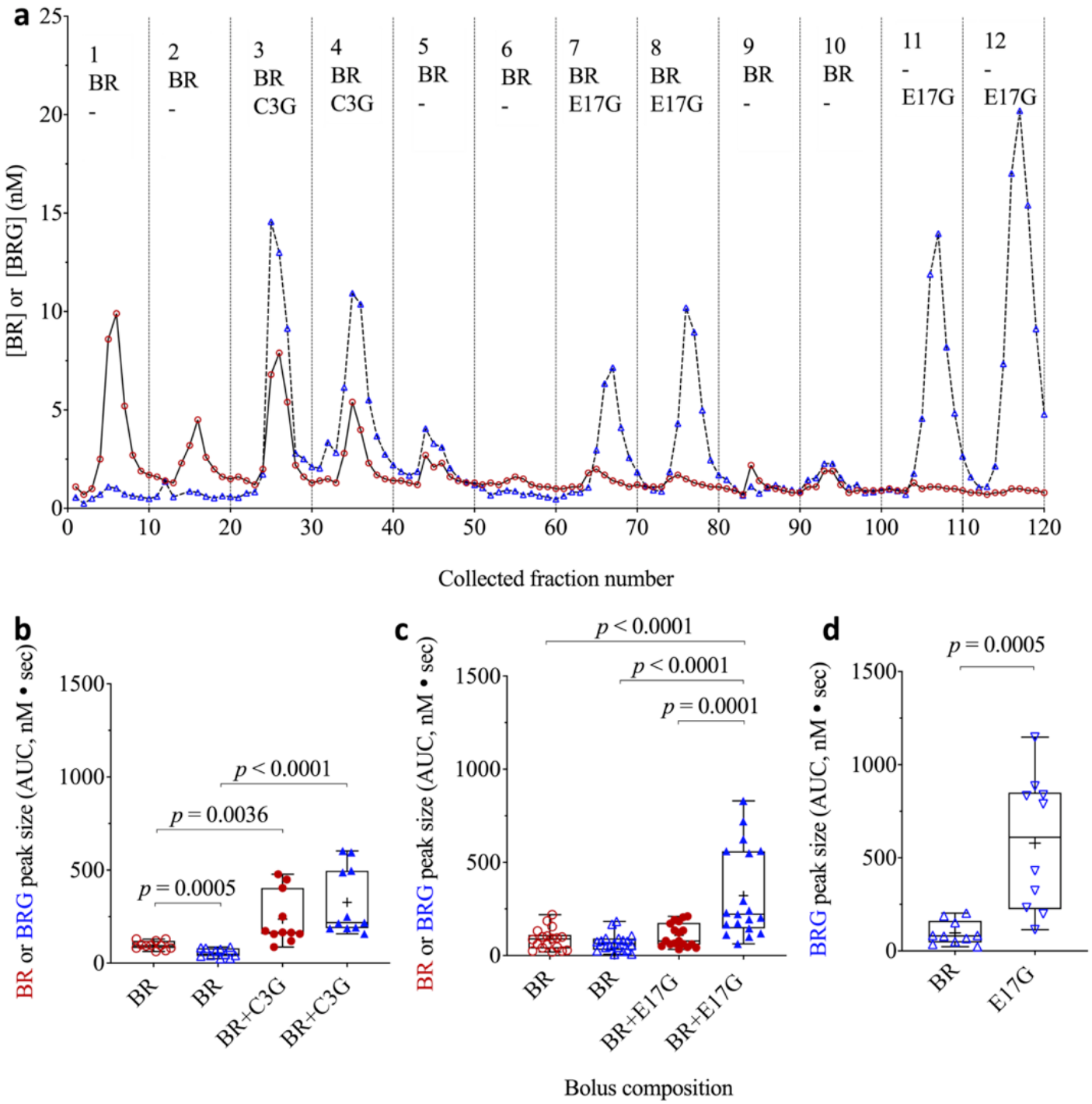

Figure 3

The effects of cyanidin 3-glucoside and estradiol-17 beta-glucuronide on the hepatic uptake of both bilirubin and bilirubin glucuronide. a Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in blue) in the venous effluent of a representative isolated perfused liver, following sequential intra-portal boli (0.2 mL) of 0.05 mM BR without or with either 2 mM cyanidin 3-glucoside (C3G) or 1 mM estradiol-17 beta-glucuronide (E17G). b Box plots of peak areas of either BR (in red) or BRG (in blue), resulting from boli of 0.05 mM BR without (empty symbols) or with 2 mM C3G (closed symbols) (livers, n = 7; peak areas, n = 11). c Box plots of peak areas of either BR (in red) or BRG (in blue), resulting from boli

of 0.05 mM BR without (empty symbols) or with 1 mM E17G (closed symbols) (livers, n = 14; peak areas, n = 19). d Box plots of BRG peak areas, resulting from boli of either 0.05 mM BR or 1 mM E17G (livers, n = 7; peak areas, n = 10).

