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Inhibition of the intestinal absorption of bile acids using cationic derivatives: Mechanism and repercussions

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ABSTRACT

To pharmacologically interrupt bile acid enterohepatic circulation, two compounds named BAPA-3 and BAPA-6, with a steroid structure and 1 or 2 positive charges, were obtained by conjugation of *N*-(3-aminopropyl)-1,3-propanediamine with one or two moieties of glycocholic acid (GC). Both BAPA-3 and BAPA-6 inhibited Na^+-dependent taurocholate (TC) uptake by *Xenopus laevis* oocytes expressing rat Asbt, with K_i values of 28 and 16 μM, respectively. BAPA-3 reduced V_{max} without affecting K_m. In contrast, BAPA-6 increased K_m, with no effect on V_{max}. Uptake of [^{14}C]-GC by the last 10 cm of the rat ileum, perfused *in situ* over 60 min, was inhibited to a similar extent by unlabeled GC, BAPA-3 and BAPA-6. However, the intestinal absorption of these compounds was lower (BAPA-6) or much lower (BAPA-3) than that of GC. When administered orally to mice, both compounds (BAPA-3 > BAPA-6) reduced the bile acid pool size, which was accompanied by up-regulation of hepatic Cyp7a1 and Hmgcr and intestinal Ostα/Ostβ. A tendency towards a decreased expression of hepatic Ntcp and an enhanced expression of intestinal Asbt was also observed. Serum biochemical parameters were not affected by treatment with these compounds, except for a moderate increase in serum triglyceride concentrations. In sum, our results suggest that these compounds, in particular BAPA-3, are potentially useful tools for inhibiting the intestinal absorption of bile acids in a non-competitive manner.

1. Introduction

Hypercholesterolemia is one of the most important risk factors for the development of cardiovascular diseases owing to the increased probability of atheromatose lesions [1]. This justifies the enormous efforts carried out in the last decades to obtain new cholesterol-lowering drugs. One of the strategies that have produced the best results has been the one based on the inhibition of the key enzyme involved in cholesterol synthesis, i.e., hydroxymethyl glutaryl coenzyme A reductase (HMGCR), by statins. However, in spite of its efficacy, this family of compounds is not the definitive solution. The long-term side effects are only partially understood and its good tolerability cannot be extended to children with genetic disorders affecting cholesterol homeostasis [2].

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Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BAPA, bile acid-polyamine derivative; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; GC, glycocholic acid; NTCP, Na^+-taurocholate-cotransporting polypeptide; OST, organic solute transporter; TC, taurocholic acid

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Alternative pharmacological approaches include the stimulation of cholesterol biotransformation into bile acids subsequent to an increased loss of these compounds due to their sequestering in the intestinal lumen using specific gels or resins. Cholesterol-7α-hydroxylase (CYP7A1), the key enzyme in bile acid synthesis [3], is down regulated by bile acids [4]. Thus, by depleting the bile acid pool an enhancement in the expression of cholesterol-7α-hydroxylase occurs, which is subsequently accompanied by enhanced removal of cholesterol, which is then biotransformed into bile acids [5]. Although the conceptual basis of this therapy is interesting, the pharmaceuticals available are difficult to manipulate and administer. Moreover they are not devoid of adverse consequences [6,7].

Since the molecular mechanisms accounting for intestinal bile acid re-absorption are being elucidated [8,9], the alternative of favouring the foecal elimination of these steroids by inhibiting the plasma membrane carriers responsible for bile acid uptake has emerged as a promising alternative. The apical sodium-dependent bile acid transporter (ASBT), located at the brush-border membrane of epithelial cells in the ileal mucosa, is a member of the family 10 of solute carriers encoded by the gene *SLC10A2* [10]. Since this is the main transport system involved in the active reabsorption of bile acids during the intestinal transit of their enterohepatic circulation, the inhibition of bile acid uptake by this carrier would result in increased fecal loss of bile acids.

Several investigators and pharmaceutical companies have developed ASBT-inhibitors with very different chemical structures. Among them, the following can be mentioned as examples: SC-635 [11], S-8921 [12], 2164U90 [13], 2,3-disubstituted-4-phenylquinolines [14]. Regarding the use of bile acid derivatives, several studies carried out in the 70 s using everted hamster gut sac as an experimental model revealed that cationic bile salt derivatives were able to interact with the ileal bile acid transport system in such a way as to inhibit the transport of natural bile salts [15]. This interaction seemed to involve two recognition components; one includes the steroid moiety, the other a coulombic interaction between the anionic bile salt and a cationic membrane site [16].

In later studies, using isolated perfused rat liver [17] and *in vivo* measurement of the absorption from the jejunum and ileum of anesthetized guinea pigs [18], it was shown that fully positively charged bile acid derivatives or zwitterionic bile acid derivatives were not appreciably taken up by the liver or the intestine.

