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Research paper

In vitro models to measure effects on intestinal deconjugation and transport of mixtures of bile acids

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ABSTRACT

Bile acid metabolism and transport are critical to maintain bile acid homeostasis and host health. In this study, it was investigated if effects on intestinal bile acid deconjugation and transport can be quantified in *vitro* model systems using mixtures of bile acids instead of studying individual bile acids. To this end deconjugation of mixtures of selected bile acids in anaerobic rat or human fecal incubations and the effect of the antibiotic tobramycin on these reactions was studied. In addition, the effect of tobramycin on the transport of the bile acids in isolation or in a mixture across Caco-2 cell layers was characterized. The results demonstrate that both the reduction of bile acid deconjugation and transport by tobramycin can be adequately detected in *vitro* systems using a mixture of bile acids, thus eliminating the need to characterize the effects for each bile acid in separate experiments. Subtle differences between the experiments with single or combined bile acids point at mutual competitive interactions and indicate that the use of bile acid mixtures is preferred over use of single bile acid given that also *in vivo* bile acids occurs in mixtures.

1. Introduction

Bile acids are produced from cholesterol and then conjugated with glycine or taurine forming conjugated primary bile acids in the liver [1,2]. The conjugated primary bile acids are secreted into the small intestine through the bile duct, after which most of the conjugated primary bile acids are reabsorbed in the ileum and channeled back via the portal vein into the liver [3]. Active reabsorption of bile acids in the ileum is important in sustaining bile acid homeostasis. More than 95% of conjugated primary bile acids are reabsorbed and returned to the liver [4,5]. This recycling is called enterohepatic circulation [6,7], and helps to maintain bile acid homeostasis [8,9]. A minor amount of conjugated primary bile acids escapes reuptake and is transferred to the large intestine, where intestinal microbiota perform deconjugation and 7α-dehydroxylation converting conjugated primary bile acids like for example taurocholic acid (TCA), via unconjugated primary bile acids like for example cholic acid (CA), into secondary bile acids like for example deoxycholic acid (DCA) [10,11]. Fig. 1 schematically presents the bile acid transformations in the large intestine of interest for the present study. Antibiotic treatment can cause profound changes in the composition and abundance of gut microbiota [12,13]. Hence, several studies focused on the impact of antibiotics on gut microbiota and some studies also characterized the resulting influences on the bile acid pool. Most of these studies applied *in vivo* animal models measuring the bile acid composition and changes therein upon oral administration of antibiotics in different *ex vivo* matrices including feces, plasma and intestinal tissues [14–16]. These studies elucidated the influence of antibiotics on bile acid profiles. In addition to the effects of antibiotics on the gut microbiota, there could also be effects on the intestinal cells and transporters involved in the reuptake underlying the changes in bile acid homeostasis induced by the antibiotics. In a previous study [17], we showed that the antibiotic tobramycin inhibits the microbial deconjugation of taurocholic acid (TCA) in anaerobic fecal incubations and also the transport of TCA over an *in vitro* Caco-2 cell layer used as a model to mimic intestinal bile acid reuptake. Thus, we showed that *in vitro* model systems could be used to study the effect of antibiotics on the gut microbial metabolism and intestinal transport using anaerobic fecal incubations and Caco-2 cell layers in a transwell system with an individual bile acid, taurocholic acid (TCA).

The aim of the present study was to investigate whether the effects on bile acid metabolism and transport upon treatment with tobramycin can also be studied in an *in vitro* model system using mixtures of bile acids instead of individual bile acids, in order to i) elucidate the effect of tobramycin on the deconjugation and intestinal transport of other bile

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acids, ii) develop a more efficient testing strategy, iii) better mimic the *in vivo* situation where bile acids also do not occur in isolation, and iv) contribute to the development of new approach methodologies focusing on the 3Rs (replacement, reduction and refinement) of animal experiments.

In the current study, the two *in vitro* model systems previously used to obtain insight into how tobramycin would modulate TCA metabolism and transport were used to study the effect of tobramycin on deconjugation and transport of a series of (conjugated) primary bile acids. For deconjugation studies anaerobic fecal incubations were used while for transport studies the Caco-2 transwell model was applied. The Caco-2 cell line is able to differentiate into cells that resemble small intestinal cells not only showing morphological characteristics of intestinal cells but also expressing typical microvillus hydrolases and nutrient transporters [18–20]. The Caco-2 cell line is a widely applied *in vitro* model to evaluate the transport of drugs and xenobiotics and has also been used to study the reuptake of bile acids [17,21,22].

Deconjugation and transport of the bile acids was studied for selected bile acids in isolation but also for a mixture of selected bile acids to further elucidate whether testing a mixture instead of single bile acids would provide an adequate way to study (effects on) bile acid transport. The results obtained provide insight into the applicability of the two *in vitro* model systems to study effects on bile acid homeostasis.

2. Materials and methods

2.1. Reagents

Tobramycin was purchased from Sigma-Aldrich (Schnelldorf, Germany). Chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA), deoxycholic acid (DCA), cholic acid (CA), glycoursodeoxycholic acid (GUDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Glycolithocholic acid (GLCA) was obtained from Cambrige isotope laboratories (Massachusetts, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile (ACN) and methanol were obtained from Biosolve BV (Valkenswaard, Netherlands). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). Fetal Bovine serum (FBS) was obtained from GE Healthcare Life Sciences Hyclone Laboratories (Logan, Utah, USA). 0.05% Trypsin-EDTA, minimum essential medium (MEM), penicillin-streptomycin-glutamine solution (PSG), sodium pyruvate, Hank's balanced salt solution (HBSS) and HEPES buffer solution were purchased from Gibco (Paisley, UK). Corning Costar 24 well transwell plates were purchased from Corning Life Sciences (Schnelldorf, Germany). 96 Well cell culture plates were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, Netherlands).

