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Novel cationic and neutral glycocholic acid and polyamine conjugates able to inhibit transporters involved in hepatic and intestinal bile acid uptake☆

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Abstract—To obtain novel drugs able to inhibit transporters involved in bile acid uptake, three compounds were synthesized by conjugating *N*-(3-aminopropyl)-1,3-propanediamine (PA) with one (BAPA-3), two (BAPA-6), or three (BAPA-8) moieties of glycocholic acid (GC) through their carboxylic group. The expected net charge in aqueous solutions was 2+ (BAPA-3), 1+ (BAPA-6), and 0 (BAPA-8). They were purified by liquid chromatography and their purity checked by HPLC before being chemically characterized by elemental analysis, NMR, and FAB-MS. Using brush-border membranes isolated from rat ileum; their ability to inhibit [^{14}C]-GC transport (BAPA-3 > BAPA-6 > BAPA-8) was suggested. This was further investigated 48 h after injecting *Xenopus laevis* oocytes with the mRNA of rat sodium/taurocholate (TC)-cotransporting polypeptide (Ntcp), rat apical sodium-dependent bile salt transporter (Asbt), or the human isoforms OATP-C/1B1 and OATP8/1B3 of organic anion-transporting polypeptides, when maximal functional expression was detected. BAPA-8, BAPA-6, and BAPA-3 induced no inhibition of OATP8/1B3-mediated [^{3}H]-TC uptake, but dramatically reduced [^{3}H]-TC uptake by OATP-C/1B1. In the cases of Ntcp- and Asbt-mediated [^{3}H]-TC uptake, these were sodium-dependent and were inhibited by BAPA-6 > BAPA-8 > BAPA-3 and BAPA-8 > BAPA-6 > BAPA-3, respectively. In conclusion, our results suggest that these compounds are potentially interesting research tools for the selective modulation of liver and intestinal uptake of bile acids and other cholephilic compounds. Moreover, they may be of pharmacological usefulness to prevent the acute toxicity of compounds reaching liver cells through specific transporters or to enhance both fecal elimination of bile acids and hence cholesterol consumption for the 'de novo' synthesis of bile acids.

1. Introduction

The liver is responsible for the elimination from the blood of a large variety of structurally unrelated compounds, including anionic, cationic, and neutral organic and inorganic compounds.[1] For some of them, the hepatobiliary pathway is by far the major excretory route and hence they are commonly known as cholephilic compounds. Some of them are taken up by the liver and excreted into the bile without undergoing major

Abbreviations: ASBT/Asbt, apical sodium-dependent bile salt transporter; BSEP, Bile salt export pump; NTCP/Ntcp, Na-taurocholate-cotransporting polypeptide; OATP, Organic anion-transporting polypeptide; OST, Organic solute transporter.

Keywords: ASBT; Enterohepatic circulation; Ileum; Liver; NTCP; OATP; Polyamine; Toxic agents; Transport; Xenobiotics.

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biotransformation. Detoxification is therefore carried out only by transport mechanisms: that is, phase 0 (uptake) and phase III (secretion). In contrast, other substances undergo chemical modification during their transcellular residence due to mechanisms involving oxidation/reduction reactions (phase I) and/or conjugation with polyatomic groups (phase II).[2]

Although some xenobiotic compounds are able to enter liver cells by simple diffusion, owing to the anionic nature of most of them, they are mainly taken up via plasma membrane carrier proteins.[3] In hepatocytes, the processes of phase 0 are performed in part by members of the family of organic anion-transporting polypeptides (OATPs)—mainly OATP-C and OATP-8, or OATP1B1 (gene symbol *SLCO1B1*) and OATP1B3 (*SLCO1B3*), respectively—according to the new nomenclature adopted by the HUGO Gene Nomenclature Committee.[4] Here we shall refer to them as OATP-C/1B1 and OATP8/1B3, respectively. Both isoforms are able to transport bile acids (BAs) and their derivatives,[5] which can be also performed by the Na^+-taurocholate-cotransporting polypeptide (NTCP, gene symbol *SLC10A1*).[6]

Substrates of these transport proteins located at the sinusoidal plasma membrane of hepatocytes include several endogenous anions, such as BAs, and many xenobiotics, such as drugs and toxic agents accidentally included in food. A good example of the latter is phalloidin a mushroom toxin taken up by hepatocytes, in which it causes fatal damage leading to liver failure and death.[7,8] Accordingly, blocking the uptake of these compounds by the liver, thus permitting their elimination by the kidney, has been suggested as a potentially useful pharmacological strategy to treat acute intoxication by these toxins.[9]

To carry out this inhibition, we have synthesized, purified, chemically characterized, and biologically assayed novel drugs, named BAPAs from BA and polyamine, because they were obtained by the conjugation of *N*-(3-aminopropyl)-1,3-propanediamine (PA) with one, two or three moieties of glycocholic acid, an endogenous cholephilic steroid commonly present in the enterohepatic circuit.[10] This polyamine was selected because it has three potential sites for conjugation with BA moieties, it is not too bulky as compared with the natural side chain but it is large enough to permit the presence of up to three BA moieties with little expected restrictions for the synthesis and the interaction with the carrier. In this respect, the different capability of these compounds to inhibit different plasma membrane transporters may afford a useful tool for use in the research of the physiology and pathophysiology of these carriers.