More recently, once the transporters involved in these processes were cloned and expressed in cell lines, it has been possible to investigate substrate specificity of sodium/bile acid co-transporters that are the main responsible for bile acid uptake by the liver (Na^+-taurocholate co-transporting polypeptide, NTCP, gene symbol *SLC10A1*) and the ileum (ASBT). Studies carried out in rabbit orthologues have suggested that the side chain of bile acids is important for their interaction with the recognition site of ASBT and NTCP [19]. Regarding human ASBT, a recent study suggests that C-24 conjugation and steroidal hydroxylation pattern modulate native bile acid interaction with human ASBT, with the effect of conjugation dominating that of steroidal hydroxylation. Moreover the results of that study indicate that bile acid binding to human ASBT may be the rate-limiting step in the apical transport of bile acids [20].

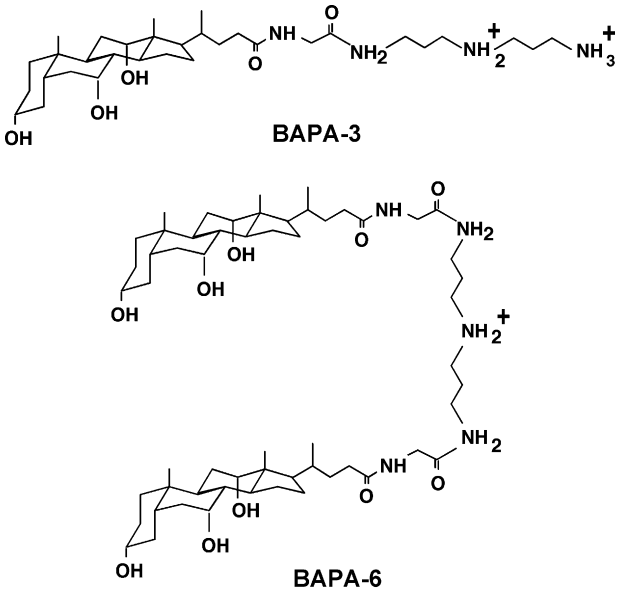

Fig. 1 – Schematic representation of the molecular structure of conjugates of N-(3-aminopropyl)-1,3-propanediamine with one or two glycocholic acid moieties to obtain BAPA-3 and BAPA-6, respectively.

Based on the results mentioned above, the conceptual base of the present work was that a negative charge in the lateral chain is needed for natural bile acids to interact with the main intestinal carrier accounting for bile acid uptake from the intestinal lumen, i.e., ASBT [18]. This prompted us to design and synthesize steroids with different sizes and net positive charges (Fig. 1) and to evaluate their ability to inhibit ASBT-mediated bile acid intestinal absorption and to affect several physiological aspects related to the enterohepatic circulation of these compounds.

2. Methods and materials

2.1. Reagents

FITC-Dextran-40 kDa, N-(3-aminopropyl)-1,3-propanediamine (PA) and sodium salts of glycocholic acid (GC), taurocholic acid (TC) and chenodeoxycholic acid (CDCA) more than 95% pure by thin-layer chromatography were purchased from Sigma–Aldrich (Madrid, Spain). [^{14}C]-GC (specific radioactivity 46.7 mCi/mmol) and [^{3}H]-TC (specific radioactivity 3.0 Ci/mmol) were obtained from Perkin-Elmer Life Science (Izasa, Barcelona, Spain). All other chemicals were from Merck Eurolab (Barcelona, Spain). They were of high purity and were used as purchased without any further purification. The GC and PA conjugates named BAPA-3 and BAPA-6 whose molecular structures are shown in Fig. 1, were obtained by an adaptation of a previously described procedure for the

synthesis of amide derivatives of bile acids [21] and purified to more than 95% by semi-preparative liquid chromatography using chloroform/methanol/acetic acid/water (65:24:15:9) (v/v) as the solvent system. The purity of final products was checked by high performance liquid chromatography in reverse phase.

2.2. Animals

Male Wistar CF rats and CD1 mice (Animal House, University of Salamanca, Spain) and mature female frogs (*Xenopus laevis*; Regine Olig, Hamburg, Germany) were fed on commercial-pelleted rat, mouse or Xenopus food (Panlab, Madrid, Spain). Lighting was controlled by a timer that permitted light between 8:00 a.m. and 8:00 p.m. All animals received humane care as outlined in ''Institutional Animal Care and Use Committee Guidebook'' (2nd ed., 2002). Experimental protocols were approved by the Ethical Committee for Laboratory Animals of the University of Salamanca.