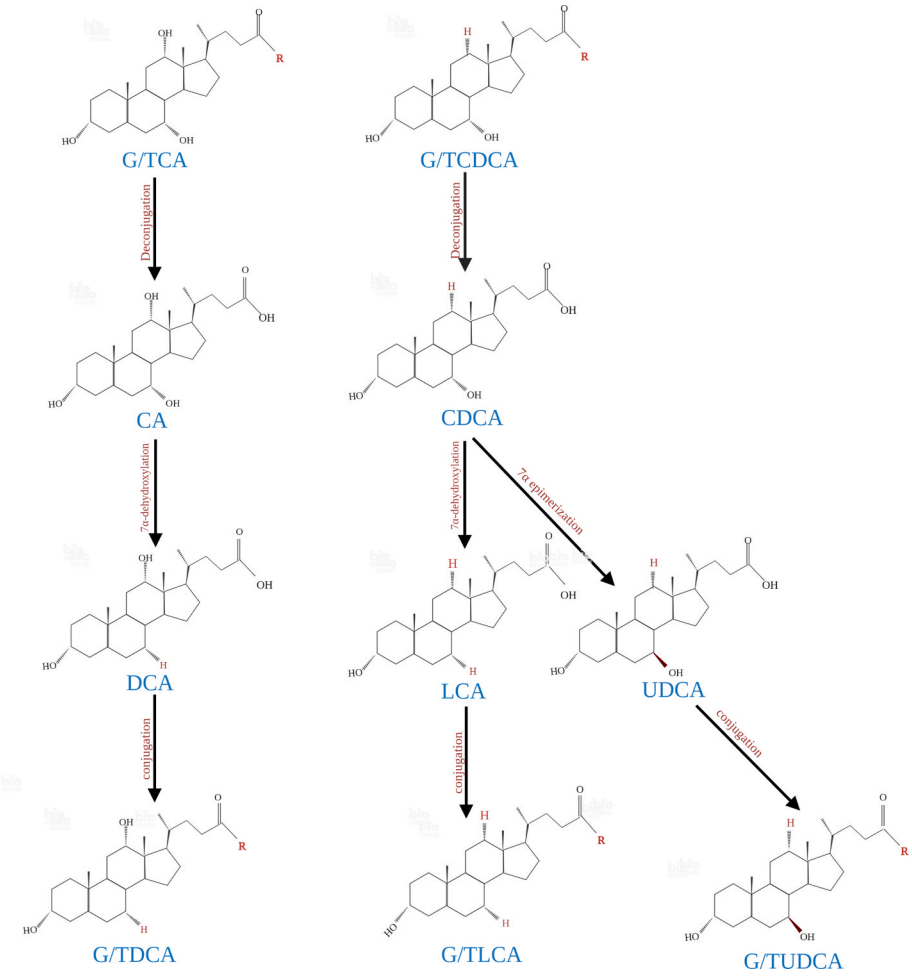

Fig. 1. Bile acid transformations in the large intestine. R means taurine/glycine.

2.2. Anaerobic incubations of bile acids with rat or human feces

2.2.1. Fecal collection and processing

Fresh rat feces from control (10 males and 10 females) Wistar rats were provided by BASF SE (Ludwigshafen, Germany). Feces were obtained by physical massage of the rectum of rats, weighed and transferred immediately into anaerobic 10% (v/v) glycerol in PBS solution, pooled and diluted to a final fecal concentration of 20% (w/v) under an anaerobic atmosphere (85% N_2, 10% CO_2, and 5% H_2) (BACTRON300 anaerobic chamber (Cornelius, USA)). Subsequently, samples were filtered using sterile gauze under anaerobic conditions, and aliquoted samples of resulting fecal slurry were stored at −80 °C until use. Human feces were collected from 12 healthy individuals without dietary limitations. They were from different gender, age and ethnicity. All of them had not taken antibiotics for at least 3 months prior to the study. After volunteers' donations, fecal samples were collected, weighed and immediately transferred to anaerobic 10% (v/v) glycerol in PBS solution, pooled and diluted to a final fecal concentration of 20% (w/v) under an anaerobic atmosphere (85% N_2, 10% CO_2, and 5% H_2) (BACTRON300 anaerobic chamber (Cornelius, USA)). A pooled sample was acquired by mixing of 12 fecal suspensions in equal amounts. The experimental protocol was evaluated and approved by the Medical Ethical Reviewing Committee of Wageningen University (METC-WU) based on the Dutch Medical Research Involving Human Subjects Act. All volunteers gave their written consent.

2.2.2. Deconjugation of fecal bile acids

Incubations to study deconjugation of mixtures of bile acids and the effect of the antibiotic tobramycin on this deconjugation were performed essentially as described previously for isolated bile acids [17]. Each incubation contained 1 μl rat fecal slurry (final concentration: 2 mg/ml fecal sample), 10 μl of a 10 times concentrated solution containing mixed conjugated bile acids (TCA, TCDCA, GCA and GCDCA) resulting in a final concentration of 500 μM for each of the bile acids tested, 79 μl PBS and 10 μl water (control) or 10 μl tobramycin from a 10 times concentrated stock solution in MilliQ water resulting in a final concentration of 45 mM tobramycin.

Anaerobic incubations were performed in the BACTRON 300 anaerobic chamber (Sheldon, Cornelius, USA) with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2, at 37 °C. Incubations were terminated by adding a similar volume (100 μL) acetonitrile after 0 h, 2 h, 4 h, 6 h and 8 h. Samples were centrifuged at 21,500 g for 15 min at 4 °C, and the supernatants were transferred to LC-MS/MS vials and stored at −80 °C until bile acid detection by LC-MS/MS.

Anaerobic incubations of human fecal slurry containing 45 mM tobramycin and mixed conjugated bile acids (TCA, TCDCA, GCA and GCDCA) at a final concentration of 500 μM for each bile acid were performed and processed in a similar way.

