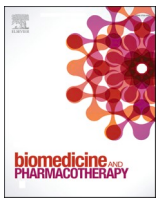
Biomedicine & Pharmacotherapy 157 (2023) 114044

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



# Cyanidin 3-glucoside targets a hepatic bilirubin transporter in rats

Paola Pelizzo [a], Marco Stebel [a,1], Nevenka Medic [a], Paola Sist [a], Andreja Vanzo [b], Andrea Anesi [c], Urska Vrhovsek [c], Federica Tramer [a], Sabina Passamonti [a,*]

[a] Department of Life Sciences, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy
[b] Department of Fruit Growing, Viticulture and Oenology, Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[c] Food Quality and Nutrition, Research and Innovation Centre, Edmund Mach Foundation, Via E. Mach 1, 38010 San Michele all'Adige, Italy

ARTICLE INFO

*Keywords:*
Bilirubin
Sinusoidal transporter
Cyanidin-3-O-beta-glucopyranoside
Pravastatin
Estradiol-17 betaglucuronide
Organic anion transporters

ABSTRACT

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, because there is no link to the protein/gene units that perform this function, 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. We treated isolated perfused livers of female rats with repeated single-pass, albumin-free bilirubin boli. We monitored both bilirubin and bilirubin glucuronide in perfusion effluent with a bio-fluorometric assay. We tested the ability of nine molecules known as substrates or inhibitors of sinusoidal membrane transporters to inhibit hepatic uptake of bilirubin. We found that cyanidin 3-glucoside and malvidin 3-glucoside were the only molecules that inhibited 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 inhibited by bilirubin, a fact reported only once in the literature. The data suggest that bilirubin and bilirubin glucuronide are transported to the liver via pharmacologically distinct membrane transport pathways. Some dietary anthocyanins may physiologically modulate the uptake of bilirubin into the liver.

## 1. 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 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

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

* Correspondence to: Dipartimento di Scienze della Vita, Università degli Studi di Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy.
*E-mail address:* spassamonti@units.it (S. Passamonti).

[1] Equal contribution

https://doi.org/10.1016/j.biopha.2022.114044
Received 7 October 2022; Received in revised form 15 November 2022; Accepted 25 November 2022
Available online 1 December 2022

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 in the female rat liver. 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].

## 2. Materials and methods

### 2.1. Isolated perfused rat liver

The isolated perfused rat liver was chosen because the hepatic architecture supporting the vectorial hepatobiliary transport processes is preserved [19]. 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 [20]. Isolated livers were prepared from Wistar rats (200–290 g) following a standard procedure [21] with modifications as described in a protocol [22]. Surgical procedures were performed between 11 am and 1 pm. Only female rats were used, as in previous work on the transport activity of bilitranslocase in vitro [23,24]. Livers were isolated from the general circulation by intraportal perfusion of 110 mL of Hank's buffered saline solution (HBSS) at a flow rate of 6 mL $min^{-1}$ and a temperature of 37 °C. The outflow from the liver was collected via a catheter inserted into the posthepatic segment of the inferior vena cava via the right atrium. When the effluent from the liver was free of blood, we replaced HBSS with phosphate-buffered saline, pH 7.4 (PBS), as this was compatible with the analysis of BR. The perfusion PBS was without serum albumin to avoid variable equilibria of the BR -albumin complex [25] and contained 2.5 mM glucose, 2 mM pyruvate and 0.2 mM lactate. Perfusion was performed without oxygenation to prevent the formation of oxygen free radicals and redox reactions with bilirubin [26] and anthocyanins [27]. It is known that short-term ischemia has no effect on the structural [28] and metabolic integrity of the organ [29]. The perfusion fluid was anoxic but not ischemic because glucose and the unequal pyruvate/lactate pair ensured both substrate-level phosphorylation and a "clamped" cellular $NAD^+/NADH + H^+$ ratio. Once the need for oxygen delivery to the liver was overcome, perfusion flow was maintained at 0.9 mL $min^{-1}$ $g^{-1}$ liver weight (6 mL $min^{-1}$), which is within the range that ensures normal portal pressure and perfusion flow in the isolated rat liver [30], and is close to physiological portal blood flow in the rat (1.25 mL $min^{-1}$ $g^{-1}$ liver weight) [31]. These conditions preserve the sinusoidal endothelium from shear stress [32], which can trigger the release of hepatic enzymes [33]. The bile duct was not cannulated, as recommended, to avoid accidental cholestasis and its unpredictable influence on the kinetics of hepatic BR uptake [34]. These conditions ensured organ viability and functional integrity for the duration of the uptake assays (1 h after isolation).

### 2.2. Viability tests on isolated perfused rat liver

The tests were performed as described in detail [22]. Liver perfusion effluent was assayed for a marker of membrane lipid oxidation (malondialdehyde), which was undetectable in contrast to oxygenated liver perfusion [33]. There was no apparent membrane permeabilization (ATP release) or cytolysis (LDH release) (Suppl. Fig. 1A), and the microcirculation was preserved, as assessed by recovery of a bovine serum albumin bolus (Suppl. Fig. 1B). The liver showed a constant release of taurocholate (Suppl. Fig. 1C), which was not affected by the portal injection of BR, C3G, or E17G. Suppl. materials

### 2.3. Hepatic bilirubin uptake: assumptions

According to data from isolated perfused rat livers [35,36], > 75% of an intra-portal bolus of 10 nmol BR is expected to be taken up, resulting in recovery of the remaining fraction in hepatic venous effluent. Variations may occur if the liver loses its ability to absorb bilirubin, as has been observed in the perfused liver of endotoxemic rats [37], or its ability to capture BR by intracellular conjugation, as has been observed in the Gunn rat, which carries an inherited bilirubin-UDP-glucuronosyl transferase deficiency [38], or eventually loses its ability to keep intracellular BRG concentration low by biliary excretion, as is the case in the Eisai rat, which carries a genetic defect in Mrp2 function [39].

If the transport of BR from the sinusoid into the hepatocyte is mediated by one or more sinusoidal membrane transporters, the membrane permeability to BR can be reduced by specific reversible inhibitors at appropriate concentrations. This would be evident in a transient increase in the concentration of BR in hepatic venous effluent. The target transporters and the compounds tested for possible inhibition of sinusoidal uptake of BR are listed in Suppl. Table 1.