2.3. Uptake studies in Xenopus laevis oocytes

After anaesthetizing 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 [22]. 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 ASBT (Asbt). This was synthesized using the T7 mMessage mMachine Ultrakit (Ambion, bioNova, Madrid, Spain) and a recombinant plasmid obtained by subcloning the DNA corresponding to the ORF of rat Asbt between the *EcoRI* and *HindIII* sites of the pSPORT 1 plasmid. The transferred DNA was obtained from pCMV5/rAsbt plasmid kindly supplied by Dr. Paul Dawson (Wake Forest University School of Medicine, Winston-Salem, NC). 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 NaCl or 100 mM cholineCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris, pH 7.5) containing the desired amount of 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 three more times before being collected and individually placed in vials. They were digested with 200 μl of 10% (w/v) SDS before measuring the radioactivity due to radiolabeled substrate [22].

2.4. In situ single-pass perfusion of the rat ileum

Overnight fasted animals were anaesthetized with sodium pentobarbital (Nembutal N.R., Abbot, Madrid, Spain) (50 mg/kg body weight, i.p.), prior to performing the surgical preparation. A catheter was placed in the common bile duct to drain bile throughout the experimental period. The pylorus was ligated to prevent any passage of stomach contents to the small intestine and two catheters were implanted in the ileum: (i) The inflow catheter at 10 cm away from the ileal-cecal junction in the ileum. (ii) The outflow catheter was implanted at ileal-cecal junction. Using a peristaltic pump, the ileal segment located between both catheters was washed with warmed (37 °C) perfusion medium (120 mM NaCl, 5 mM KCl, 0.65 mM $MgSO_4{\cdot}7H_2O$, 1.17 mM KH_2PO_4, 1.29 mM $CaCl_2{\cdot}2H_2O$, 25 mM $NaHCO_3$, 100 mg/l gentamycin, pH 7.40) at 0.2 ml/min, until the effluent was colourless (approximately 10 min). Then, an additional period of 10 min perfusion was allowed before starting the experimental period with a 15 min administration period during which the perfusion medium was replaced by a similar one containing substrate alone or together with inhibitors (0–15 min). Fresh perfusion medium was used to perfuse the ileal segment for the next 45 min period. Throughout the experimental period, outflowing perfusate was collected in pre-weighed vials every 10 min. Volume was determined gravimetrically and the uptake of the compounds was measured by the difference in contents between the inflow and the outflow perfusate. Using appropriate calibration curves for each compound, GC, BAPA-3 and BAPA-6 were measured by enzymatic detection of the 3α-hydroxyl group [23], which is present in these three compounds.

2.5. In vivo experiments in mice

To investigate the effect on the bile acid pool, this was labeled as follows: 3-month-old male CD1 mice of approximately 30–40 g body weight received an intragastric administration of 0.23 μCi [^{3}H]-TC in 500 μl saline (time 0 h). After 24 h, the animals received in a similar way 20 μmol BAPA-3 or BAPA-6 or vehicle alone (500 μl saline:ethanol, 9:1, v/v). Mice were fasted from 48 to 72 h and then anaesthetized with sodium pentobarbital (i.p., 0.5 mg/10 g body weight) before undergoing laparotomy, blood extraction (≈1 ml) from the cava vein, and subsequent removal of the liver, the gallbladder, the small intestine and the mesenterium. These organs were washed with ice-cold saline, weighed, and homogenized in saline (1:2; w/v). Radioactivity in the homogenate was measured and used as an internal standard to follow the extraction procedure. In brief, after addition of two volumes of ethanol to the homogenized tissues and incubation at 65 °C for 2 h, bile acids were extracted in the supernatant resulting from centrifugation at 2200 × *g* for 10 min. The process was repeated with the pellet and the two supernatants pooled together and filtered through filter paper. Bile acid concentrations were measured enzymatically [23]. Bile acid pool size was calculated after correcting by the yield of the extraction procedure using values of radioactivity measured in the initial homogenate and in the final extraction solution. The average yield was 93.6%. In control animals, the average amount of radioactivity in the homogenate was 65.4% of the dose given 3 days before. In separate similar experiments, the mice did not receive radiolabeled bile acids and they were used to determine serum biochemical parameters by routine biochemical analysis and the liver

Table 1 – Gene-specific primers used for quantitative real-time RT-PCR analysis of mouse mRNA