Moreover, the major transporter involved in active BA uptake by the intestinal mucosa is the apical sodium-dependent bile salt transporter or ASBT (gene symbol *SLC10A2*).[11] The ability of this carrier to transport bile acid derivatives obtained by coupling the active agent to the hydroxyl group at C3 of the BA moiety, but not at the carboxyl group, which interacts with a deep region of the substrate-binding site of the transporter, has been reported.[12]

Here, we investigated whether BA derivatives, in which the side chain was used to link a polyamine with or without additional BA moieties, were able to inhibit the ASBT-mediated transport of natural BAs. This is of great pharmacological interest as indicates the considerable effort that is being invested in obtaining new inhibitors of intestinal BA absorption with the aim of enhancing fecal loss of these steroids and stimulating their 'de novo' synthesis by the liver from cholesterol, which are expected to reduce cholesterol levels.[13]

2. Results and discussion

2.1. Chemistry

The synthesis of amide derivatives of glycocholic acid (GC) and *N*-(3-aminopropyl)-1,3-propanediamine was carried out following standard procedures for the formation of the amide linkage, using EEDQ as a coupling agent and dimethylformamide as solvent[14] (Fig. 1). Based on preliminary studies to improve the yield of amide products and to obtain a less complex reaction mixture (data not shown), the best results were obtained when a 10:1 ratio of PA:GC was used. Under these conditions, three amide derivatives of GC were isolated by a combination of insolubilization with diethyl ether and liquid chromatography. The purity of the compounds was checked by HPLC (Fig. 2). These amides were named BAPA-3, BAPA-6, and BAPA-8 following their increasing R_F in TLC (decreasing polarity) (Fig. 2D).

The compounds were characterized spectroscopically, the presence of both, BA and PA, moieties being observed, although the differences between the three compounds were very small. Among the five possible amides, the obtained products were identified as those depicted in Figure 1, whose ^1H and ^{13}C-NMR are listed in Tables 1 and 2. In all three cases, only one set of signals for the glycocholanic residues were observed: in BAPA-3 with only one glycocholanic moiety; in BAPA-6 with two equal moieties, and in BAPA-8 with two identical and one different moiety, the differences among the spectra being very small and only observable in the PA residue. The definitive data for the structural determination of the three compounds were the MS (data not shown), which revealed molecular ions in agreement with their molecular formulae, such as for example 1048 $[M + Na]^+$ for BAPA-6 carrying two glycocholanic residues.

The deduced structures of the synthesized compounds are also in agreement with the higher reactivity of the terminal amino groups in comparison with the central amino group of the starting *N*-(3-aminopropyl)-1,3-propanediamine.[15] The most abundant products isolated were those resulting from the reaction of one GC moiety at one terminal amino group (BAPA-3, ≈8.5%) or two GC moieties at both terminal amino groups