2.3. Cell culture

Human colon adenocarcinoma Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells (passage number 10–20) were cultured in the medium including MEM with 20% FBS, 1% sodium pyruvate and 1% penicillin-streptomycin-glutamine. The cells were grown in 75 cm^2 flask in an incubator at 37 °C, 5% CO_2 and 100% humidity. Cells were subcultured at 50%–60% confluence with 0.05% trypsin-EDTA.

2.4. Bile acid transport

To study bile acid transport across Caco-2 cell layers, 100 μl of a cell suspension containing 1.8×10^5 cells/cm^2 was seeded at the apical sides of a Corning 24 well transwell plate (Product no. CLS3413), and 600 μl MEM medium was added to the basolateral compartments. Cells were cultured for 18 days in order to differentiate into intestinal cell layers, while medium was changed every other day. MEM medium free of penicillin-streptomycin was applied when cells were seeded in the 24 well transwell plate in order to eliminate potential impact on the effects induced by the antibiotic tobramycin. After 18 days cultivation of the Caco-2 cells in the transwells, cell layers were exposed to 45 mM tobramycin or solvent control for 48 h. Following this pre-incubation, the integrity of the Caco-2 cell layers was measured by using trans-epithelial electrical resistance (TEER). The TEER was measured with a Millicell® ERS-2 Volt-Ohm Meter (Millipore, Amsterdam, Netherlands). The TEER value was detected before, during and after the transport assay, and expressed in Ω × cm^2. Only cell layers with a TEER value above 500 Ω × cm^2 were used for transport experiments and it was also checked that tobramycin pre-exposure did not affect the TEER value compared to the control, indicating there was no effect on cell layer integrity.

After the pre-exposure to tobramycin (or solvent control), the exposure medium was removed and the cell layers were cultured in transport medium (HBSS supplemented with 10 mM HEPES) for 30 min. After this 30 min incubation in transport medium, medium was removed and fresh transport medium containing 5 μM (final concentration) TCA, TCDCA, GCA or GCDCA, either individually or as a mixture, were added to the apical part of the wells. 50 μL samples were collected from the basolateral compartment at 1 h, 2 h and 3 h, and the compartment was refilled with the same amount of transport medium after each collection. In the collected samples, the amount of the bile acids was quantified by LC-MS/MS.

Additionally, similar transport experiments for a mixture of 17 bile acids (UDCA, HDCA, CDCA, DCA, CA, GLCA, GUDCA, GDCA, GCDCA, GCA, TCDCA, TCA, TLCA, TUDCA, THDCA, TDCA and LCA) each at a final concentration of 5 μM were performed. Samples were collected as described above and measured by LC-MS/MS.

Concentrations of bile acids in the intestinal tract and plasma have been reported to be in the high micromolar or low millimolar range [23,24]. The overall concentrations used in the combined bile acid experiments of the present study amounted to 20 mM and 85 mM for the experiment with 4 or 17 bile acids combined and thus fall in the lower range of the physiological levels, while potential relative differences between different bile acids were not taken into account testing all bile acids at an equimolar concentration also to allow comparison.

2.5. Bile acid profiling by LC-MS/MS

A triple quadrupole LCMS-8045 (Shimadzu Corporation, Japan) was used to perform bile acid analysis. The detection method was able to measure 20 bile acids. A Kinetex C18 column (1.7 μm × 100 A × 50mm × 2.1 mm, Phenomenex 00B-4475-AN) was used to separate bile acids. Mobile phase A (0.01% formic acid in MilliQ water) and mobile phase B (50% acetonitrile/50% methanol) were applied. 1 μL sample was injected onto the column equilibrated with 70% mobile phase A and 30% mobile phase B at a flow rate of 0.4 mL/min. The following gradient was used: 0–10 min 30–70% B, kept at 70% B for 1 min; 11–19 min 70–98% B, kept at 98% B for 1min; and then 20–25 min 98-30% B followed by 10 min equilibration at 30% B before the next injection. The mass spectrometer (MS) used electrospray ionization (ESI) in negative ion mode. The ESI parameters were as follows: Nebulizing gas flow 3 L/min; drying gas flow and heating gas flow 10 L/min; interface voltage 3 kV; interface current 0.9 μA; interface temperature 300 °C; Desolvation temperature 526 °C; DL temperature 250 °C; heat block temperature 400 °C; Conversion Dynode Voltage 10 kV; detector voltage 2.16 kV; IG Vacuum 1.8e-03 Pa; PG Vacuum 9.4e+01 Pa; CID Gas 230 kPa. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were applied for the bile acid detection. Both SIM and MRM were used for detection of the bile acid to obtain adequate sensitivity for all the bile acids. Specific bile acid identification MS values are presented in Table S1 in the supplementary information.

2.6. Data processing and analysis

Bile acid profile data were analyzed using the Labsolutions software in the LC-MS/MS system. Graphical figures were drawn using Graphpad Prism 5 (San Diego, USA). Statistical analysis was performed by Student's *t*-test. $P < 0.05$ was considered significant. Fig. 1 was drawn by Biorender (San Francisco, USA). All the results were expressed as mean ± SD from three independent measurements.

3. Results

3.1. The impact of tobramycin on the deconjugation of conjugated bile acids when tested in isolation or in a mixture

In our previous study [25], the effect of tobramycin on the gut microbial deconjugation of TCA, TCDCA, GCA and GCDCA when tested in isolation was reported. Table 1 summarizes the rates of deconjugation derived from these previous data and reveals that the deconjugation of TCDCA and GCDCA was faster than that of TCA and GCA, and that tobramycin inhibited the deconjugation of all 4 conjugated bile acids. The results in Fig. 2A–D shows the effect of tobramycin compared to the control on the time dependent deconjugation of TCA, TCDCA, GCA and GCDCA when tested in a mixture. Fig. 2E and F presents the accompanying formation of the deconjugated metabolites CA, formed from TCA and GCA, and CDCA, formed form TCDA and GCDA. Table 1 also presents the rates of deconjugation derived from these data. From the results obtained it follows that when tested in a mixture, the rates of deconjugation were either comparable or at most up to about 2-fold lower than when testing the bile acids in isolation. Under both conditions the inhibition of bile acid deconjugation by tobramycin was readily observed with the effect being even somewhat more pronounced when the bile acids were tested in a mixture. Also when tested in a mixture deconjugation of TCDCA and GCDCA was faster than that of TCA and GCA which was also reflected by the faster rate of CDCA metabolite formation as compared to the rate of CA formation. The total mass balance for these rat fecal incubations at different timepoints is shown in supplementary information S1 A. The total mass recovery decreased from 94.4% at 0 h to 56.8% at 8 h, indicating that CA and CDCA were further degraded into other metabolites which were not included in the present LC-MS analyses.