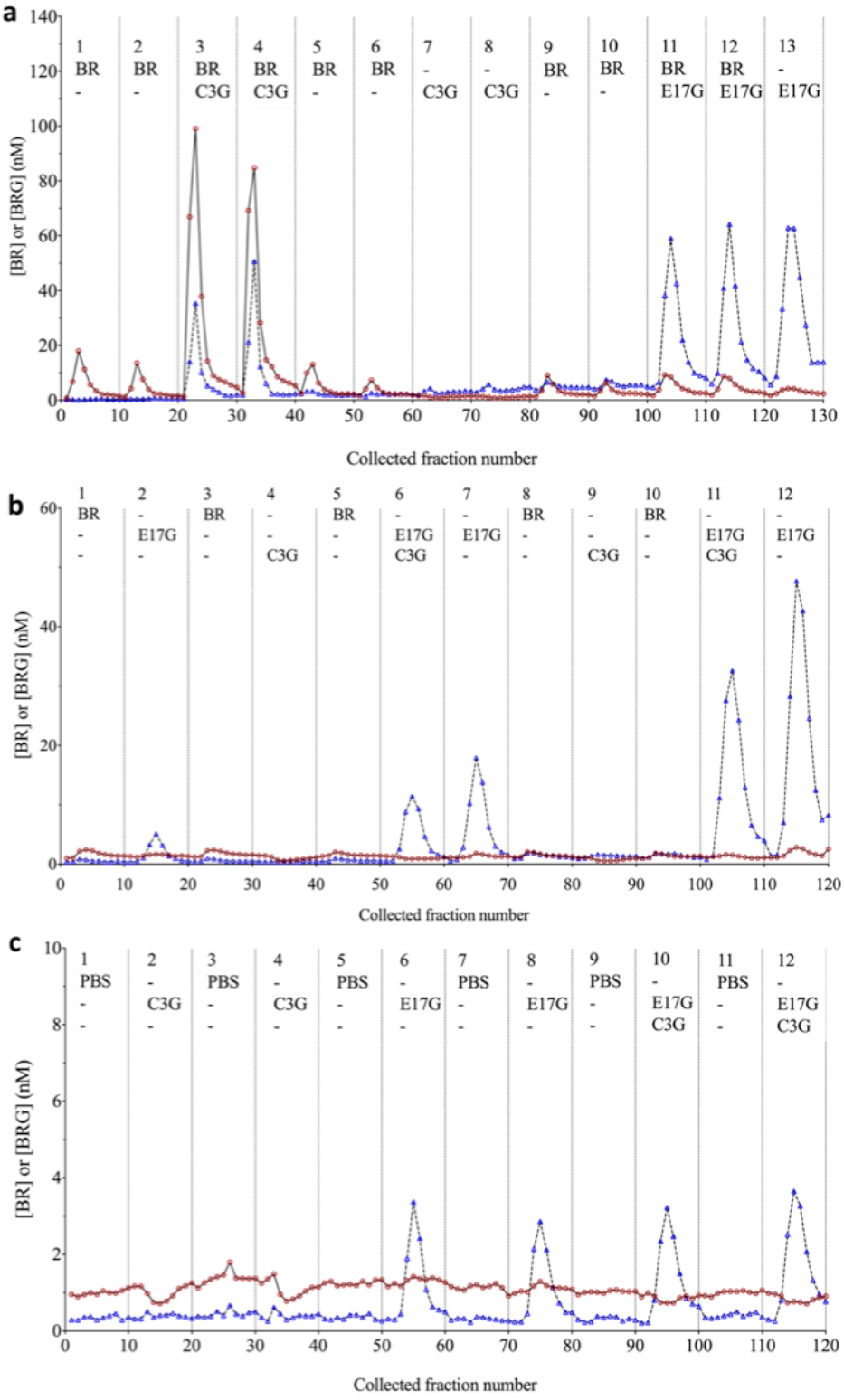

Figure 4

The effects of cyanidin 3-glucoside and estradiol-17 beta-glucuronide on the hepatic uptake of bilirubin, when injected without bilirubin. Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in

blue) in the venous effluent of representative isolated perfused rat livers. a A series of 6 intra-portal boli (10 nmol BR in 0.2 mL, each), containing 0.05 mM BR without or with 2 mM cyanidin 3-glucoside (C3G), was followed by 2 intra-portal boli of 2 mM C3G solo; then, a series of 4 intra-portal boli, containing 0.05 mM BR without or with 1 mM estradiol-17 beta-glucuronide (E17G), was followed by a bolus of 1 mM E17G solo. b A series of 6 intra-portal boli (0.2 mL) starting with only one 0.05 mM BR bolus, followed by boli of 1 mM E17G, or 2 mM C3G, or both, without co-injection of BR. c Sequential intra-portal boli (0.2 mL) of 2 mM C3G or 1 mM E17G or both, without pre- or co-injection of BR.