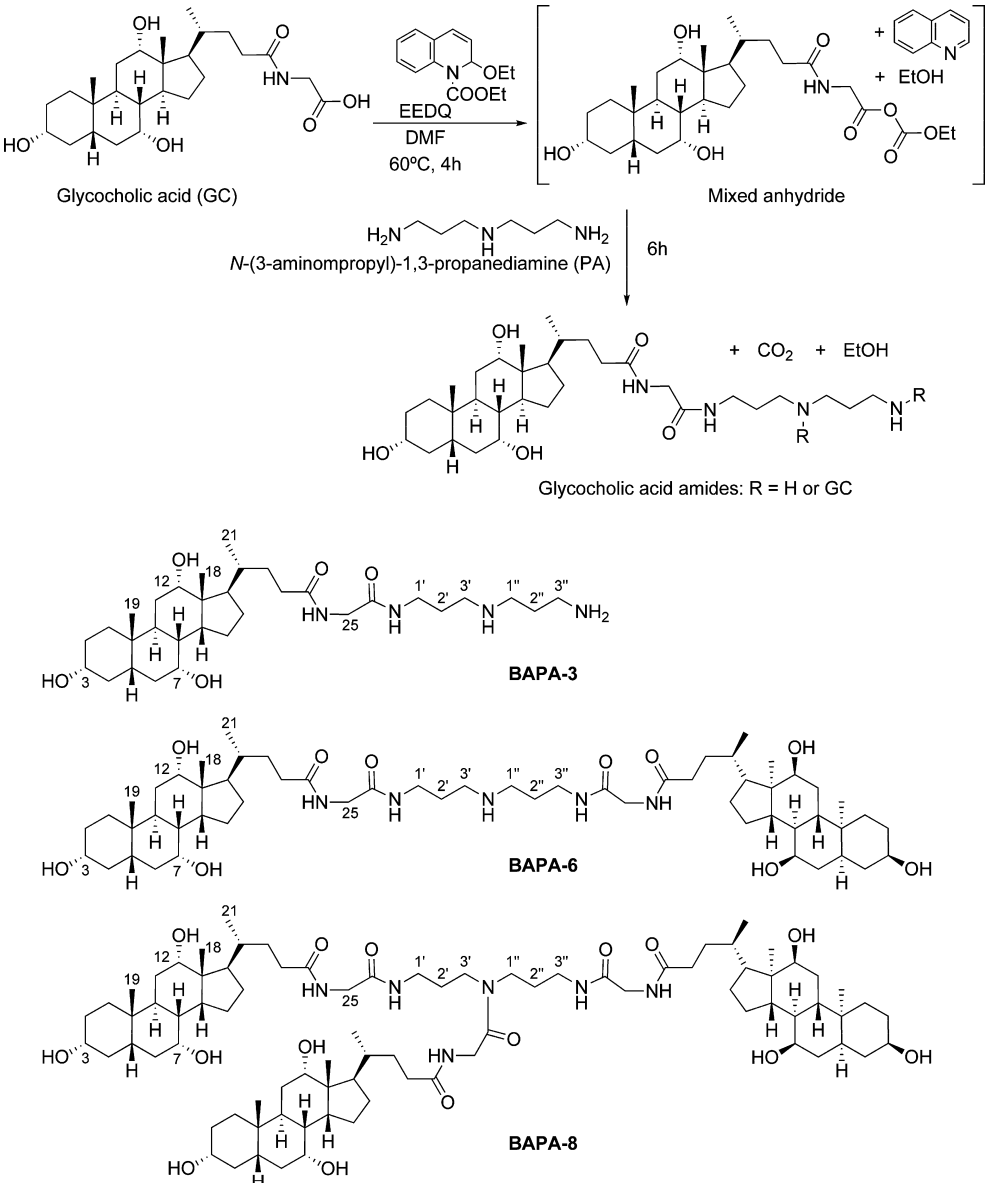

Figure 1. Scheme of the synthesis process of conjugates of *N*-(3-aminopropyl)-1,3-propanediamine with one, two or three moieties of glycocholic acid and molecular structure of BAPA-3, BAPA-6, and BAPA-8, respectively.

(BAPA-6, ≈6%), whereas the product formed by reaction at the less reactive central amino group (BAPA-8, ≈2%) was only produced in minor amounts and after the reaction at the other two more reactive positions. The expected net charge in aqueous solution in the physiological range of pH is 2+, 1+, and 0 for BAPA-3, BAPA-6, and BAPA-8, respectively.

2.2. Inhibition of bile acid transporters

As indicated by specific membrane markers (Table 3), preparations of apical ileal plasma membrane vesicles (aIPMV) were more than eight times enriched in apical membranes as compared with basolateral membranes, with slight contamination by intracellular membranes. Using these aIPMV, evidence for the ability of BAPA-3, BAPA-6, and BAPA-8 to inhibit BA transport was found (Fig. 3). Because several transporters were present, and hence were probably involved, in BA uptake by this mixed population of inside-out and right-side out vesicles from different subcellular membranes, a more appropriate model was used to further evaluate the effect of these compounds on BA transport by the major carrier proteins involved in the uptake of BA by the intestine from the intestinal lumen, that is, ASBT, and by the liver from sinusoidal blood, that is, NTCP, OATP-C/1B1, and OATP8/1B3.[5]

The results obtained in studies on the time-course of the functional expression of rat Asbt (Fig. 4A), Ntcp (Fig. 5A) and human OATP-C/1B1 and OATP8/1B3 (data not shown) in *X. laevis* oocytes revealed that the highest [^{3}H]-TC uptake rate was obtained 2 days after injecting the mRNA of all these transporters. Therefore, subsequent uptake experiments were carried out at this time after injection. Suitable expression of these carrier

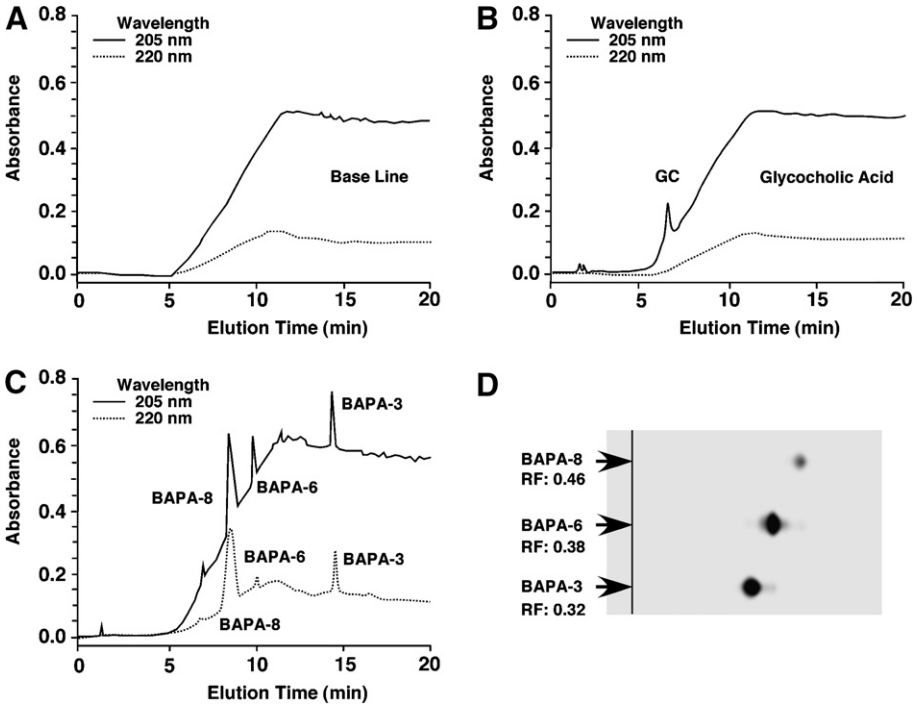

Figure 2. Method used to check the purity of BAPA-3, BAPA-6, and BAPA-8 by high performance liquid chromatography (HPLC) using reverse phase column of C18 and solvent gradient from 50% methanol and 50% of 10 mM phosphate buffer, pH 7.5, for 1 min to 100% methanol in 7 min, which was maintained for an additional 15 min period. Characteristic chromatograms of absorbance recorded at 205 nm (solid line) and 220 nm (dashed line) wavelength of base line (A), pure glycocholic acid (B), and a mix of BAPA-3, BAPA-6, and BAPA-8 (C). Fractions collected during synthesis and purification procedures were checked by thin-layer chromatography (TLC) using chloroform/methanol/acetic acid/water (65:24:15:9) (vol/vol) as the solvent system. Here the result of final products subjected to TLC is shown (D).