After showing that the effect of tobramycin on bile acid deconjugation by rat gut microbiota can be elucidated in anaerobic fecal incubations with a mixture of the primary bile acids, the effects of tobramycin on bile acid deconjugation by human gut microbiota was investigated. Fig. 3 shows the results obtained in anaerobic human fecal incubations with and without tobramycin and a mixture of the 4 conjugated bile acids (TCA, TCDCA, GCA and GCDCA). Table 2 presents the rate for bile acid deconjugation derived from these data. These data reveal that rates for deconjugation by rat and human feces were comparable and that, similar to the rat fecal incubations, the deconjugation of TCDCA and GCDCA by human feces was faster than that of TCA and GCA. Also, the inhibition of bile acid deconjugation by tobramycin was comparable for rat and human fecal incubations. The total mass recovery of the bile acids in the human fecal incubation at different incubation timepoints are presented in the supplementary information S1 B. The total mass recovery decreased from 93.14% at 0 h to 67.5% at 8 h. This indicates that also in the anaerobic human fecal incubations CA and CDCA were further degraded into other metabolites which were not included in the present LC-MS analyses.

3.2. Effect of tobramycin on bile acid transport across the Caco-2 cell layer when bile acids are tested in isolation or in a mixture

In further experiments, the effects of tobramycin pre-exposure on the transport of bile acids in a Caco-2 transwell system when tested in isolation or as a mixture was investigated. First, Caco-2 cell integrity was confirmed by measuring TEER values before, during and after the bile acid transport experiment to confirm the barrier integrity of Caco-2 cell layers. The TEER values of the cell layers were consistent both in the control group and upon tobramycin pre-treatment and in the presence or absence of the bile acids (Figs. S2–S4 in supplementary information).

Fig. 4 shows the time dependent transport of the 4 selected primary bile acids tested in isolation across the Caco-2 cell layers with or without the tobramycin pre-treatment. The apparent permeability coefficient Papp expressed in cm/s was estimated based on the data at 1 h and the Papp values thus obtained are presented in Table 3. From these data it follows that tobramycin pre-incubation results in a significant reduction of the transport of all 4 tested conjugated bile acid across the Caco-2 cell layers. The results also reveal that transport of GCDCA and TCDCA across the control as well as the tobramycin treated cell layers was about 2-fold higher than that of GCA and TCA. Transport of TCA and GCA was comparable and that also holds for the transport of TCDCA and GCDCA.

Fig. 5 presents the results of the experiment when the bile acids were tested as a mixture. From these data it follows that both when testing bile acids in isolation or as an equimolar mixture, the Papp values for especially transport of GCA and GCDCA were comparable while those for TCA and TCDCA seemed somewhat lower when testing the mixture. Both conditions, however equally well detected the effect of tobramycin pre-treatment of the Caco-2 cells resulting in substantial and significantly decreased transport of all 4 conjugated bile acids compared with control. Also, when tested as a mixture the transport of TCA and GCA was comparable and the transport of TCDCA was comparable to that of GCDCA. Also similar to the results obtained with the individual bile acids, transport of GCDCA and TCDCA across the control as well as the tobramycin pre-treated cell layers appeared to be about 2- fold higher than that of GCA and TCA.

After it was shown that the effect of tobramycin pre-exposure on bile acid transport could be equally well detected by testing 4 selected bile acids in a mixture, the effect of tobramycin on the transport of a mixture of 17 conjugated and unconjugated bile acids was tested. Fig. 6 shows the results obtained for a selected number of representative bile acids while the remaining figures are presented in Fig. S5 in the supplementary information. From the results obtained it follows that, in line with what was observed when testing the conjugated bile acids in isolation or in a mixture of 4, tobramycin pre-treatment significantly affected the transport of the conjugated bile acids TCA, TCDCA, GCA, GCDCA and

Table 1
Deconjugation rate (in μmol/h/g feces) of conjugated bile acids tested in isolation or in an equimolar mixture in anaerobic rat fecal slurry incubations in the absence (control) or presence of tobramycin (*$P < 0.05$). Data shown are mean ± SD, n = 3.

	Deconjugation rate (μmol/h/g feces)			Deconjugation rate (μmol/h/g feces)		
	Rat feces, individual bile acids[a]			Rat feces, mixture bile acids		
Bile acid	Control	Tobramycin	% of control	Control	Tobramycin	% of control
TCA	59.6 ± 7.8	37.4 ± 4.4*	62.8	26.5 ± 1.6	10.9 ± 6.1*	41.1
TCDCA	47.8 ± 2.5	38.8 ± 3.5*	81.2	50.5 ± 5.8	29.1 ± 10.8*	57.6
GCA	39.2 ± 5.4	22.8 ± 3.2*	58.2	28.2 ± 4.2	7.6 ± 3.2*	27.0
GCDCA	58.6 ± 10.2	32.8 ± 11.9*	56.0	47.1 ± 6.1	17.1 ± 1.1*	36.3

[a] Data derived from Figs. 5 and 6 in Ref. [17].