### 2.4. The general experimental design for testing hepatic bilirubin uptake

Because the focus of this study was to characterize the pharmacology of hepatic single-pass BR uptake, which occurs in < 0.5 min [35,36], we administered small intraportal BR boli, each corresponding to approximately 3.6% of the dose (38.5 nmol $g^{-1}$ liver) that would be taken up by the liver at half-maximal rate [35]. Up to 12 repeated BR boli (0.2 mL; 0.05 mM in PBS: DMSO, 80:20 vol/vol) were injected into the portal vein using a 1 mL syringe connected to a port of the cannula inserted into the portal vein. Thus, BR boli were administered under constant perfusion flow. After each bolus, ten fractions (0.35 mL) of hepatic venous effluent were collected in Eppendorf tubes at 4 s/fraction intervals. Thereafter, the effluent was collected in bulk (3.5 mL) for 40 s. During this time, the 1-mL syringe was prepared for delivery of the next BR bolus and collection of hepatic venous outflow continued. The entire test took approximately 25 min. Known inhibitors/substrates of hepatic sinusoidal transporters were injected or co-injected with BR in the same vehicles as above at the concentrations indicated in Suppl. Table 1B and in the captions. See protocol [22] for further details.

### 2.5. Assay of bilirubin and bilirubin glucuronide

The BR assay was performed using the HUG fluorometric assay [9]. 96-well plates (Nunc) containing 0.010 mL HUG (0.4 mg/mL) were used. A freshly prepared BR standard solution (0–0.05 mM in PBS: DMSO, 97:3, vol/vol; 0.2 mL) was used to calibrate the fluorescence of the HUG-BR complex, which was recorded in a multiplate reader (Synergy H1; BioTek Winooski, VT) at $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 528 nm, yielding a lower limit of detection LoD = 0.74 nM and a lower limit of quantification LoQ = 2.25 nM (n = 40) (Suppl. Fig. 2). Controls were performed to evaluate the potential interference of molecules

co-injected with BR by adding them to serially diluted standard solutions of BR (Suppl. Fig. 2). Immediately after collection, fractions of hepatic venous effluent (0.2 mL) were transferred to the multi-well plates containing HUG (0.4 mg/mL) to prevent decay of trace amounts of BR in the perfusion solution. The covered multi-well plate was incubated at T = 25 °C for 1 h and the emission fluorescence was recorded. Then, BRG present in the same samples was analyzed by adding 0.01 mL (8 IU) of β-glucuronidase (Sigma) to each well, including the wells containing the standard solutions BR, and keeping the plate at T = 25 °C. Emission fluorescence was recorded 2 h later as described above. The multiwell plate was stored covered at 4 °C overnight and the fluorescence was recorded again (Suppl. Fig. 2). The value of the last reading represents the total bilirubin concentration (BR + BRG) and allows the calculation of the BRG concentration. See protocol [22] for further details.

### 2.6. Analysis of anthocyanins and taurocholate

C3G and peonidin 3-glucoside (P3G) in perfusion effluent were analyzed using HPLC-DAD [40]. Samples (0.1 mL) were mixed with methanol (0.1 mL) and stored at − 20 °C. At the time of analysis, samples were acidified with 1 μL of 70% perchloric acid, filtered through a 0.22 μ PDVF filter (Millipore Corporation, MA, USA) and injected directly into a Purospher C18 column (250 ×4.6 mm, 5 μm; Merck, Darmstadt, Germany) with a Purospher C18 guard column (4 ×4 mm, 5 μm) on an 1100 HPLC-DAD (Agilent Technologies, Palo Alto, USA). Anthocyanins were detected and quantified using a slightly modified method as described in [40]. The mobile phase consisted of 0.3% perchloric acid in Milli-Q water (phase A) and 100% methanol (phase B). The separation was carried out at T = 35 °C. Linear gradient conditions started at 28.5% B, reached 42% B in 25 min, then 100% B in 1 min, and remained at 100% B for 3 min. The column was equilibrated for 6 min before each analysis. The flow rate was 0.55 mL min-1 and the injection volume was 70 μL. UV-Vis spectra were recorded from 200 to 700 nm. Identification of the peaks was based on both the retention times and the UV-Vis spectra of the pure reference compounds. Quantification of C3G and P3G was based on the peak areas at λ = 520 nm and concentrations were estimated using the external standard method.

An Ultra High Performance Liquid Chromatography-ElectroSpray Ionization-tandem mass spectrometry method (UHPLC-ESI-MS /MS) was used for the simultaneous analysis of taurocholate, C3G, and P3G in the perfusion effluent fractions. Samples (0.18 mL) were added to a 96-well plate together with 0.02 mL of 1 ppm taurocholate-d5 in methanol and mixed on an Eppendorf shaker (Eppendorf, Milan, Italy) at 300 rpm for 5 min. Calibration curves (15 points) were prepared in methanol by serial dilution of the mother solution (5 ppm taurocholate, 1.5 ppm taurocholate-$d_5$, and 2.5 ppm of both C3G and P3G). Analyses were performed using a AB Sciex 6500 + triple quadrupole coupled to a Shimadzu LC-30 CE pump (Ab Sciex, Milan, Italy). Chromatographic separation was performed on Acquity Premier HSST3 2.1 × 150 mm (1.8 μm particle size) (Waters, Milan, Italy). The mobile phase was 0.1% formic acid, either in water (phase A) or acetonitrile (phase B). The linear gradient at a constant flow of 0.4 mL $min^{-1}$ started at 20% B and reached 100% B at 2 min. It was continued for 1.30 min, then the column was re-equilibrated at the original conditions. The total analysis time was 5.80 min. ESI was run in positive ion mode to detect cyanidin, C3G, peonidin and P3G and in negative mode to detect taurocholate and taurocholate-$d_5$. The curtain gas (CUR) was set at 35 PSI, the ion source (IS) gas 1 and 2 were set at 45 and 55 PSI, respectively; CUR and IS were air; collision gas was nitrogen. The source temperature was 400 °C; the ion spray voltage was 5500 V in positive ion mode and 4500 V in negative ion mode. Compound-dependent parameters, such as Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE), and Collision-cell exit potential (CXP), were determined by direct infusion of the pure standard. Analytical details can be found in Suppl. Table 2.

### 2.7. 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.

## 3. Results

### 3.1. 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. information, 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, showing the extensive uptake capacity of this liver preparation. 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. Fig. 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. 1 C 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. No 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).