Target DNA	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Accession number
Slc10a1	GGCCACAGACACTGCGCT	AGTGAGCCTTGATCTTGCTGAACT	101	NM_011387
Slc10a2	TTGCACAGCACAAGCAGTGA	TGCATTGAAGTTGCTCTCAGGT	103	NM_011388
OSTα	CAGATCGCTTGCTCACCTCC	GGTCCAAGCCACTCTCCTCA	200	NM_145932
OSTβ	GATGCGGCTCCTTGGAATTA	TTCGATTTCTGTTTGCCAGGAT	103	NM_178933
Cyp7a1	TGAGACCTCCGGGCCTTC	CGTTAGATATCCGGCTTCAAACA	110	NM_007824
Cyp27a1	GGCCTGGATAGGGCTCATAGT	TCCAGGAGCGTCCATCTCA	102	NM_024264
Hmgcr	TGCCTGGATGGGAAGGAGTA	TCGAGTCATCCCATCTGCAA	138	XM_127496

Slc10a1, Ntcp (Na^+-taurocholate cotransporting polypeptide); *Slc10a2*, Asbt (apical sodium-dependent bile acid transporter); *OST*, organic solute transporter; *Cyp7a1*, cholesterol 7α-hydroxylase; *Cyp27a1*, sterol 27-hydroxylase; *Hmgcr*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

and the small intestine were employed to measure gene expression.

2.6. Quantitation of gene expression by real-time RT-PCR

Since bile acids play a crucial role in the regulation of enzymes and transporters involved in bile acid and cholesterol homeostasis the degree of expression of some of these proteins was indirectly determined by measuring steady-state levels of their mRNA. Freshly obtained mouse tissues were immersed in the RNAlater RNA-stabilization reagent (Ambion, bioNova Cientifica, Madrid) and stored at −80 °C until use. Total RNA was isolated from these samples using RNeasy spin columns from Qiagen (Izasa, Barcelona). After treatment with RNase-free DNase I (Roche Diagnostics, Barcelona), RNA was quantified fluorimetrically with the RiboGreen RNA-Quantitation kit (Molecular Probes, Leiden, The Netherlands). DNA was synthesized using random nonamers and avian myeloblastosis virus reverse transcriptase (enhanced Avian RT-PCR kit; Sigma–Genosys, Cambridge, UK). Primer oligonucleotides obtained from Sigma–Genosys were designed with the assistance of Primer Express software (Perkin-Elmer Applied Biosystems, Madrid) for DNA fragments in described sequences, and their specificity was checked using BLAST. Their nucleotide sequences for mouse genes are shown in Table 1 and those for human ASBT, OSTα and OSTβ have been reported elsewhere [24]. Real-time quantitative PCR was then performed using AmpliTaq Gold polymerase (Perkin-Elmer Applied Biosystems) in an ABI-Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The thermal cycling conditions were: one cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. Detection of amplification products was carried out using SYBR Green I (Perkin-Elmer Applied Biosystems). The absence of artifacts or non-specific products of PCR, which was checked using 2.5% agarose gel electrophoresis and melting temperature curves, permitted the use of SYBR Green I detection in all cases. The results of mRNA abundance for each target gene in each sample were normalized on the basis of its 18S rRNA content, which was measured with the TaqMan® Ribosomal RNA Control Reagents kit (Perkin-Elmer Applied Biosystms).

2.7. Statistical analysis

Results are expressed as mean ± S.D. To calculate the statistical significance of the differences among groups the Student's *t*-test or the Bonferroni method of multiple-range testing were used, as appropriate. Regression lines in kinetic studies were calculated by the least squares method.