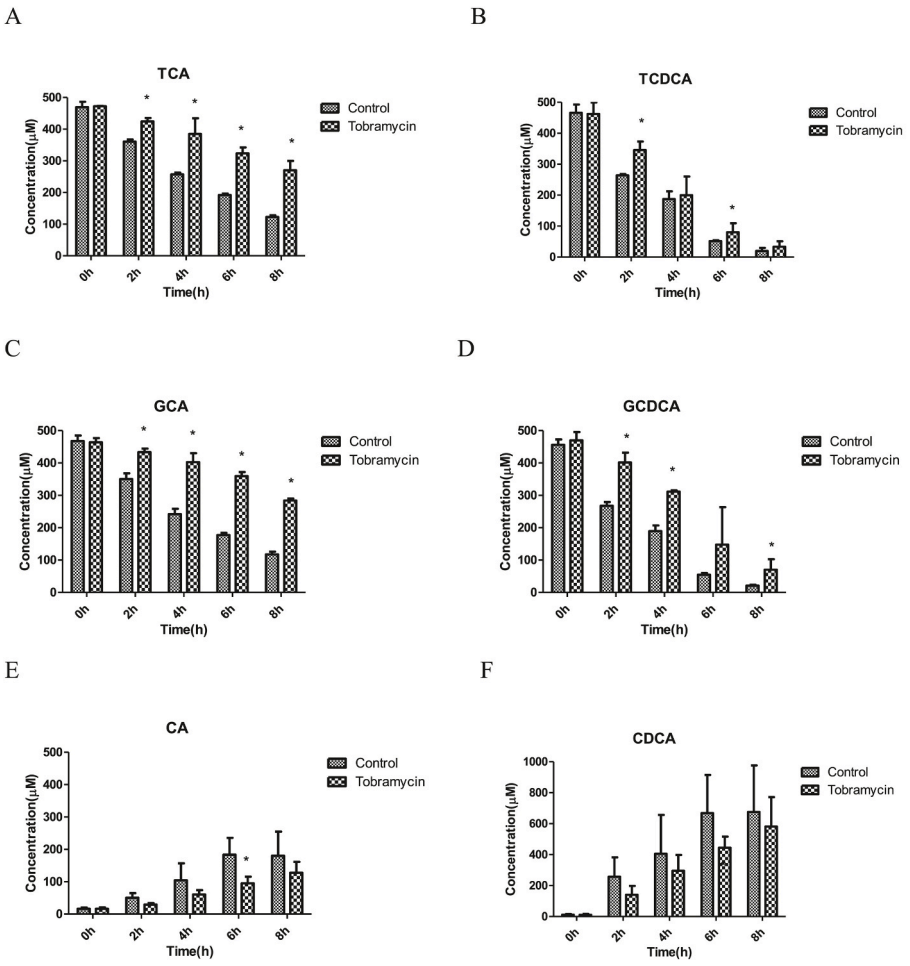

Fig. 2. Time dependent deconjugation of a mixture of taurine and glycine conjugated bile acids (TCA, TCDCA, GCA and GDCA) (Figure A–D) to their unconjugated metabolites CA or CDCA (Figure E,F) in anaerobic rat fecal incubations with or without (control) tobramycin (*$P < 0.05$). Results are shown as mean ± SD, n = 3.

-not tested in the mixture of 4 bile acids- TDCA. It also appeared that, in contrast to an effect on the transport of the conjugated bile acids, tobramycin did not have an effect on transport of the unconjugated bile acids including UDCA, CA, CDCA, HDCA, DCA, and LCA. For the other conjugated bile acids tested including GLCA, GUDCA, GDCA TUDCA and THDCA (with the exception of TLCA), there was a decrease in transport upon tobramycin pre-treatment of the Caco-2 cell layer, albeit not significant. Table 3 presents the Papp values derived from this experiment for the 4 conjugated bile acids that were also tested in isolation and in a mixture of 4. Comparison of the Papp values obtained when testing these bile acids in a mixture of 17 bile acids reveals that especially for GCA and GCDCA the results are similar to the results obtained when testing the bile acids in isolation or in a mixture of 4, while for TCA and TCDCA testing in a mixture of 17 resulted in transport that was comparable to what was observed when the bile acids were tested in a mixture of 4 while transport was about 2-fold lower than when testing these two bile acids in isolation. Similar to data obtained when testing bile acids in a mixture of 4, also when testing them in a mixture of 17, the transport of TCDCA and GCDCA was similar and about 2-fold higher than that of TCA and GCA, which were also transported to a similar extent. Also the effect of tobramycin pre-treatment of the Caco-2 cells was similar resulting in a significant about 2-fold reduction of the transport.

4. Discussion

In our previous study, it was shown in *vitro* model systems that tobramycin significantly inhibited the deconjugation of the individual bile acids TCA, TCDCA, GCA or GCDCA and the intestinal transport of TCA [17]. To facilitate further studies on the effect of oral exposure to antibiotics and other drugs or food-borne chemicals on bile acid homeostasis, it is of interest to investigate whether the effects can also be detected when testing bile acids in a mixture. This does not only enable the concomitant detection of effects on a large number of bile acids but also can be expected to more closely mimic the *in vivo* situation where bile acids will also not occur in isolation. The results of the present study reveal that in a qualitative way the experiments with mixtures of bile acids give results that are similar to those obtained when testing individual bile acids. When testing mixtures subtle differences compared to the results obtained with isolated bile acids were also observed. Deconjugation of the 4 conjugated primary bile acids in anaerobic fecal incubations was 1.5- to 2-fold slower when tested as a mixture, and their transport across a Caco-2 cell layer was 1.5- to 3-fold slower when tested as a mixture. These differences can best be ascribed to the involvement of enzymes and active transporters for which different bile acids may act in competition with one another thereby resulting in mutual inhibition of the deconjugation and transport.