#### 3.1.1. 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 [41].

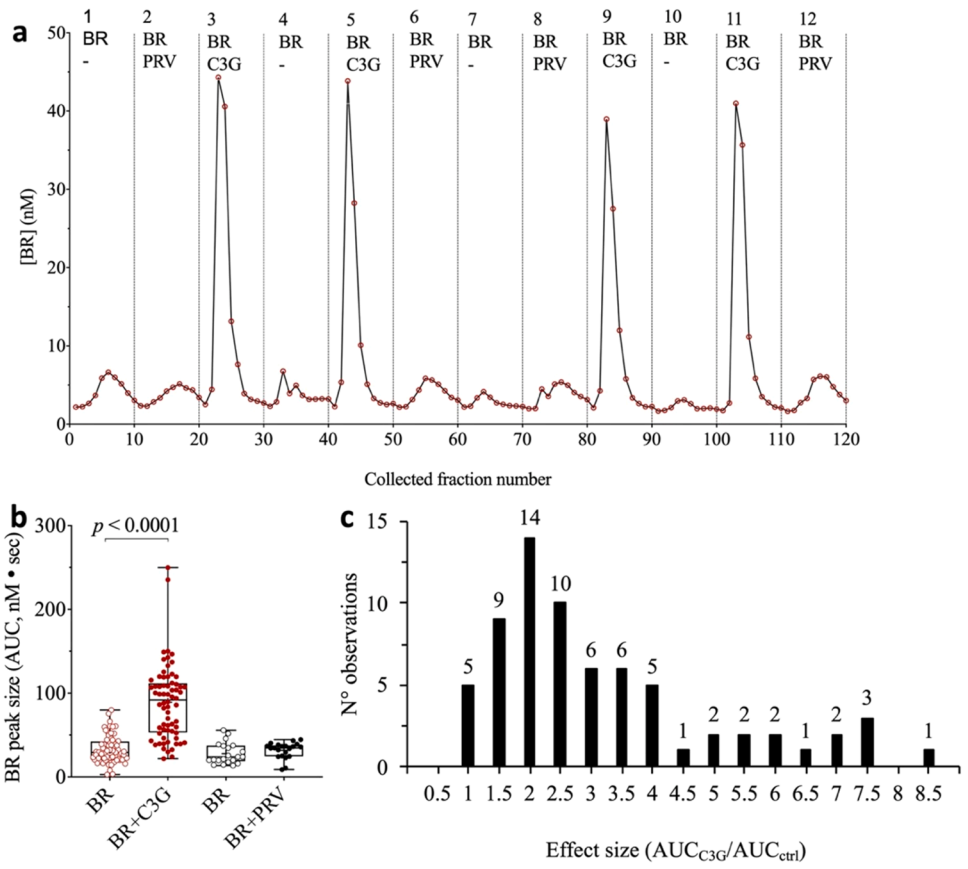



**Fig. 1. The effects of cyanidin 3-glucoside and pravastatin on the hepatic uptake of bilirubin.** (A) Concentrations of bilirubin (BR) in the venous effluent of a representative isolated perfused rat liver, following sequential intra-portal boli (0.2 mL) of 0.05 mM BR without and with either 2 mM cyanidin 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 (AUC$_{C3G}$) vs each respective control BR peak area (AUC$_{Ctrl}$) (livers, n = 21; peak pairs, n = 68).

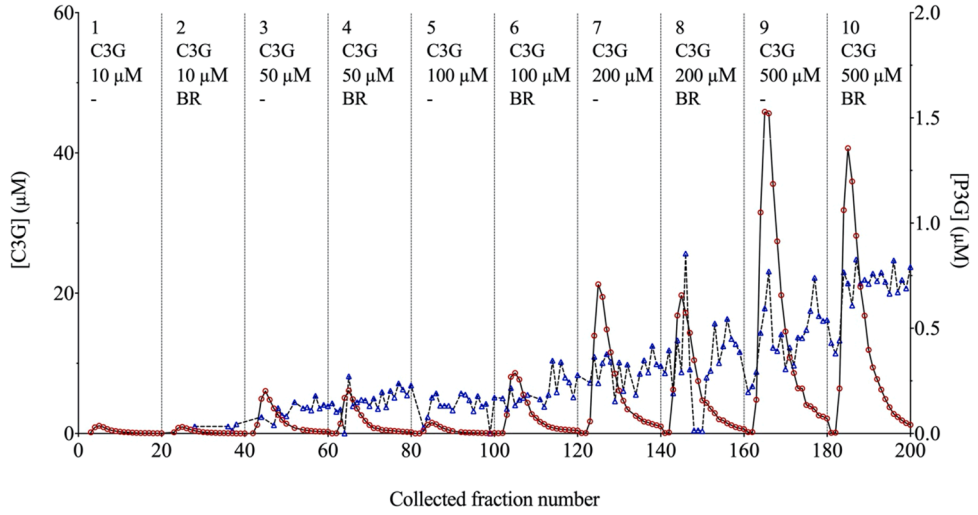



**Fig. 2. The effect of bilirubin on the hepatic uptake of cyanidin 3-glucoside and efflux of peonidin 3-glucoside.** Concentration of C3G (in red) and P3G (in blue) in the venous effluent of an isolated perfused rat liver, following sequential boli (0.2 mL) with increasing concentrations of C3G (0.01, 0.05, 0.1, 0.2, and 0.5 mM) without or with 0.05 mM BR.

### *3.2. 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 [42]. The efflux of BRG through sinusoidal Mrp3 and its downstream reuptake by sinusoidal Oatp is referred to as BRG hopping [43]. 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),