Table 1. ^{1}H-NMR assignments of most representative protons of compounds BAPA-3, BAPA-6, and BAPA-8 (400 MHz) in CD_3OD

H	BAPA-3	BAPA-6	BAPA-8
3	3.40 (1H, m)	3.32 (1H, m)	3.25 (1H, m)
7	3.78 (1H, sa)	3.77 (1H, sa)	3.77 (1H, sa)
12	3.95 (1H, sa)	3.92 (1H, sa)	3.93 (1H, sa)
18	0.71 (3H, s)	0.68 (3H, s)	0.70 (3H, s)
19	0.91 (3H, s)	0.90 (3H, s)	0.91 (3H, s)
21	1.03 (3H, d, J = 6.2)	1.01 (3H, d, J = 6.2)	1.02 (3H, d, J = 6.2)
25	3.76 (2H, sa)	3.77 (2H, sa)	3.78 (2H, sa)
1′	3.2–3.6 (m)	3.1–3.4 (m)	3.2–3.4 (m)
2′	1.5–1.9 (m)	1.5–1.9 (m)	1.5–1.9 (m)
3′	2.75–2.95 (m)	2.90 (m)	2.87 (2H,t, J = 6.2)
1″	2.75–2.95 (m)		
2″	1.5–1.9 (m)		
3″	2.75–2.95 (m)		

proteins in *X. laevis* oocytes was confirmed by the enhanced ability to take up [^{3}H]-TC in an Na^{+}-dependent manner by oocytes injected with the mRNA of rat Asbt (Figs. 4B and 6A), rat Ntcp (Figs. 5B and 6B) and in an Na^{+}-independent manner by oocytes injected with human OATP-C/1B1 and OATP8/1B3 (Figs. 6C and D).

Unlabeled TC was able to significantly reduce [^{3}H]-TC uptake by all these transporters (Fig. 6), which is consistent with self-inhibition due to a competitive process. However different BAPAs had different effects on [^{3}H]-TC uptake by these transporters. BAPA-3 induced a milder degree of inhibition on Asbt- and Ntcp-mediated [^{3}H]-TC uptake than that of unlabeled TC (Figs. 6A and B). In contrast, the inhibition induced on these transporters by BAPA-6 and BAPA-8 was stronger than that caused by BAPA-3 (Figs. 6A and B). Regarding OATP-C/1B1, all three compounds, BAPA-3, BAPA-6, and BAPA-8, induced a dramatic inhibition of [^{3}H]-TC uptake, which was markedly stronger than that caused by unlabeled TC (Fig. 6C). However, neither BAPA-3, BAPA-6 nor BAPA-8 was able to significantly inhibit OATP8/1B3-mediated [^{3}H]-TC uptake (Fig. 6D).

The implications of these results in research fields related to toxicology, pharmacology, and physiology are important because OATPs have partially overlapping and partially different substrate preferences for a wide range of amphipathic organic solutes, including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, xenobiotics, and many drugs such as gadodexate, ouabain, iloprost, Gd-B 20790, methotrexate, rifampicin, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-220, the opioid receptor agonists [D-penicillamine (2,5) enkephalin] (DPDPE) and deltorphin II, the angiotensin-converting enzyme inhibitors enalapril and temocaprilat, the HMG-CoA reductase inhibitor pravastatin and the antihistamine fexofenadine,[16] in addition to several cytostatic derivatives obtained by coupling BA moieties to chlorambucil[17] or cisplatin.[18] Some isoforms of this family have also been shown to transport bulky organic cations, such

Table 2. ^{13}C-NMR assignments of compounds BAPA-3, BAPA-6, and BAPA-8 (102.6 MHz) in CD_3OD

C		BAPA-3	BAPA-6	BAPA-8
1	CH_2	36.5	36.1	36.5
2	CH_2	31.3	30.9	31.2
3	CH	72.6	72.1	72.9
4	CH_2	37.7	40.1	40.5
5	CH	43.0	42.5	43.0
6	CH_2	36.0	35.6	35.9
7	CH	68.6	68.1	69.0
8	CH	41.0	40.5	41.0
9	CH	27.8	27.3	27.9
10	C	35.8	35.7	35.9
11	CH_2	29.7	29.3	29.6
12	CH	73.5	73.2	74.0
13	C	47.4	47.3	47.5
14	CH	43.1	42.6	43.2
15	CH_2	24.2	23.8	24.2
16	CH_2	28.7	28.3	28.7
17	CH	47.7	46.4	48.0
18	CH_3	13.2	12.8	13.0
19	CH_3	23.3	23.0	23.2
20	CH	36.9	36.4	37.0
21	CH_3	17.9	17.6	17.7
22	CH_2	33.8	33.4	33.8
23	CH_2	32.9	32.5	33.0
24	C	172.4	172.7	172.9
25	CH_2	45.6	45.7	43.8
26	C	176.9	177.5	177.7
1′	CH_2	37.7	36.6	35.9
2′	CH_2	25.4	26.9	28.6
3′	CH_2	43.7	43.3	46.8
1″	CH_2	46.1		
2″	CH_2	27.4		
3″	CH_2	40.6		

as *N*-methylquinine and *N*-methylquinidine, *N*-(4,4-azo-*n*-pentyl)-21-deoxy-ajmalinium and rocuronium.[19]