Deconjugation of taurine or glycine conjugated bile acids is known to be rapid and to proceed via bile salt hydrolases (BSH) provided by a wide variety of gut microbiota such as for example *Bacteroides fragilis*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Enterococcus* and others [11,26,27]. Conjugated bile acids when present as a mixture may compete with each other at the active site of these enzymes performing the deconjugation. The results also show that deconjugation of glycine conjugated GCA and GCDCA is faster than that of taurine conjugated

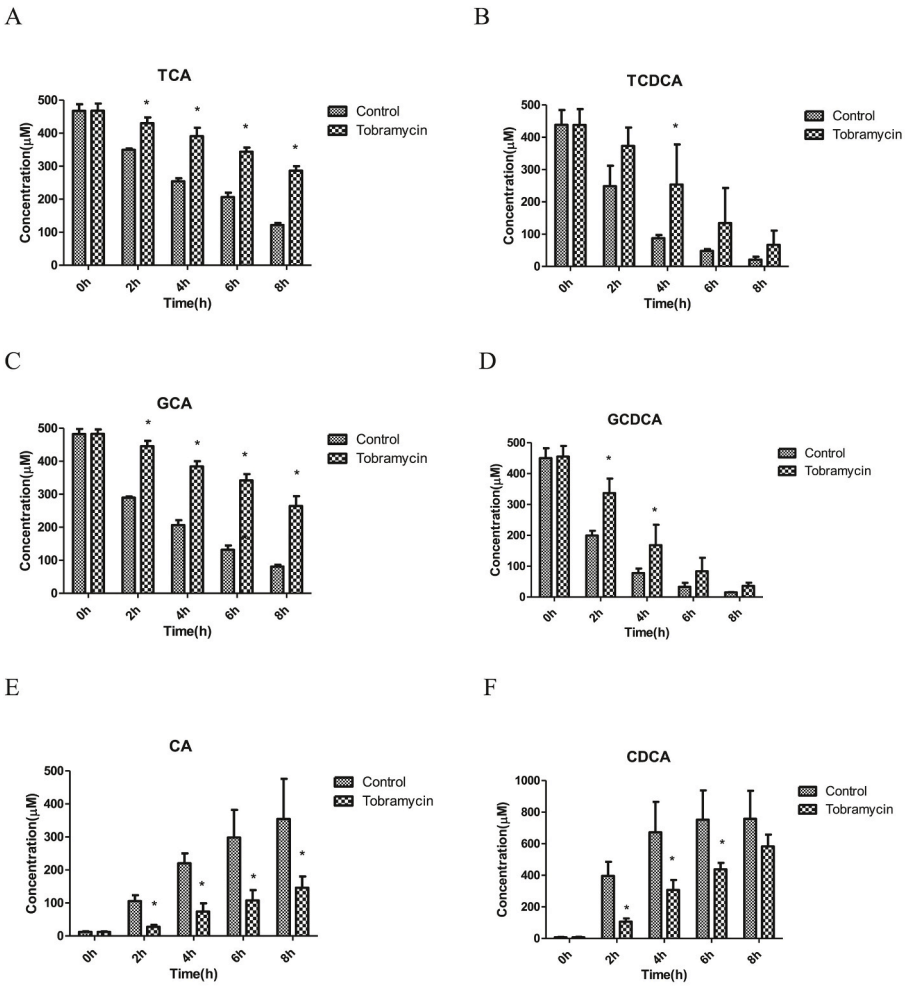

Fig. 3. Time dependent deconjugation of an equimolar mixture of taurine and glycine conjugated bile acids (TCA, TCDCA, GCA and GCDCA) (Figure A–D) to their unconjugated metabolites CA or CDCA (Figure E,F) in anerobic human fecal incubations with or without (control) tobramycin (*$P < 0.05$). Results are shown as mean ± SD, n = 3.

Table 2
Deconjugation rate of mixed conjugated bile acids in human fecal slurry in the absence (control) or presence of tobramycin (*$P < 0.05$). Data shown are mean ± SD, n = 3.

	Deconjugation rate (μmol/h/g feces)		
	Human feces, mixture bile acid		
Bile acid	Control	Tobramycin	% of control
TCA	26.7 ± 2.4	9.6 ± 1.2*	36.0
TCDCA	44.4 ± 5.3	19.9 ± 4.3*	44.8
GCA	33.8 ± 3.6	9.5 ± 1.4*	28.1
GCDCA	62.7 ± 5.2	29.6 ± 4.6*	47.2

TCA and TCDCA in anaerobic incubations with both human and rat gut microbiota, while there was no species difference in these deconjugations. This observation can likely be ascribed to the fact that a wide range of gut microbiota and/or enzymes may be involved in the deconjugation of these primary bile acids by human and rat gut microbiota

Studying transport of the bile acids in isolation or in mixtures in a Caco-2 transwell system revealed that under both conditions the transport of GCDCA and TCDCA was faster than that of GCA and TCA. This may be due to the fact that GCDCA and TCDCA are dihydroxy bile acids while TCA and GCA are trihydroxy bile acids [28,29]. The apical sodium-dependent bile acid transporter (ASBT) is located at the apical membrane of enterocytes and is responsible for reabsorption of the bile acids from the intestine back to the liver [30,31]. Also, in Caco-2 cells the ASBT transporter is responsible for bile acid transport across the cell layer [32,33]. It is known that dihydroxy bile acids have a greater affinity to the apical sodium-dependent bile acid transporter (ASBT) than trihydroxy bile acids [34–36]. It is also known that the passive diffusion for these conjugated bile acids is inversely related to the number of hydroxyl groups of bile acids [37]. Given that the bile acids were all tested at a similar concentration the greater affinity of the dihydroxy bile acids can be expected to result in faster transport by the ASBT transporter of GCDCA and TCDCA than what is observed for the trihydroxy bile acids TCA and GCA, while for GCDCA and TCDCA also passive diffusion is expected to be higher than for GCA and TCA. Together these characteristics may explain the faster rate of transport for GCDCA and TCDCA than what is observed for the trihydroxy bile acids TCA and GCA.

The experiments also showed that pre-incubation of the Caco-2 cells with tobramycin resulted in substantial reduction of intestinal transport for the 4 conjugated bile acids (TCA, TCDCA, GCA or GCDCA) compared to the transport across the control Caco-2 cell layers, either when tested in isolation or in a mixture. In a quantitative way also in these transport experiments testing of the bile acids as a mixture resulted in somewhat lower transport rates likely due to mutual competition.