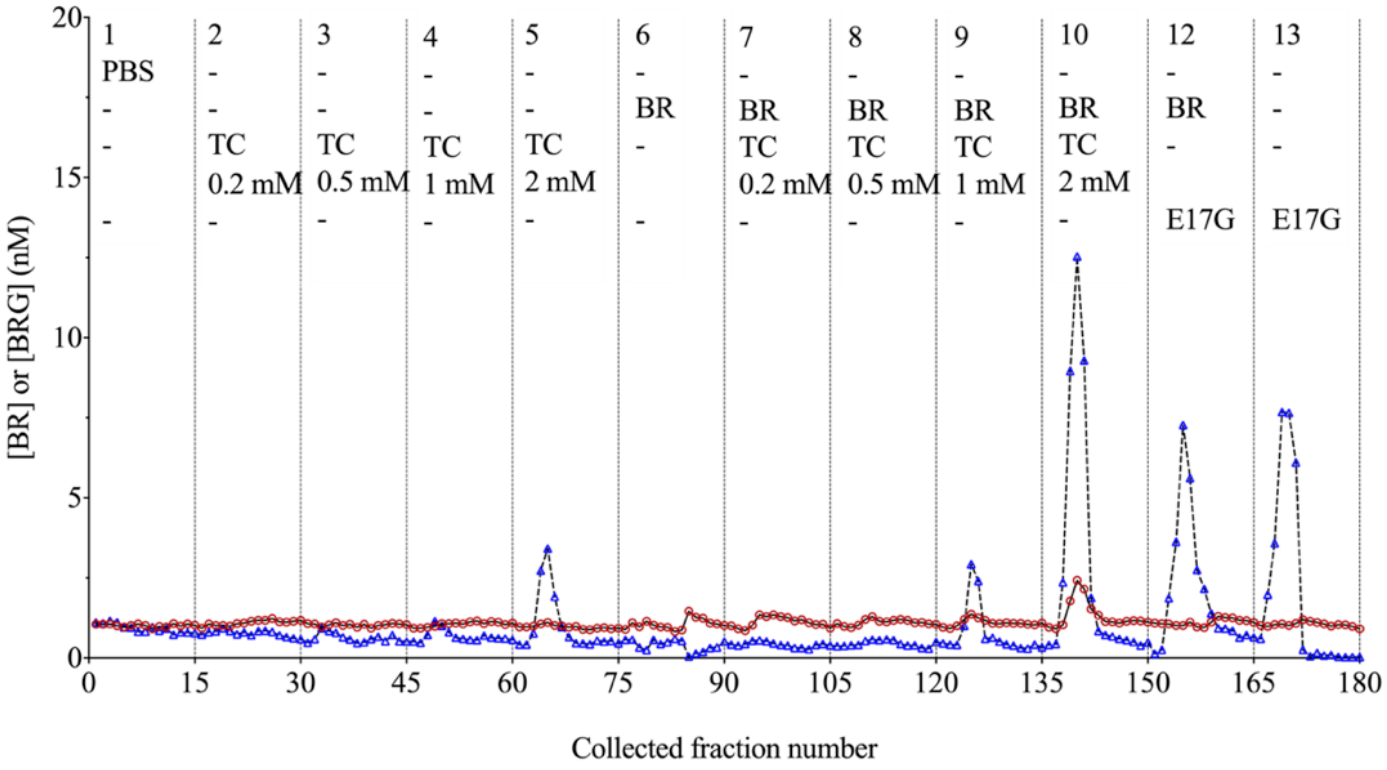

Figure 5

The dose-dependent effect of taurocholate on the hepatic uptake of both bilirubin and bilirubin glucuronide. Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in blue) in the venous effluent of a representative isolated perfused liver, following sequential intra-portal boli (0.2 mL) of 0.05 mM (bilirubin) BR without or with 0.2, 0.5, 1.0, 2.0 mM taurocholate (TC); boli of 1 mM estradiol-17 beta-glucuronide (E17G) were injected with 0.05 mM BR (bolus 12) or alone (bolus 13).