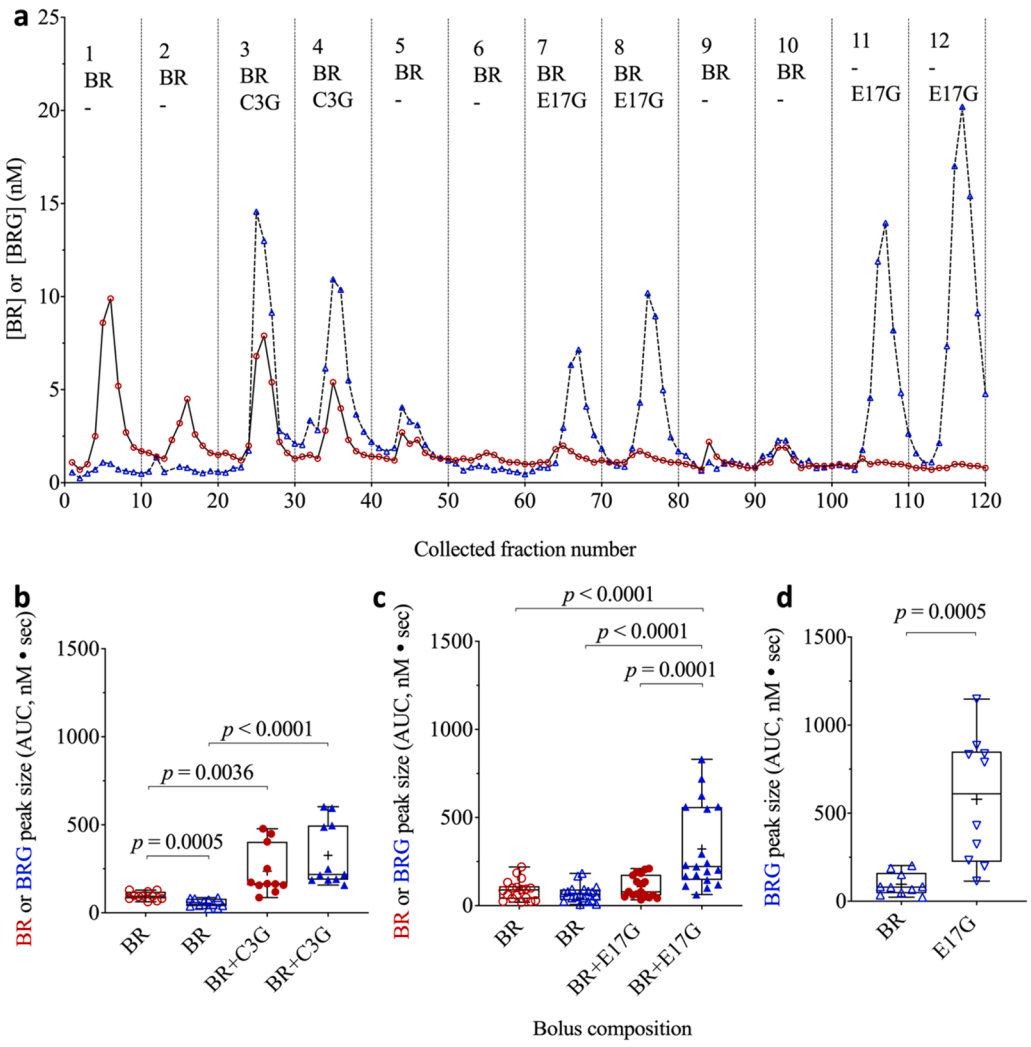



**Fig. 3. The effects of cyanidin 3-glucoside and estradiol-17 beta-glucuronide on the hepatic uptake of both bilirubin and bilirubin glucuronide.** (A) Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in blue) in the venous effluent of a representative isolated perfused liver, 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).

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.

### *3.3. 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 [44]. 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.

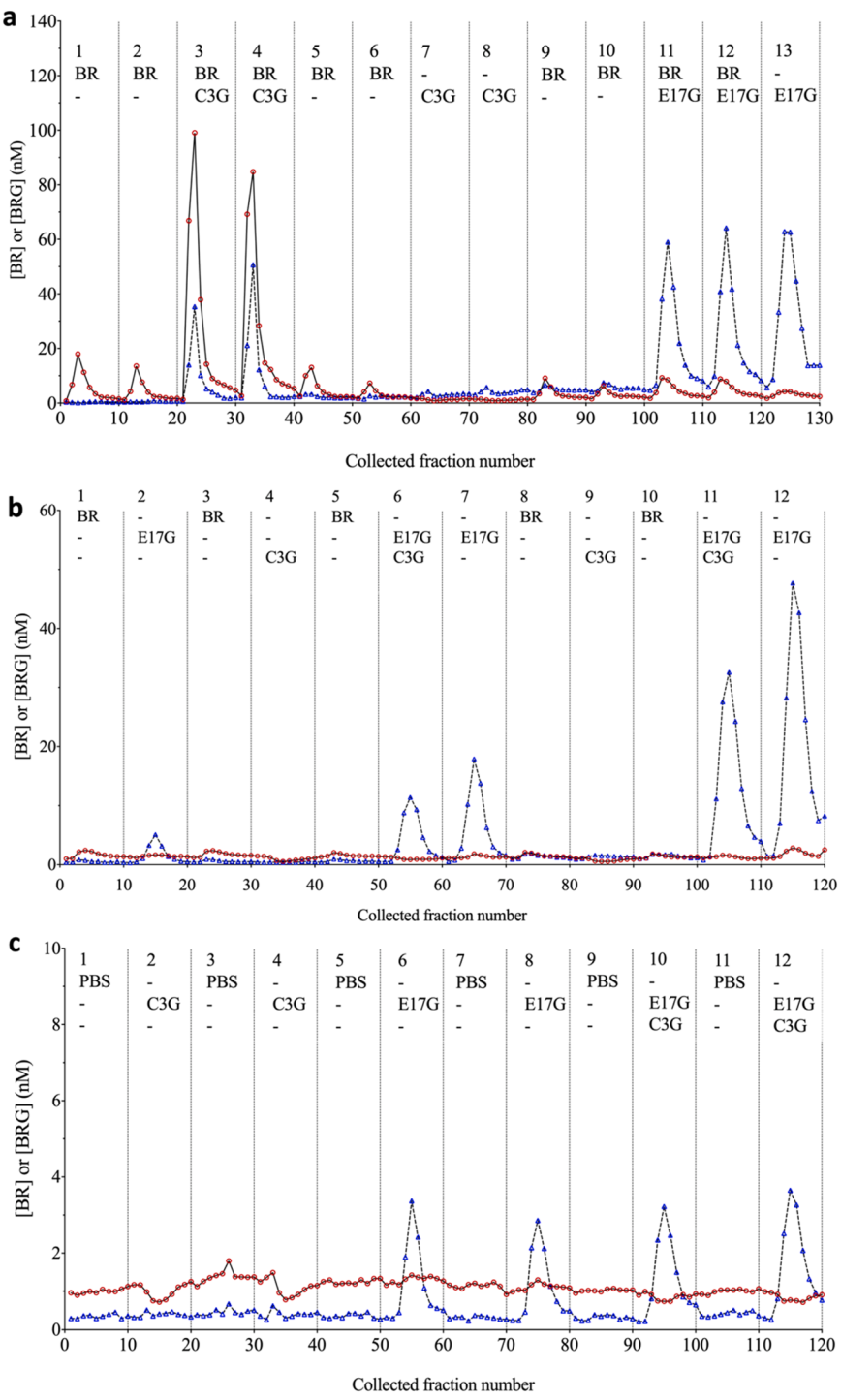



**Fig. 4. The effects of cyanidin 3-glucoside and estradiol-17 beta-glucuronide on the hepatic uptake of bilirubin, when injected without bilirubin.** Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in blue) in the venous effluent of representative isolated perfused rat livers. (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.

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.

### *3.4. 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 [18,45]. 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 [46]. We found that the Oat2 substrates ketoprofen or indomethacin (Suppl. Table 1B) did not affect the uptake of BR and BRG (Suppl. Fig. 9).