In the normal *in vivo* situation, more than 50 bile acids are present in the systemic and the enterohepatic circulation [38,39]. Thus, we also

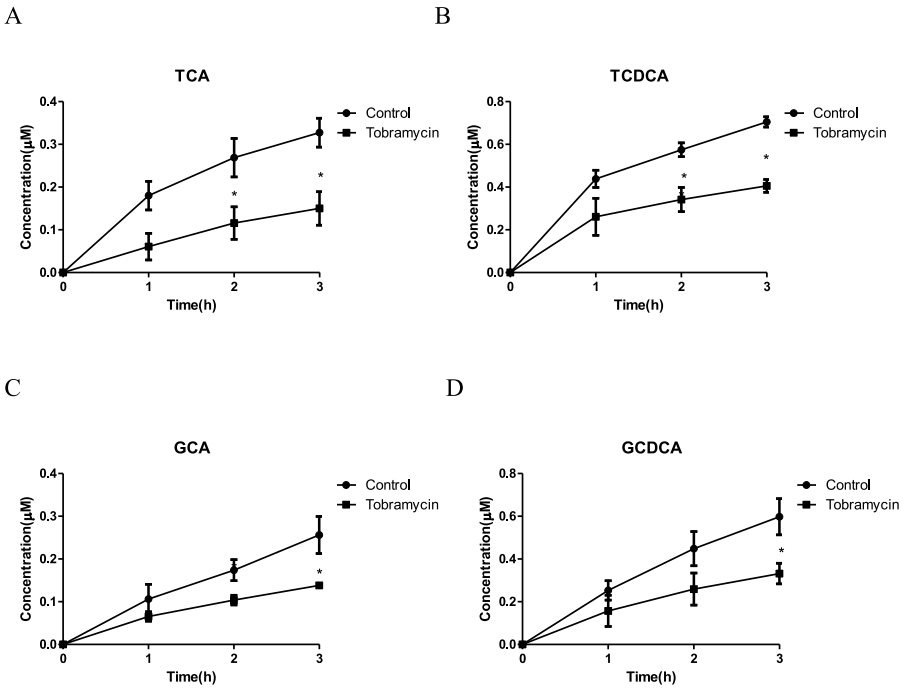

Fig. 4. Time dependent transport of individual bile acids including A) TCA, B) TCDCA,C) GCA or D) GCDCA) across a Caco-2 cell layer upon pre-exposure with or without (control) tobramycin, when tested in isolation (*$P < 0.05$). Results are expressed as mean ± SD, n = 3.

Table 3
Transport rate (Papp) of 4 selected conjugated bile acids when tested in isolation or in a mixture of 4 or 17 bile acids across Caco-2 cell layers upon pre-exposure without (control) or with tobramycin (*$P < 0.05$). Data shown are mean ± SD, n = 3.

	Papp ($\times 10^{-5}$ cm/s)			Papp ($\times 10^{-5}$ cm/s)			Papp ($\times 10^{-5}$ cm/s)		
	Individual bile acids			Mixture of 4 bile acids			4 from a mixture of 17 bile acids		
Bile acid	Control	Tobramycin	% of control	Control	Tobramycin	% of control	Control	Tobramycin	% of control
TCA	1.8 ± 0.3	0.5 ± 0.1*	27.8	1.2 ± 0.1	0.5 ± 0.1*	41.7	0.8 ± 0	0.4 ± 0.1*	50.0
TCDCA	4.4 ± 0.4	2.7 ± 0.4*	61.4	2.2 ± 0.1	1.4 ± 0*	63.6	1.6 ± 0.1	1 ± 0.2*	62.5
GCA	0.9 ± 0.2	0.7 ± 0.1*	77.7	1.1 ± 0.1	0.4 ± 0.1*	36.4	0.9 ± 0.1	0.5 ± 0.1*	55.6
GCDCA	2 ± 0.3	1.3 ± 0.4*	65.0	2.3 ± 0.3	0.9 ± 0.1*	39.1	1.6 ± 0.1	1 ± 0.2*	62.5

studied the effect of tobramycin on the transport of a mixture of more than the 4 primary conjugated bile acids, including 17 common bile acids, including the 4 conjugated primary bile acids tested in the earlier mixtures. Thus, the mixture consisted of TCA, GCA, GCDCA and TCDCA, as well as 7 other conjugated secondary bile acids including GLCA, GUDCA, GDCA, TDCA, TLCA, TUDCA and THDCA and 6 unconjugated bile acids including UDCA, HDCA, CDCA, DCA, CA, LCA. It was of interest to note that for the unconjugated bile acids there was no difference in transport across the Caco-2 cell layer either with or without tobramycin pre-treatment. This observation is consistent with the consensus that unconjugated bile acids are reabsorbed via passive diffusion [40–42]. For the 6 conjugated secondary bile acids including GLCA, GUDCA, GDCA, TLCA, TUDCA and THDCA the transport upon the tobramycin pre-treatment of the Caco-2 cells was lower than in the control group albeit not significant. This observation is in line with the consideration that also some conjugated bile acids are known to be mainly transported via passive or facilitated diffusion [43]. Thus, only the transport of the 4 conjugated primary bile acids TCA, TCDCA, GCA and GCDCA and 1 taurine conjugated secondary bile acid TDCA appeared to be significantly inhibited upon the pre-treatment of the cells with tobramycin, an observation that can be related to an effect of tobramycin on the activity of the ASBT transporter, which has been reported to represent the rate-limiting step in bile acid reuptake [35]. Tobramycin is a bactericidal drug able to affect protein synthesis inside the bacterial cells [44–46]. It is also reported that aminoglycoside antibiotics affect protein synthesis in human cells thus leading to a reduction in normal functional protein formation [47]. Tobramycin belongs to the aminoglycosides, hence, it may inhibit ASBT synthesis at the protein level thus resulting in decreased bile acid transport. The synthesis of another important bile acid transporter which is located in the basolateral cell membrane – the organic solute transporter OSTα-OSTβ may also be inhibited by tobramycin further reducing the active transport of the bile acids across the intestinal cell layer [48–50].