The above implies the possibility of transporter-mediated interactions among OATP substrates and BAPAs, similar to those reported for the competition for OATP-C/1B1 between cyclosporin A or gemfibrozil and HMG-CoA reductase inhibitors, such as cerivastatin.[20] This type of interaction has been also described between drugs, toxins, and normal food components. Among the examples of drug-toxin transporter-mediated interactions is the ability of drugs such as bromosulfophthalein, cyclosporin A, and rapamycin to inhibit the OATP-C/1B1-mediated uptake of the mycotoxin phalloidin from *Amanita phalloides*. This inhibition protects the hepatocytes from the severe damage caused by this toxin.[9] In this respect, our results suggest that BAPAs could be considered as interesting drugs for blocking the acute hepatotoxicity of agents taken up by the hepatocytes via OATP-C/1B1, whereas the uptake of endogenous compounds that share the entry pathway to these cells would be not affected (in the case of OATP8/1B3) or only moderately reduced (in the case of NTCP).

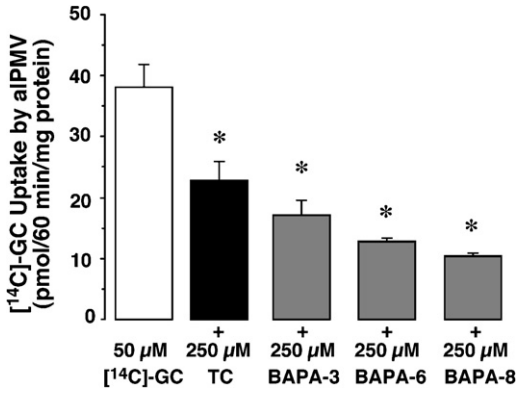

Figure 3. Uptake of [^{14}C]-GC (50 μM) by apical plasma membrane vesicles from rat ileum (aIPMV) in the presence (250 μM) or absence of taurocholate, BAPA-3, BAPA-6 or BAPA-8 at 37 °C for 60 min. Values are means ± SD from measurement carried out in triplicate on vesicles from three different preparations. $*P < 0.05$, on comparing with controls with the paired *t* test.

The interest in inhibiting intestinal BA absorption in attempts to reduce serum cholesterol levels justifies the important efforts devoted to developing a large number of drugs able to inhibit ASBT-mediated Na^+-dependent BA transport across the brush-border membrane of intestinal mucosa cells.[13] Two representative examples of these types of inhibitors are 2164U90 and S-8921.[21–24] The benzothiazepine-based compound 2164U90 [(3R, 5R)-trans-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4- benzothiazepine 1,1-dioxide] was the first compound found to be able to competitively inhibit Na^+-dependent BA transport across the plasma membrane of rat, monkey, and human ileal cells and to stimulate the elimination of exogenously loaded BAs. Moreover, 2164U90 inhibited the increase in the levels of lipoproteins VLDL plus LDL induced by diets containing cholesterol-CA (in rats) and cholesterol-CA-coconut oil (in mice).[21,22] S-8921 (methyl-1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8- trimethoxy-2-naphthoate) is able to inhibit ASBT. Owing to this ability, when administered in the diet this drug caused a dose-dependent hypocholesterolemic action

Table 3. Characterization of the purity and contamination of preparations of apical ileal plasma membrane vesicles

Marker	Ileum homogenate	Apical plasma membrane vesicles	Enrichment versus homogenate
Alkaline phosphatase (mU/mg protein)	13.2 ± 1.8	79.2 ± 11	6.11 ± 0.52
Sucrase (mU/mg protein)	12.7 ± 2.4	75.9 ± 11.0	6.05 ± 0.33
Glucose-6-phosphatase (mU/mg protein)	5.4 ± 0.6	16.7 ± 1.6	3.10 ± 0.52
Lysosomal acid phosphatase (mU/mg protein)	12.5 ± 1.4	11.9 ± 1.4	0.96 ± 0.13
Succinate dehydrogenase (mU/mg protein)	1.51 ± 0.11	0.44 ± 0.12	0.29 ± 0.04
Ouabain binding (pmol/mg protein)	48.4 ± 5.2	35.9 ± 4.0	0.74 ± 0.08

Results are means ± SD of measurements carried out in triplicate on three different preparations of apical plasma membrane vesicles.