### 3.5. 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: Ntcp, Oatp1, and OSTα-OSTβ (Suppl. Table 1). By using taurocholate, we aimed primarily to inhibit Ntcp, which is not targeted by E17G or pravastatin (Suppl. Table 1) but the human orthologue NTCP transports indocyanine green [47]. In human subjects carrying mutant forms of Ntcp [48], hypercholanemia may be associated with elevated blood levels of indirect bilirubin (BR) [49] and direct bilirubin (BRG) [50,51].

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 [35].

### 3.6. 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 [52] and in human systemic circulation [53]. 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 [54]. 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.

## 4. Discussion

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.

### 4.1. Extensive bilirubin uptake

We observed that perfused livers released endogenous BR in the sinusoid, which occurred in the very first fractions of all experiments (1.2 ± 0.5 nM, n = 24). This could be due to the efflux of the intracellular "mobile" BR, i.e. the protein-free fraction [2]. Conversely, livers were able to absorb repeated boli of 10 nmol BR almost completely, with a residual amount of BR (0.13%, n = 68) recovered in the liver perfusion effluent. Simultaneous injection of C3G with BR had considerable effects on the occurrence of BR in the effluent, but had a negligible effect on the uptake balance, as the proportion of BR recovered in the effluent increased to only 0.36% (n = 68), demonstrating an overwhelming driving force for the uptake of BR from the sinusoid.

This result arises from the combination of the biophysical properties of BR membrane transport, the geometric property of this model, and perfusion flow rate. BR diffusion across the sinusoidal membrane of the hepatocyte is a rapid, transporter-mediated process, as shown by pharmacokinetic observations in the rat [4], later demonstrations in HepG2 cell cultures exposed to nM BR in an albumin-free buffer where an anti-bilitranslocase antibody blocked uptake [55], and the data obtained in this study. Because the vascular volume of the rat liver is approximately 5% of the liver volume [56], the volume in which BR could be distributed exceeds the volume of the portal vein bed by 2 orders of magnitude. Therefore, the fraction of the injected BR that should be recovered in the venous effluent at the equilibrium must be a few percent of the injected dose. Finally, the relatively low perfusion flow rate contributed to the quasi-total extraction of BR, as this parameter is directly dependent on flow [57]. Other factors contribute to the driving force for BR uptake, including the intracellular association of BR and BRG with glutathione S-transferase (Ligandin) [2] and the rapid BR glucuronidation to BRG, which implies, in turn that the liver has sufficient availability of UDP-glucuronic acid.

### 4.2. Hopping of bilirubin glucuronide in isolated perfused rat liver

Livers exhibited a basal net release of BRG that remained essentially unchanged by repeated administrations of BR and independent of the rate of any intracellular BR conjugation. This resulted from efflux via an uncharacterized efflux pathway that appeared to be independent of Mrp3 (since it was not inhibited by E17G) or other sinusoidal membrane transporters targeted by the inhibitors used. Importantly, hepatic reuptake of BRG occurred, as demonstrated by specific transient inhibition

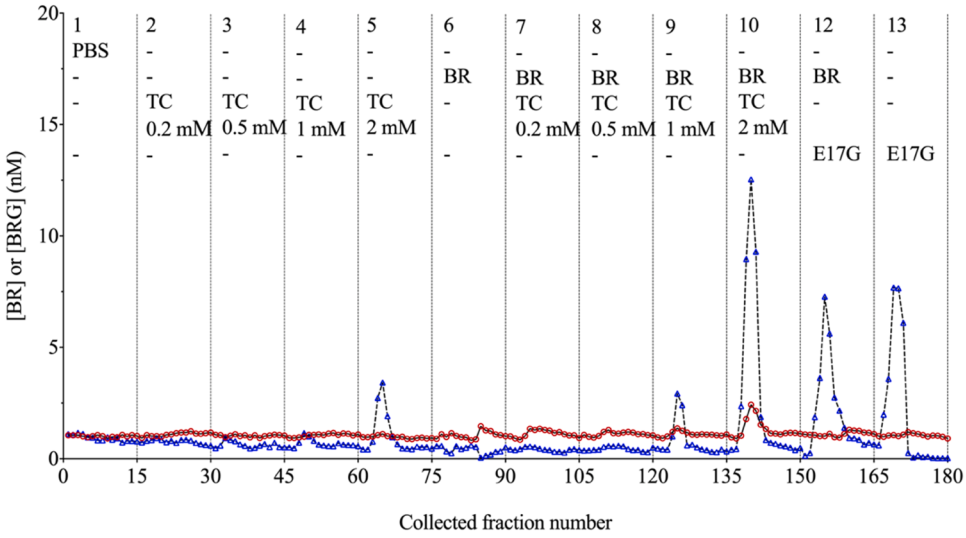



**Fig. 5. The dose-dependent effect of taurocholate on the hepatic uptake of both bilirubin and bilirubin glucuronide.** Concentrations of bilirubin (BR, in red) and bilirubin glucuronide (BRG, in blue) in the venous effluent of a representative isolated perfused liver, following sequential intra-portal 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 11) or alone (bolus 12).

by E17G, pravastatin, and taurocholate. Though these molecules may have acted by trans-activating sinusoidal efflux of BRG, the latter process takes dozen minutes to its maximum [58], rather than a dozen seconds as in our model, and is likely negligible under our liver perfusion conditions. Thus, in the isolated organ, we reproduced the phenomenon of sinusoidal reuptake of BRG previously described in knockout mice with defective expression of Oatp [59,60]. Overall, we consider BRG hopping in the isolated liver preparations as evidence of their viability and functional capacity.

### 4.3. 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. 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 [23,61], for the functional evaluation of bilitranslocase transporter [62]. 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 [62], and it appears that this is the unique specificity of bilitranslocase compared to all other BSP transporters such as Oatp [63], Ntcp [64], Oat [65], and Ostα-Ostβ [66]. 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 [67] and two anti-sequence antibodies [12,68]. 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 [24], 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.

### 4.4. 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.

## 5. Conclusions

In conclusion, this study has shed light on previously unknown functional properties of the liver. Though only female animals were used as liver donors, the same tests should be repeated in male rats to explore if sexual dimorphism exists for hepatic bilirubin uptake. 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.

## Funding

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

## CRediT authorship contribution statement

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

## Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Primary data sets are available in Mendeley Data at this link: https://data.mendeley.com/datasets/c3k9s4jw6j/draft?a= 5de8e6a8-f84e-4dfe-bac2–5f85d8752671.

## Acknowledgments

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

*Research data for this article*

Primary data sets are available in Mendeley Data at https://data.mendeley.com/datasets/c3k9s4jw6j/draft?a= 5de8e6a8-f84e-4dfe-bac2–5f85d8752671.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114044.