Taking all together the results obtained support that the effects of the selected model compound tobramycin on bile acid deconjugation and transport can be adequately detected in the *in vitro* systems using a mixture of bile acids, thus eliminating the need to characterize the effects for each bile acid in separate experiments. Subtle differences between the experiments with single or combined bile acids point at mutual competitive interactions and indicate that the use of bile acid mixtures is preferred over use of single bile acid given that also *in vivo* bile acids occur in mixtures.

CRediT authorship contribution statement

Nina Zhang: Methodology, Experiments, Measurement, Formal analysis, Writing – original draft, Visualization. **Weijia Zheng:** Methodology, Software, Measurement, Writing – review & editing. **Wouter Bakker:** Software, Measurement, Writing – review & editing. **Bennard van Ravenzwaay:** Writing – review & editing. **Ivonne M.C.M.**

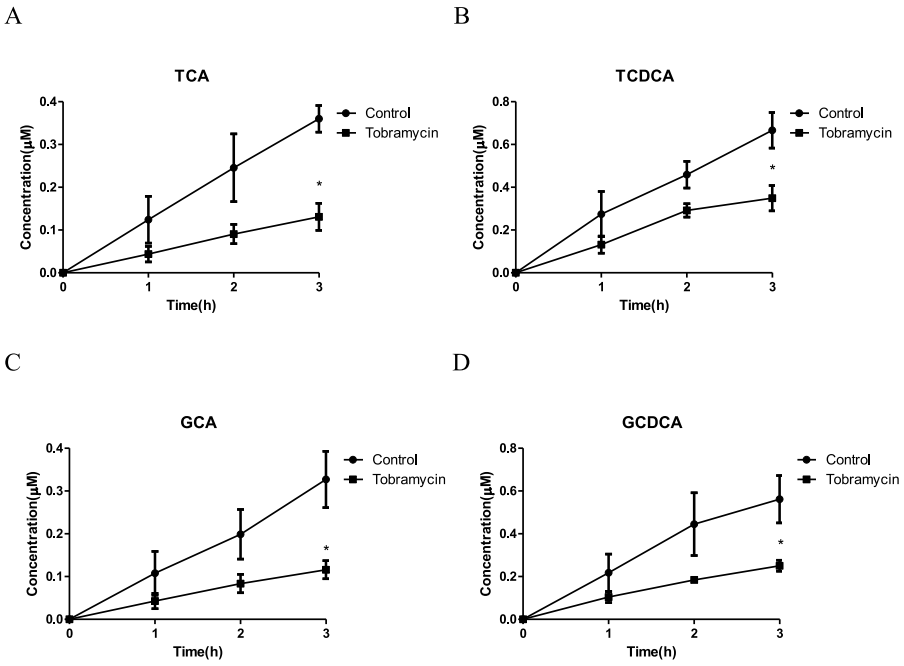

Fig. 5. Time dependent transport of bile acids including A) TCA, B) TCDCA, C) GCA and D) GCDCA) across a Caco-2 cell layer upon pre-exposure with or without (control) tobramycin, when tested as an equimolar mixture (*$P < 0.05$). Results are expressed as mean ± SD, n = 3.

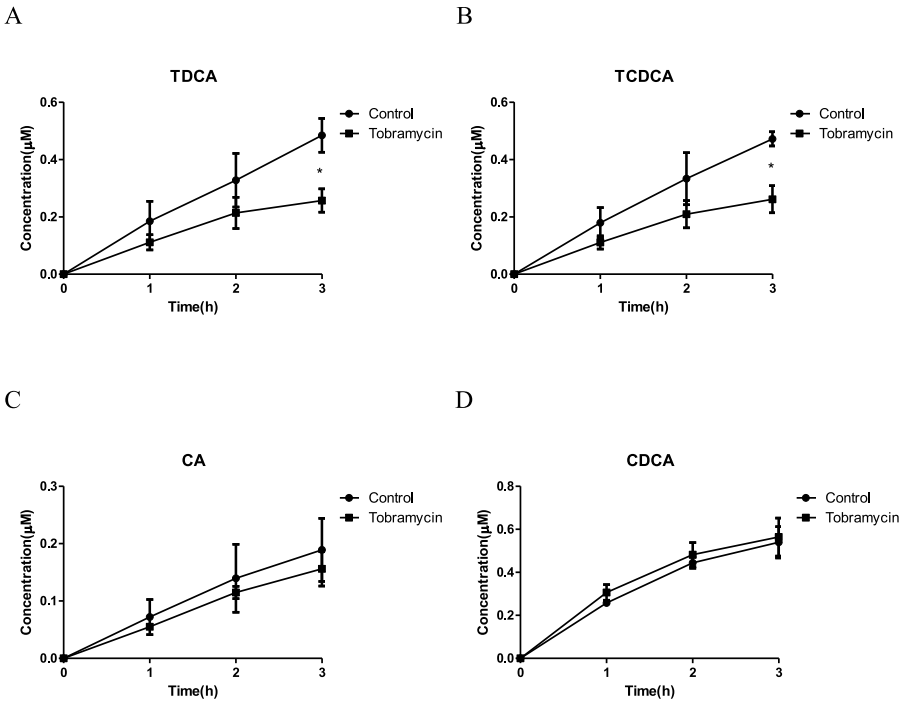

Fig. 6. Time dependent transport of 4 selected bile acids (TDCA, TCDCA, CA and CDCA) when tested in a mixture of 17 bile acids, across a Caco-2 cell layer upon pre-exposure without (control) or with tobramycin (*$P < 0.05$). Results are expressed as mean ± SD, n = 3.

Rietjens: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2023.110445.

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