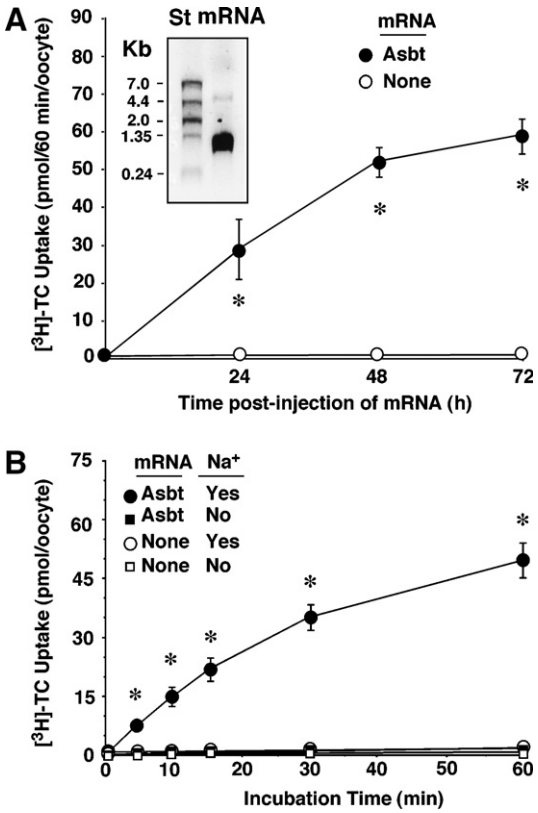

Figure 4. (A) Uptake of [^{3}H]-TC by *Xenopus laevis* oocytes expressing rat Asbt. [^{3}H]-TC uptake was measured at different times after the injection of 9 ng Asbt mRNA to determine the maximum functional expression time. The cells were incubated with 10 μM [^{3}H]-TC with 100 mM NaCl at 25 °C for 60 min. (B) Sensitivity of Asbt-mediated [^{3}H]-TC uptake to the sodium gradient 48 h after the injection of mRNA. The oocytes were incubated with 10 μM [^{3}H]-TC with 100 mM NaCl or CholineCl at 25 °C for the indicated time. Values are means ± SD ($n \geqslant 24$ oocytes from three different frogs for each experimental condition or incubation time). The inset in (A) depicts agarose gel electrophoresis of the mRNA product of the transcription reaction from linearized plasmid containing cDNA of rat Asbt. St, standard RNA ladder. *$P < 0.05$, on comparing with controls by the paired t test.

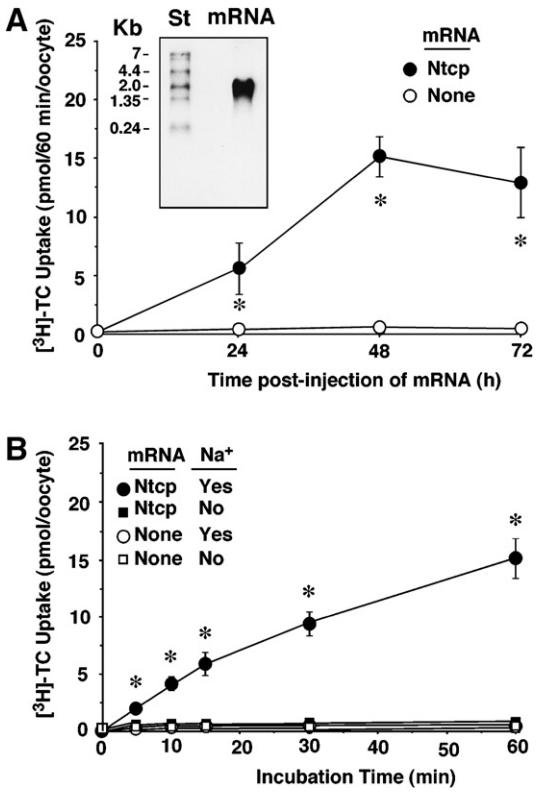

Figure 5. (A) Uptake of [^{3}H]-TC by *Xenopus laevis* oocytes expressing rat Ntcp. [^{3}H]-TC uptake was measured at different times after the injection of 9 ng Ntcp mRNA to determine the maximum functional expression time. The cells were incubated with 10 μM [^{3}H]-TC with 100 mM NaCl at 25 °C for 60 min. (B) Sensitivity of Ntcp-mediated [^{3}H]-TC uptake to the sodium gradient 48 h after the injection of mRNA. The oocytes were incubated with 10 μM [^{3}H]-TC with 100 mM NaCl or CholineCl at 25 °C for the indicated time. Values are means ± SD ($n \geqslant 24$ oocytes from three different frogs for each experimental condition or incubation time). The inset in (A) depicts agarose gel electrophoresis of the mRNA product of the transcription reaction from the linearized plasmid containing cDNA of rat Ntcp. St, standard RNA ladder. *$P < 0.05$, on comparing with controls by the paired t test.

accompanied by increased fecal excretion of BAs in hamsters[23] and rats.[24] Based on the promising results of this drug in preventing atherosclerosis in heterozygous Watanabe heritable hyperlipidemic rabbits,[25] phase I trials were begun.[26]

In conclusion, in the present study, we have obtained three new BA conjugates that can be used as molecular tools able to inhibit BA transport by hepatic and intestinal transporters and that are potentially interesting for research and pharmacological purposes.

3. Experimental

3.1. Chemicals and animals

BAs, GC, taurocholic acid (TC), and PA, more than 95% pure by thin-layer chromatography, were purchased from Sigma–Aldrich (Madrid, Spain). [^{3}H]-TC (specific radioactivity 3.0 Ci/ mmol) and [^{14}C]-GC (specific radioactivity 46.7 mCi/ mmol) were obtained from New England Nuclear (Pacisa, Madrid, Spain). All other chemicals were from Merck Eurolab (Barcelona, Spain). They were of high purity and were used as purchased without any further purification.

Male rats (200 g) from the University of Salamanca Animal House and mature female frogs (*Xenopus laevis*; Bedarf, Hamburg, Germany) were used. All animals received humane care as outlined in 'Guide for the Care and Use of Laboratory Animals' (NIH Publication 80–23, revised 1985). Experimental protocols were approved by the Ethical Committee for Laboratory Animals, University of Salamanca.