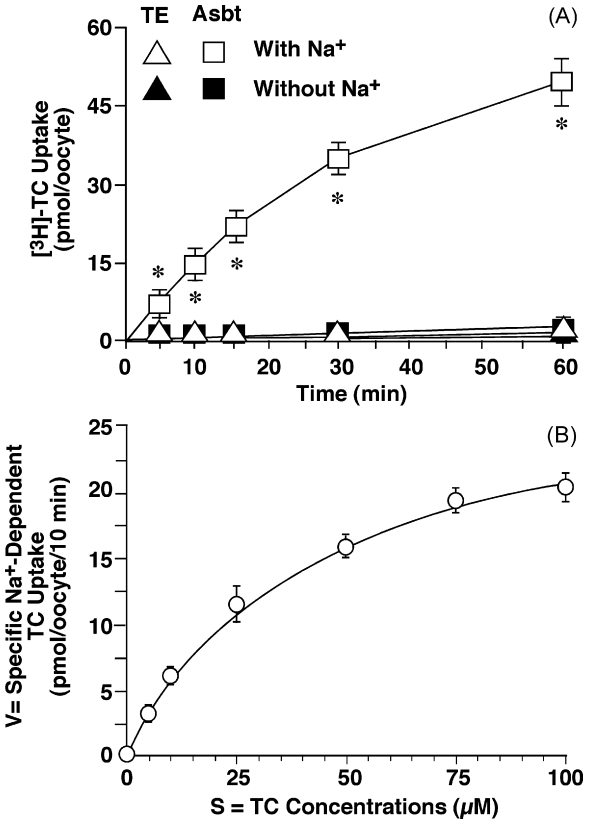

Fig. 2 – (A) Time-course of [^{3}H]-taurocholic acid ([^{3}H]-TC) uptake by *Xenopus laevis* oocytes microinjected with vehicle (TE buffer, triangles) or with 9 ng of rat Asbt mRNA (squares) and incubated 2 days later with 10 μM [^{3}H]-TC in the presence (open symbols) or absence (closed symbols) of sodium. (B) Effect of substrate concentration (*S*) on specific sodium-dependent [^{3}H]-TC uptake (*V*) as defined as the difference in uptake in the presence and absence of sodium in the incubation medium of oocytes expressing rat Asbt mRNA. Uptake of [^{3}H]-TC by oocytes injected with TE buffer alone was subtracted in each condition. Uptake values are mean ± S.D. from measurements carried out in 30 oocytes from three different frogs per data-point.

3. Results

3.1. Studies in Xenopus laevis oocytes expressing rat Asbt

Studies on the time-course of TC uptake by *Xenopus laevis* oocytes revealed that the expression of rat Asbt and the presence of sodium in the extracellular medium markedly enhanced this process (Fig. 2A). Moreover, these results suggested that 10 min was the right time to determine TC uptake under the initial velocity (*V*) conditions. Under these circumstances, TC uptake was dependent on substrate concentrations (*S*), resulting in a typical plot for saturable processes (Fig. 2B). Indeed, the best fit was a Michaelis–Menten equation whose V_{max} and K_m values were approximately 11–25 pmol/oocyte/10 min and 30–70 μM, respectively. When BAPA-3 or BAPA-6 was added to the incubation medium, TC uptake was markedly reduced. Kinetic studies based on Dixon approaches [25] were carried out to characterize this inhibition. In a first step, TC uptake at varying substrate concentrations was measured in the presence or absence of a fixed amount of BAPA-3 or BAPA-6. Upon plotting *V* versus *V/S* and *S/V* versus *S*, statistically significant ($P < 0.001$) correlations were found (Fig. 3). These plots suggested that BAPA-3 induced a decrease in V_{max} without affecting K_m, which is the typical behavior for a non-competitive inhibitor. In contrast, BAPA-6 induced a competitive inhibition, as suggested by the absence of effect on V_{max}, together with an enhancement in the value of K_m. To calculate the inhibition constant (K_i), a different set of experiments was carried out, measuring TC uptake by oocytes incubated with varying concentrations of BAPA-3 or BAPA-6 and two different substrate concentrations (Fig. 4). By plotting 1/V versus inhibitor concentrations, it was possible to obtain two curves (both $P < 0.001$) whose intersection and extrapolation to the *X*-axis permitted us to calculate K_i for BAPA-3 and BAPA-6. These values were 28 and 16 μM, respectively.

3.2. Bile acid uptake by the in situ perfused rat ileum

Preparations of *in situ* perfused rat ileum maintained their viability throughout the experimental period, as indicated by the relative steady-state in the outflow perfusion rate (Fig. 5A). Moreover, in some experiments the absence of marked changes in epithelial integrity and net water balance between the luminal content and the animal was confirmed by including in the perfusion medium the non-absorbable fluorescent compound FITC-dextran-40 kDa. The concentration of this compound in the outflow remained relatively steady and similar to that seen in the inflow perfusate throughout the perfusion period (data not shown). When [^{14}C]-GC was administered over 15 min, the radioactivity found in