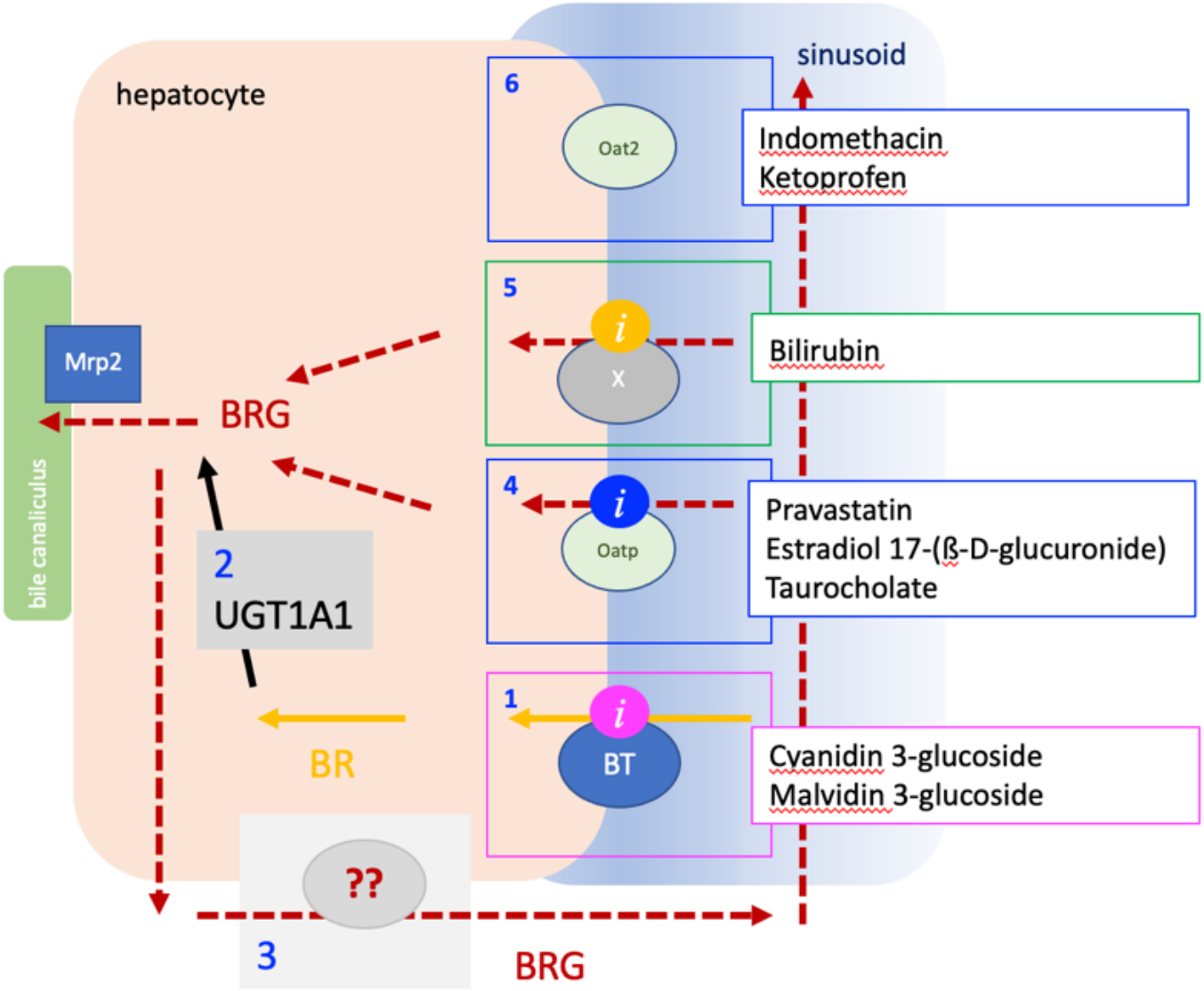

Figure 6

Scheme 1 Membrane transport pathways for the liver uptake of bilirubin and bilirubin glucuronide. A bilirubin transporter (BT) performs the uptake of bilirubin (BR, yellow arrow), which is inhibited (i) by 2 anthocyanin molecules (1). The enzyme UGT1A1 catalyzes the BR glucuronidation reaction, forming bilirubin glucuronide (BRG) (2). An unknown molecular pathway determines the efflux of a small portion of BRG (red arrow) from the liver into the sinusoid (3). Oatp1b2 transporters perform selective sinusoidal BRG uptake, which is inhibited by pravastatin, estradiol-17 beta-glucuronide, and taurocholate (4). BRG is taken up by an unknown transporter that is inhibited by high BR concentrations, triggered by inhibition of the bilirubin transporter by cyanidin-3-glucoside (5). Indomethacin and ketoprofen, substrates of Oat2, do not inhibit BR or BRG uptake.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Pelizzoetal.2021Supplementarydata.docx