## References


[1] L. Vítek, C. Tiribelli, Bilirubin: the yellow hormone? J. Hepatol. (2021) https://doi.org/10.1016/j.jhep.2021.06.010.

[2] D.G. Levitt, M.D. Levitt, Quantitative assessment of the multiple processes responsible for bilirubin homeostasis in health and disease, Clin. Exp. Gastroenterol. 7 (2014) 307–328, https://doi.org/10.2147/CEG.S64283.

[3] L. Lin, S.W. Yee, R.B. Kim, K.M. Giacomini, SLC transporters as therapeutic targets: emerging opportunities, Nat. Rev. Drug Discov. 14 (2015) 543–560.

[4] B.F. Scharschmidt, J.G. Waggoner, P.D. Berk, Hepatic organic anion uptake in the rat. J. Clin. Invest 56 (1975) 1280–1292, https://doi.org/10.1172/JCI108204.

[5] J. Čvorović, S. Passamonti, Membrane transporters for bilirubin and its conjugates: a systematic review, Front. Pharmacol. 8 (2017) 887, https://doi.org/10.3389/fphar.2017.00887.

[6] T. Watanabe, M. Miyake, T. Shimizu, M. Kamezawa, N. Masutomi, T. Shimura, R. Ohashi, Utility of bilirubins and bile acids as endogenous biomarkers for the inhibition of hepatic transporters, Drug Metab. Dispos. 43 (2015) 459–466.

[7] I. Takehara, T. Yoshikado, K. Ishigame, D. Mori, K. Furihata, N. Watanabe, O. Ando, K. Maeda, Y. Sugiyama, H. Kusuhara, Comparative Study of the Dose-Dependence of OATP1B Inhibition by Rifampicin Using Probe Drugs and Endogenous Substrates in Healthy Volunteers, Pharm. Res. 35 (2018) 138, https://doi.org/10.1007/s11095-018-2416-3.

[8] M. Sorokina, M. Stam, C. Medigue, O. Lespinet, D. Vallenet, Profiling the orphan enzymes, Biol. Direct 9 (2014) 10, https://doi.org/10.1186/1745-6150-9-10.

[9] P. Sist, A. Bandiera, R. Urbani, S. Passamonti, Macromolecular and solution properties of the recombinant fusion protein HUG, Biomacromolecules 23 (2022) 3336–3348, https://doi.org/10.1021/acs.biomac.2c00447.

[10] X. Wu, G.R. Beecher, J.M. Holden, D.B. Haytowitz, S.E. Gebhardt, R.L. Prior, Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J. Agric. Food Chem. 54 (2006) 4069–4075, https://doi.org/10.1021/jf060300l.

[11] S. Passamonti, U. Vrhovsek, F. Mattivi, The interaction of anthocyanins with bilitranslocase, Biochem Biophys. Res Commun. 296 (2002) 631–636, https://doi.org/10.1016/S0006-291X(02)00927-0.

[12] L. Battiston, S. Passamonti, A. Macagno, G.L. Sottocasa, The bilirubin-binding motif of bilitranslocase and its relation to conserved motifs in ancient biliproteins. Biochem. Biophys. Res. Commun. 247 (1998) 687–692, https://doi.org/10.1006/bbrc.1998.8868.

[13] B. Stieger, Z.M. Mahdi, W. Jager, Intestinal and hepatocellular transporters: therapeutic effects and drug interactions of herbal supplements, Annu. Rev. Pharmacol. Toxicol. 57 (2017) 399–416, https://doi.org/10.1146/annurev-pharmtox-010716-105010.

[14] X. Lu, T. Chan, C. Xu, W.V. Ng, L. Zhu, F. Zhou, The interactions of herbal compounds with human organic anion/cation transporters, J. Pharm. Pharm. 5 (2014) 645–2153.

[15] X. Wang, A.W. Wolkoff, M.E. Morris, Flavonoids as a novel class of human organic anion-transporting polypeptide OATP1B1 (OATP-C) modulators, Drug Metab. Dispos. 33 (2005) 1666–1672, https://doi.org/10.1124/dmd.105.005926.

[16] J. Riha, S. Brenner, A. Srovnalova, L. Klameth, Z. Dvorak, W. Jager, T. Thalhammer, Effects of anthocyans on the expression of organic anion transporting polypeptides (SLCOs/OATPs) in primary human hepatocytes, Food Funct. 6 (2015) 772–779, https://doi.org/10.1039/c4fo00977k.

[17] Y. Li, J. Revalde, J.W. Paxton, The effects of dietary and herbal phytochemicals on drug transporters, Adv. Drug Deliv. Rev. 116 (2017) 45–62, https://doi.org/10.1016/j.addr.2016.09.004.

[18] M. Shupeck, A.W. Wolkoff, B.F. Scharschmidt, J.G. Waggoner, P.D. Berk, Studies of the kinetics of purified conjugated bilirubin-3H in the rat, Am. J. Gastroenterol. 70 (1978) 259–264.

[19] B. Stieger, Z.M. Mahdi, Model systems for studying the role of canalicular efflux transporters in drug-induced cholestatic liver disease, J. Pharm. Sci. 106 (2017) 2295–2301.

[20] N.P. du Sert, A. Ahluwalia, S. Alam, M.T. Avey, M. Baker, W.J. Browne, A. Clark, I. C. Cuthill, U. Dirnagl, M. Emerson, Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0, PLoS Biol. 18 (2020).

[21] M. Bessems, N.A. 't Hart, R. Tolba, B.M. Doorschodt, H.G.D. Leuvenink, R.J. Ploeg, T. Minor, T.M. van Gulik, The isolated perfused rat liver: standardization of a time-honoured model, Lab. Anim. 40 (2006) 236–246, https://doi.org/10.1258/002367706777611460.

[22] M. Stebel, N. Medic, P. Pelizzo, P. Sist, F. Tramer, S. Passamonti, Protocol for the study of hepatic bilirubin uptake in the isolated perfused rat liver, 2021. https://doi.org/10.21203/rs.3.pex-1698/v1.

[23] S. Passamonti, F. Tramer, E. Petrussa, E. Braidot, A. Vianello, Electrogenic bromosulfalein transport in isolated membrane vesicles: implementation in both animal and plant preparations for the study of flavonoid transporters, Methods Mol. Biol. 643 (2010) 307–335, https://doi.org/10.1007/978-1-60761-723-5_21.

[24] S. Zuperl, S. Fornasaro, M. Novic, S. Passamonti, Experimental determination and prediction of bilitranslocase transport activity, Anal. Chim. Acta 705 (2011) 322–333, https://doi.org/10.1016/j.aca.2011.07.004.