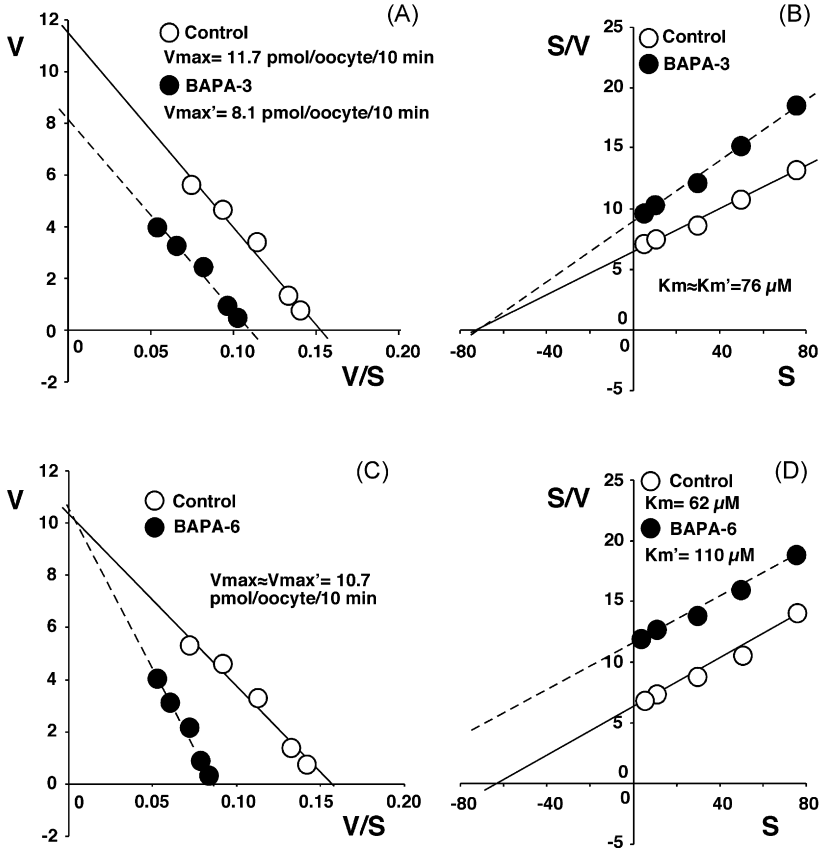

Fig. 3 – Effect of the presence of 25 μM BAPA-3 (A and B) or BAPA-6 (C and D) on maximal velocity of transport (V_{max}) and affinity constant (K_m) obtained by plotting the results of specific sodium-dependent [^{3}H]-taurocholic acid ([^{3}H]-TC) uptake (*V*) vs. substrate concentrations (*S*) in two different ways: *V* vs. *V/S* to calculate V_{max} (A and C), and *S/V* vs. *S* to calculate K_m (B and D). Uptake values are mean ± S.D. from measurements carried out in 30 oocytes from three different frogs per data-point. S.D. bars are not seen because they are smaller than the symbols.

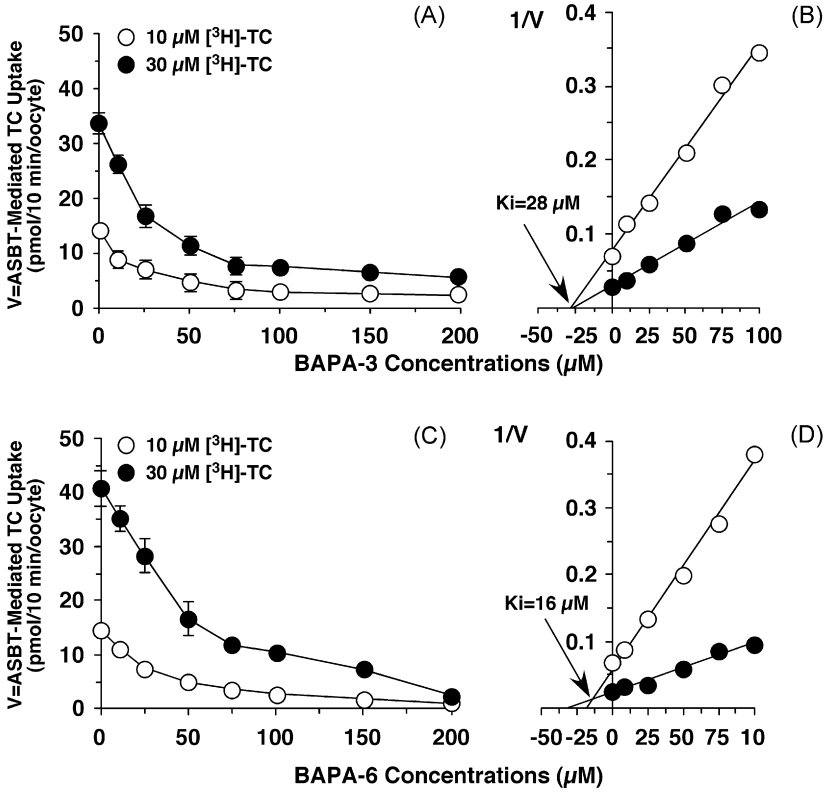

Fig. 4 – Effect of the presence of varying concentrations of BAPA-3 (A and B) or BAPA-6 (C and D) on specific sodium-dependent [^{3}H]-taurocholic acid ([^{3}H]-TC) uptake (*V*), which was measured using two different substrate concentrations (10 and 30 μM). Uptake values are mean ± S.D. from measurements carried out in 30 oocytes from three different frogs per data-point. When S.D.-bars are not seen is because they are smaller than the symbols. The absolute value of the inhibition constant (K_i) was calculated by the intersection of both curves.

the outflowing pefusate for the 60 min that followed the start of the administration period was 46% of the dose administered (0.15 μmol) (Fig. 5A). Thus, under these experimental circumstances the absorption of [^{14}C]-GC was calculated to be 0.081 μmol. This was confirmed by determination of the radioactivity in bile samples collected during this time, which was 98% of the amount of [^{14}C]-GC removed from the ileal perfusate (data not shown). When [^{14}C]-GC was administered together with 1.5 μmol of unlabeled GC, BAPA-3 or BAPA-6, a marked inhibition in [^{14}C]-GC absorption was found (Fig. 5B). Measurement by an enzymatic technique [23], as described above, of the difference between the amount of these compounds in the inflow and outflow perfusate revealed that the absorption of BAPA-6 was lower than that of GC, whereas that of BAPA-3 was much lower (Fig. 5C).