3.2. Synthesis and purification

The GC and PA conjugates, named BAPA-3, BAPA-6, and BAPA-8, and whose molecular structures are shown in Figure 1, were obtained by an adaptation of a previ-

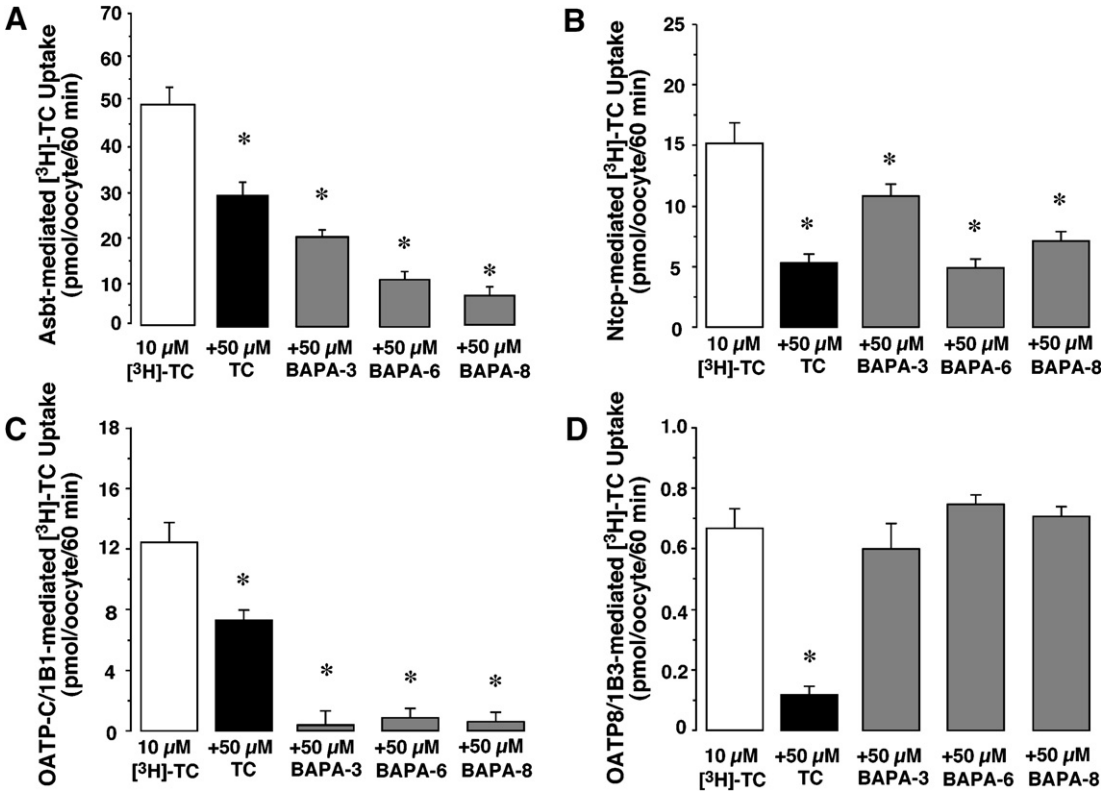

Figure 6. Inhibition of [^{3}H]-TC uptake by *Xenopus laevis* oocytes mediated by rat Asbt (A), rat Ntcp (B), human OATP-C/1B1 (C), and human OATP8/1B3 (D). [^{3}H]-TC uptake was measured in cells that were incubated with 10 μM [^{3}H]-TC with 100 mM NaCl (for Asbt and Ntcp) or 100 mM cholineCl (for OATP-C/1B1 and OATP8/1B3) at 25 °C for 60 min in the presence of 50 μM unlabeled TC, BAPA-3, BAPA-6 or BAPA-8. Inhibitors were dissolved in DMSO, whose final concentration in the incubation medium was 0.2%. Control oocytes were also incubated with this amount of DMSO. Values are means ± SD ($n \geqslant 24$ oocytes from three different frogs for each experimental condition or incubation time). *$P < 0.05$, on comparing with controls by the paired t test.

ously described procedure for the synthesis of amide derivatives of BAs.[14] In brief, GC dissolved in dimethylformamide (DMF) was activated with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinolin (EDDQ) in an N_2 atmosphere at 60 °C for 4 h. PA was then added and the reaction was maintained under similar conditions for 6 h. The reaction products were separated by precipitation with diethyl ether. The pellet was dried and redissolved in methanol. The resulting solution was further purified by semi-preparative liquid chromatography using chloroform/methanol/acetic acid/water (65:24:15:9) (vol/vol) as the solvent system. The synthesis and separation procedures were checked by thin-layer chromatography (TLC) on silica gel plates (60 F254) using the same solvent system (Fig. 2). The purity of final products was checked by high performance liquid chromatography (HPLC) in reverse phase, using a Waters C-18 RCM column (5 μm, 10 mm × 25 cm) with a gradient pump module and a Photo-Diode-Array detector set simultaneously at 205 and 220 nm. The system was controlled using System Gold software from Beckman. The column was equilibrated with 10 mM KH_2PO_4/methanol 1:1 (v/v), pH 7.5 (solvent A), and eluted with an isocratic system with solvent A for 1 min and then with a linear gradient to 100% methanol in 7 min, which was maintained for an additional 15 min period. The solvent rate was 1 ml/min. The yields of the final products purified to more than 95% by HPLC as described above were 8.5%, 6.0%, and 2.2% for BAPA-3, BAPA-6, and BAPA-8, respectively.

3.3. Analytical methods

Chemical analyses for C, H, and N were performed on a Perkin-Elmer 2400 elemental analyzer. Mass spectrometry studies were carried out on a VG-Autospec (Universidad Autonoma, Madrid, Spain), using L-SIMS ionization in the FAB^+ mode (Cs ion emission) and m-NBA as matrix. ^{1}H (400 MHz) and ^{13}C (102.6 MHz) NMR spectra were obtained in CD_3OD and DMSO-d_6 solutions on a Bruker DX400 instrument. The carbon resonances were distinguished by DEPT-90 and DEPT-135 experiments. TMS was used as internal standard for ^{1}H and ^{13}C-NMR spectra.