[25] C.M. Bowman, L.Z. Benet, An examination of protein binding and protein-facilitated uptake relating to in vitro-in vivo extrapolation, Eur. J. Pharm. Sci. 123 (2018) 502–514, https://doi.org/10.1016/j.ejps.2018.08.008.

[26] S.F. Asad, S. Singh, A. Ahmad, N.U. Khan, S.M. Hadi, Prooxidant and antioxidant activities of bilirubin and its metabolic precursor biliverdin: a structure–activity study, Chem. Biol. Interact. 137 (2001) 59–74, https://doi.org/0.1016/s0009-2797(01)00209-5.

[27] H. Yamasaki, H. Uefuji, Y. Sakihama, Bleaching of the red anthocyanin induced by superoxide radical, Arch. Biochem. Biophys. 332 (1996) 183–186, https://doi.org/10.1006/abbi.1996.0331.

[28] B. González-Flecha, J.C. Cutrin, A. Boveris, Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. J. Clin. Invest 91 (1993) 456–464, https://doi.org/10.1172/JCI116223.

[29] T.S. Chan, S. Cassim, V.-A. Raymond, S. Gottschalk, G. Merlen, C. Zwingmann, P. Lapierre, P. Darby, C.D. Mazer, M. Bilodeau, Upregulation of Krebs cycle and anaerobic glycolysis activity early after onset of liver ischemia, PLoS One 13 (2018), e0199177, https://doi.org/10.1371/journal.pone.0199177.

[30] I.A. Sherman, J.A. Dlugosz, F. Barker, F.M. Sadeghi, K.S. Pang, Dynamics of arterial and portal venous flow interactions in perfused rat liver: an intravital microscopic study, Am. J. Physiol. Liver Physiol. 271 (1996) G201–G210, https://doi.org/10.1152/ajpgi.1996.271.1.G201.

[31] M.J. Daemen, H.H. Thijssen, H. van Essen, H.T. Vervoort-Peters, F.W. Prinzen, H. A. Struyker Boudier, J.F. Smits, Liver blood flow measurement in the rat. The electromagnetic versus the microsphere and the clearance methods. J. Pharmacol. Methods 21 (1989) 287–297, https://doi.org/10.1016/0160-5402(89)90066-1.

[32] A.W. Wolkoff, K.L. Johansen, T. Goeser, The isolated perfused rat liver: preparation and application, Anal. Biochem. 167 (1987) 1–14, https://doi.org/10.1016/0003-2697(87)90127-8.

[33] J.A. Díaz-Juárez, R. Hernández-Muñoz, Rat liver enzyme release depends on blood flow-bearing physical forces acting in endothelium glycocalyx rather than on liver damage, Oxid. Med. Cell. Longev. 2017 (2017), 1360565, https://doi.org/10.1155/2017/1360565.

[34] K. Kato, Y. Hasegawa, K. Iwata, T. Ichikawa, T. Yahara, S. Tsuji, M. Sugiura, J.-I. Yamaguchi, Recommendation to exclude bile-duct-cannulated rats with hyperbilirubinemia for proper conduct of biliary drug excretion studies, Drug Metab. Dispos. 44 (2016) 1180–1183, https://doi.org/10.1124/dmd.116.070532.

[35] G. Paumgartner, J. Reichen, Kinetics of hepatic uptake of unconjugated bilirubin, Clin. Sci. Mol. Med 51 (1976) 169–176, https://doi.org/10.1042/cs0510169.

[36] Y.R. Stollman, U. Gartner, L. Theilmann, N. Ohmi, A.W. Wolkoff, Hepatic bilirubin uptake in the isolated perfused rat liver is not facilitated by albumin binding. J. Clin. Invest 72 (1983) 718–723, https://doi.org/10.1172/JCI111021.

[37] H. Roelofsen, C.N. van der Veere, R. Ottenhoff, B. Schoemaker, P.L. Jansen, R.P. Oude Elferink, Decreased bilirubin transport in the perfused liver of endotoxemic rats, Gastroenterology 107 (1994) 1075–1084, https://doi.org/10.1016/0016-5085(94)90232-1.

[38] J.R. Chowdhury, P.L. Jansen, E.B. Fischberg, A. Daniller, I.M. Arias, Hepatic conversion of bilirubin monoglucuronide to diglucuronide in uridine diphosphate-glucuronyl transferase-deficient man and rat by bilirubin glucuronoside glucuronosyltransferase. J. Clin. Invest 62 (1978) 191–196, https://doi.org/10.1172/JCI109105.

[39] M. Hirouchi, H. Suzuki, Y. Sugiyama, Treatment of hyperbilirubinemia in Eisai hyperbilirubinemic rat by transfecting human MRP2/ABCC2 gene, Pharm. Res. 22 (2005) 661–666, https://doi.org/10.1007/s11095-005-2502-1.

[40] A. Vanzo, M. Terdoslavich, A. Brandoni, A.M. Torres, U. Vrhovsek, S. Passamonti, Uptake of grape anthocyanins into the rat kidney and the involvement of bilitranslocase, Mol. Nutr. Food Res. 52 (2008) 1106–1116.

[41] S. Fornasaro, L. Ziberna, M. Gasperotti, F. Tramer, U. Vrhovsek, F. Mattivi, S. Passamonti, 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. 6 (2016) 22815, https://doi.org/10.1038/srep22815.

[42] J.L. Barnhart, R. Clarenburg, Factors determining clearance of bilirubin in perfused rat liver, Am. J. Physiol. Content 225 (1973) 497–507.

[43] D. Iusuf, E. Van De Steeg, A.H. Schinkel, Hepatocyte hopping of OATP1B substrates contributes to efficient hepatic detoxification, Clin. Pharmacol. Ther. 92 (2012) 559–562.

[44] S. Passamonti, L. Battiston, G.L. Sottocasa, Bilitranslocase can exist in two metastable forms with different affinities for the substrates–evidence from cysteine and arginine modification, Eur. J. Biochem. 253 (1998) 84–90.

[45] Y. Adachi, J. Roy-Chowdhury, N. Roy-Chowdhury, R. Kinne, T. Tran, H. Kobayashi, I.M. Arias, Hepatic uptake of bilirubin diglucuronide: analysis by using sinusoidal plasma membrane vesicles, J. Biochem 107 (1990) 749–754.

[46] N. Morita, H. Kusuhara, Y. Nozaki, H. Endou, Y. Sugiyama, Functional involvement of rat organic anion transporter 2 (Slc22a7) in the hepatic uptake of the nonsteroidal anti-inflammatory drug ketoprofen, Drug Metab. Dispos. 33 (2005) 1151–1157.