3.3. *Effect of administration of BAPA-3 and BAPA-6 to mice in vivo*

To investigate the *in vivo* physiological repercussions in mice of the inhibition of bile acid absorption by the intestine, the bile acid pool size, biochemical parameters and the expression levels of the key enzymes and transporters involved in bile acid homeostasis were measured 2 days after intra-gastric administration of BAPA-3 or BAPA-6. Both compounds were able to reduce the bile acid pool size without affecting serum biochemical parameters, except for a moderate increase in serum triglyceride concentrations, which was only statistically significant for BAPA-6 (Table 2). Similar hypertriglyceridemia has been also observed to accompany several strategies able to lower the transhepatic flux of bile acids [26]. Regarding the effects on liver expression (Fig. 6), no significant change in the levels of mRNA for Cyp27a1 was found. A tendency for Ntcp to be reduced was observed, but the differences were not significant. However, the expression of Cyp7a1 and Hmgcr was clearly increased after treatment with BAPA-3, and less markedly also with BAPA-6. In the small intestine, a progressive increase from proximal to distal regions in the expression of Asbt, Ostα and Ostβ was found (Fig. 7). The regional distribution of these transporters was not affected by BAPA-3 or BAPA-6, but the expression of these transporters was enhanced by both compounds, mainly in the distal segment, i.e., that closest to the ileo-caecal valve. The up-regulation induced by BAPA-3 was more marked than that induced by BAPA-6. In both cases, these were Ostα ≫ Ostβ ≫ Asbt (Fig. 7).

4. Discussion

Several pharmacological strategies aimed at reducing the bile acid pool size are available. Some of them are based on the use

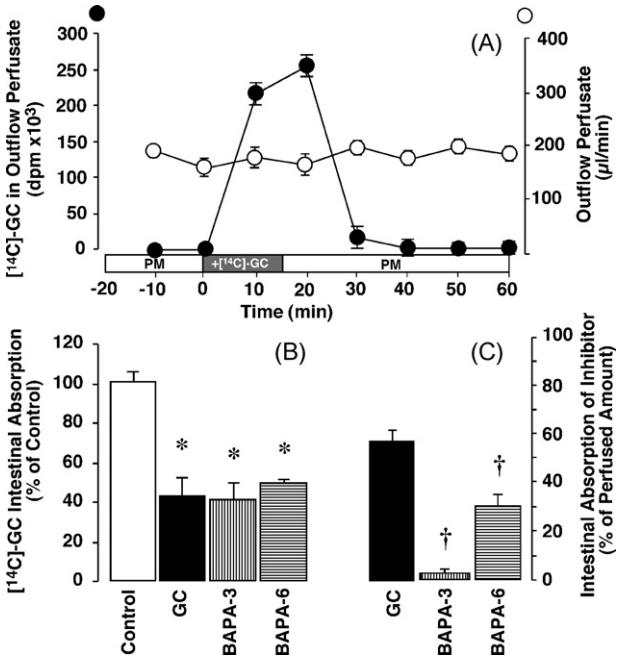

Fig. 5 – (A) Recovery of volume (open circles) and radioactivity (closed circles) due to [^{14}C]-glycocholic acid ([^{14}C]-GC) in the outflow perfusate from the last 10 cm of the rat ileum perfused *in situ* at 200 μl/min with perfusion medium (PM) alone or containing 0.15 μmol [^{14}C]-GC over 15 min. (B) Cumulative [^{14}C]-GC uptake by the rat ileum perfused *in situ* with 0.15 μmol [^{14}C]-GC alone or together with 1.5 μmol GC, BAPA-3 or BAPA-6. (C) Cumulative uptake of inhibitors according to their concentrations measured enzymatically in the PM before and after passing through the ileum. Values are mean ± S.D. from five different preparations. $^{*}P < 0.05$ as compared with controls. $^{\dagger}P < 0.05$ as compared with GC. The Bonferroni method of multiple range testing was used for comparisons.

of sequestrant agents able to trap bile acids in the intestine and partly prevent their intestinal re-absorption. However, the efficiency of these formulations is moderate, their handling and administration present some difficulties and their use is frequently accompanied by undesirable side effects [6,7]. The present study constitutes an additional contribution to the numerous efforts carried out by several groups to develop a variety of compounds aimed at enhancing the fecal excretion of bile acids by reducing their intestinal re-absorption [11,13,14].