3.4. Studies with apical ileal plasma membrane vesicles (aIPMV)

Overnight-fasted rats were used to obtain aIPMV according to an adaptation of previously described methods.[27] In brief, ileum resection was carried out under sodium pentobarbital anesthesia. The length of small intestine was measured before preparing 10 segments of approximately equal size in phosphate-buffered saline containing 0.1% (vol/vol) protease inhibitor cocktails (P2714 and P8340 from Sigma). They were inverted

in order to detach the mucosa by scraping the inner side of the intestine, which was collected in 10 ml buffer H (100 mM mannitol, 5 mM EDTA, and 5 mM Tris/HCl, adjusted to pH 7.00 with 200 mM Hepes and supplemented with 0.1% (vol/vol) protease inhibitor cocktails). After homogenization, the suspension was supplemented with $MgCl_2$ (10 mM final concentration) and centrifuged at 1000*g* for 15 min. The supernatant was further centrifuged at 30,000*g* for 30 min. The pellet was resuspended in buffer C (300 mM mannitol, 0.1 mM $MgSO_4 \cdot 7\ H_2O$, and 20 mM Hepes/Tris, pH 7.50, supplemented with 0.1%—vol/vol—protease inhibitor cocktails) and further homogenized. After centrifugation at 30,000*g* for 20 min, the pellet was resuspended in 20 ml buffer C by 10 passages through a 25-gauge needle. A final centrifugation at 30,000*g* for 30 min was carried out. The resulting pellet was resuspended in loading buffer (250 mM mannitol, 100 mM KNO_3, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 10 mM Hepes/Tris, pH 7.40, supplemented with 0.1%—vol/vol—protease inhibitor cocktails) by sequentially passing it through a 25-gauge hypodermic needle (10 times) and a 28-gauge needle (5 times), divided into aliquots, and stored in liquid nitrogen until use.

To carry out uptake studies on the inhibition of bile acid transport by aIPMV, a previously described adaptation[28] of the rapid filtration technique[29] was used. In brief, frozen vesicles were rapidly thawed at 37 °C, passed through a 25-gauge needle six times, and then placed on ice. Uptake was initiated by adding 80 μl of incubation buffer (50 μM [^{14}C]-GC, 250 mM mannitol, 100 mM KNO_3, 10 mM $MgCl_2$, 0.2 $CaCl_2$, 10 mM Hepes/Tris, pH 7.40, with or without 250 μM of inhibitor) to 20 μl of membrane vesicles (≈100 μg protein). Uptake was stopped with 4 ml of ice-cold stop medium (250 mM KCl, 25 mM $MgSO_4$, 0.1 mM cholic acid, and 10 mM Hepes/Tris, pH 7.40). Vesicles were separated from the medium by rapid filtration through 0.65 μm-pore size nitrocellulose filters. They were washed three additional times with ice-cold stop medium before the radioactivity retained was measured with a liquid scintillation counter (LS-6500-Beckman/Beckman Instruments España, S.A., Madrid, Spain) using the Universol Scintillation Cocktail from ICN (Biolink, Barcelona, Spain) as scintillant. All determinations were carried out in triplicate, using at least four separate membrane preparations.

3.5. Uptake studies in *X. laevis* oocytes

After anesthetizing the frogs by intramuscular administration of 12.5 mg ketamine in the leg (Imalgène 500; Rhône Mérieux, Barcelona, Spain), the harvesting and preparation of oocytes were carried out as described elsewhere.[18] The oocytes were then microinjected with TE buffer (1 mM EDTA, 10 mM Tris, pH 8.0) alone (Control) or with 9 ng of the mRNA of the rat orthologue of NTCP synthesized using the T7 mMessage mMachine kit (Ambion, bioNova, Madrid, Spain) and a pBluescript-Ks+-rNtcp recombinant plasmid containing the open-reading frame of this transporter, kindly supplied by Drs. P. Meier, B. Stieger, and B. Hagenbuch (University of Zurich, Switzerland), except for rat Asbt, which was contained in a recombinant plasmid obtained by subclonning between the *Eco*RI and *Hin*dIII sites of the pSPORT 1 plasmid the ORF of this transporter that had been cloned in the pCMV5/rIbat plasmid and kindly supplied by Dr. Paul Dawson (Wake Forest University School of Medicine, Winston-Salem, North Carolina). Oocytes were used 2 days after RNA injection, when—on the basis of preliminary experiments on the time-course of functional expression for this carrier—the uptake rate was highest (data not shown).

Uptake studies were carried out using groups of 8–10 oocytes per data point. Experiments were repeated three times using different frogs. The oocytes were washed with substrate-free uptake medium and incubated with 100 μl of uptake medium (100 mM sodium chloride or 100 mM cholineCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes/Tris, pH 7.0) containing the desired amount of the substrate and inhibitor to be tested at 25 °C for the indicated time. Uptake was stopped by the addition of 4 ml ice-cold uptake medium. The oocytes were washed a further three times before being collected and individually placed in vials for dissolution in 200 μl of 10% (w/v) SDS and further measurement of the radioactivity due to radiolabeled TC.[18]

3.6. Statistical methods

Results are expressed as individual values or as means ± SD. To calculate the statistical significance of the differences between groups, the paired *t* test or the Bonferroni method for multiple-range testing was used, as appropriate.

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