[47] W. de Graaf, S. Häusler, M. Heger, T.M. van Ginhoven, G. van Cappellen, R. J. Bennink, G.A. Kullak-Ublick, R. Hesselmann, T.M. van Gulik, B. Stieger, Transporters involved in the hepatic uptake of 99mTc-mebrofenin and indocyanine

green, J. Hepatol. 54 (2011) 738–745, https://doi.org/10.1016/j.jhep.2010.07.047.
[48] J.L. Boyer, C.J. Soroka, Bile formation and secretion: an update, J. Hepatol. (2021), https://doi.org/10.1016/j.jhep.2021.02.011.
[49] J.-W. Qiu, M. Deng, Y. Cheng, R.-M. Atif, W.-X. Lin, L. Guo, H. Li, Y.-Z. Song, 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 8 (2017) 106598–106607, https://doi.org/10.18632/oncotarget.22503.
[50] F.M. Vaz, C.C. Paulusma, H. Huidekoper, M. de Ru, C. Lim, J. Koster, K. Ho-Mok, A. H. Bootsma, A.K. Groen, F.G. Schaap, R.P.J. Oude Elferink, H.R. Waterham, R.J. A. Wanders, Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype, Hepatology 61 (2015) 260–267, https://doi.org/10.1002/hep.27240.
[51] Y.Y. Yan, M.X. Wang, J.Y. Gong, L.L. Liu, K.D.R. Setchell, X.B. Xie, N.L. Wang, W. Li, J.-S. Wang, Abnormal Bilirubin Metabolism in Patients With Sodium Taurocholate Cotransporting Polypeptide Deficiency. J. Pediatr. Gastroenterol. Nutr. 71 (2020) e138–e141, https://doi.org/10.1097/MPG.0000000000002862.
[52] S. Passamonti, U. Vrhovsek, A. Vanzo, F. Mattivi, The stomach as a site for anthocyanins absorption from food, FEBS Lett. 544 (2003) 210–213.
[53] R.M. de Ferrars, C. Czank, Q. Zhang, N.P. Botting, P.A. Kroon, A. Cassidy, C.D. Kay, The pharmacokinetics of anthocyanins and their metabolites in humans, Br. J. Pharmacol. 171 (2014) 3268–3282, https://doi.org/10.1111/bph.12676.
[54] J. Ahlberg, B. Angelin, I. Björkhem, K. Einarsson, Individual bile acids in portal venous and systemic blood serum of fasting man, Gastroenterology 73 (1977) 1377–1382.
[55] S. Passamonti, M. Terdoslavich, A. Margon, A. Cocolo, N. Medic, F. Micali, G. Decorti, M. Franko, Uptake of bilirubin into HepG2 cells assayed by thermal lens spectroscopy: Function of bilitranslocase, FEBS J. 272 (2005) 5522–5535.
[56] T.V. Masyuk, E.L. Ritman, N.F. LaRusso, Hepatic artery and portal vein remodeling in rat liver: vascular response to selective cholangiocyte proliferation, Am. J. Pathol. 162 (2003) 1175–1182, https://doi.org/10.1016/S0002-9440(10)63913-2.
[57] J.M. Pries, A.B. Staples, R.F. Hanson, The effect of hepatic blood flow on taurocholate extraction by the isolated perfused rat liver. J. Lab. Clin. Med 97 (1981) 412–417.
[58] R.D. Schnegelberger, B. Steiert, P.J. Sandoval, B. Hagenbuch, Using a competitive counterflow assay to identify novel cationic substrates of OATP1B1 and OATP1B3, Front. Physiol. (2022) 1803.
[59] E. van de Steeg, E. Wagenaar, C.M.M. van der Kruijssen, J.E.C. Burggraaff, D.R. de Waart, R.P.J.O. Elferink, K.E. Kenworthy, A.H. Schinkel, Organic anion transporting polypeptide 1a/1b–knockout mice provide insights into hepatic handling of bilirubin, bile acids, and drugs, J. Clin. Invest 120 (2010) 2942, https://doi.org/10.1172/JCI42168.
[60] E. van de Steeg, V. Stránecký, H. Hartmannová, L. Nosková, M. Hřebíček, E. Wagenaar, A. van Esch, D.R. de Waart, R.P.J.O. Elferink, K.E. Kenworthy, Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver, J. Clin. Invest 122 (2012) 519, https://doi.org/10.1172/JCI59526.
[61] G. Baldini, S. Passamonti, G.C. Lunazzi, C. Tiribelli, G.L. Sottocasa, Cellular localization of sulfobromophthalein transport activity in rat liver, Biochim. Biophys. Acta 856 (1986) 1–10.
[62] G.L. Sottocasa, S. Passamonti, L. Battiston, L. Pascolo, C. Tiribelli, Molecular aspects of organic anion uptake in liver, J. Hepatol. 24 (Suppl 1) (1996) 36–41.
[63] E. Jacquemin, B. Hagenbuch, B. Stieger, A.W. Wolkoff, P.J. Meier, Expression cloning of a rat liver Na(+)-independent organic anion transporter, Proc. Natl. Acad. Sci. U. S. A 91 (1994) 133–137.
[64] S. Hata, P. Wang, N. Eftychiou, M. Ananthanarayanan, A. Batta, G. Salen, K. S. Pang, A.W. Wolkoff, Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake, Am. J. Physiol. Liver Physiol. 285 (2003) G829–G839, https://doi.org/10.1152/ajpgi.00352.2002.
[65] H. Saito, S. Masuda, K. Inui, Cloning and functional characterization of a novel rat organic anion transporter mediating basolateral uptake of methotrexate in the kidney, J. Biol. Chem. 271 (1996) 20719–20725.
[66] D.J. Seward, A.S. Koh, J.L. Boyer, N. Ballatori, Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta, J. Biol. Chem. 278 (2003) 27473–27482, https://doi.org/10.1074/jbc.M301106200.
[67] S. Passamonti, L. Battiston, G.L. Sottocasa, Arylsulfonylation of bilitranslocase in plasma membranes from rat liver enables to discriminate between natural and artificial substrates, Biochim. Biophys. Acta - Biomembr. 1323 (1997) 130–136.
[68] S. Passamonti, A. Cocolo, E. Braidot, E. Petrussa, C. Peresson, N. Medic, F. Macri, A. Vianello, Characterization of electrogenic bromosulfophthalein transport in carnation petal microsomes and its inhibition by antibodies against bilitranslocase. FEBS J. 272 (2005) 3282–3296, https://doi.org/10.1111/j.1742-4658.2005.04751.x.