One of the interesting findings of the present study concerns the mechanism of inhibition caused by BAPA-3 and BAPA-6 on bile acid transport by rat Asbt. The fact that BAPA-3 induced non-competitive inhibition whereas BAPA-6 induced competitive inhibition was surprising. Owing to similarity of these compounds with natural bile acids as regards their steroid moiety, competition with the active site of rat Asbt could be expected. However, the non-competitive inhibition observed for the di-cationic compound BAPA-3 suggests a more complex interaction with this transporter. Our results are consistent with, although they do not prove, the existence in this carrier protein of a regulatory site, different to the one involved in transport activity, to which BAPA-3 could bind and modify bile acid transport. The question therefore arises as to whether the site of Asbt able to interact with BAPA-3 is also sensitive to endogenous compounds, such as natural bile acids, food components or drugs, and whether this plays a role in regulating the overall transport capability under physiological and pathological circumstances. The findings that BAPA-6 induced competitive inhibition of bile acid transport by Asbt and that when given through the *in situ* perfused rat ileum a certain amount of BAPA-6 was taken up by the intestine, although to a lesser extent than natural bile acids, suggest that this compound may be taken up in part via Asbt. In contrast, accordingly with this hypothesis, BAPA-3, which induced non-competitive inhibition of Asbt, was poorly absorbed by the intestine. Moreover, the possibility that these compounds may interact with natural bile acids to form lipophilic ion-pairs which may influence the recognition of bile acids by relevant intestinal transporters cannot be ruled out.

Since the highest expression of bile acid transporters was found to be located in the distal ileum (Fig. 7), we used this segment of the rat small intestine to investigate the ability of BAPA-3 and BAPA-6 to inhibit intestinal bile acid absorption. Using a modification of the *in situ* perfused rat ileum preparation previously described [27–29], we observed here that both BAPA-3 and BAPA-6 were able to inhibit GC uptake. These results are consistent with the reduction in the bile acid pool size induced by the administration of these compounds to mice. The repercussions of this effect include an increased expression of enzymes responsible for cholesterol and bile acid biosynthesis in the liver. Regarding the expression of transporters, the main change was a compensatory response of the ileal mucosa to increase its ability to transfer bile acids from epithelial cells toward the portal blood by up-regulation of Asbt and, more markedly, the Ostα/Ostβ heterodimer, which is responsible for the exit of bile acids across the basal plasma membrane [9]. These results are consistent with the concept that bile acids induce negative feedback regulation of ileal bile acid transporters [30]. By preventing intestinal uptake of endogenous bile acids and hence interaction with nuclear receptors [31], BAPA-3 and BAPA-6 are able to induce up-regulation of these transporters. The fact that together with the increased expression of enzymes favouring cholesterol metabolism the expression of transporters associated with the intestinal bile acid absorption was also increased by the treatment could be disadvantageous to the objective of lowering enterohepatic recycling of bile acids and hence hepatic cholesterol level. This suggests that combined therapy with drugs inhibiting mechanisms involved in feedback regulation of ileal expression of ASBT would probably enhance the efficacy of therapies based on the administration of inhibitors of the intestinal absorption of bile acids.

Drug-induced reduction in the overall intestinal absorption of bile acids could take place at different steps, which include: (i) Interaction with ASBT at the brush-border apical plasma membrane of the ileal mucosa [32,33]. (ii) Inhibition of the efflux across the basal plasma membrane, a process that is believed to be mediated by the heterodimer OSTα/OSTβ, with a

Table 2 – Effect of BAPA-3 or BAPA-6 administration to mice on the bile acid pool size and serum biochemical parameters

	Control ($n = 5$)	BAPA-3 ($n = 6$)	BAPA-6 ($n = 7$)
Bile acid pool size			
Total (μmol)	26.9 ± 1.0	15.8 ± 2.9*	19.1 ± 2.1*
Normalized (μmol/g body weight)	0.77 ± 0.03	0.43 ± 0.09*	0.53 ± 0.06*
Serum biochemical parameters			
Glucose (mg/dl)	90 ± 20	94 ± 13	97 ± 10
Total bilirubin (mg/dl)	0.42 ± 0.05	0.60 ± 0.04	0.71 ± 0.12
GOT-ASAT (UI/l)	71 ± 8	51 ± 5	57 ± 12
GPT-ALAT (UI/l)	14 ± 2	11 ± 2	19 ± 5
Alkaline phosphatase (UI/l)	54 ± 4	64 ± 5	70 ± 13
Urea (mg/dl)	30 ± 6	31 ± 4	24 ± 3
Cholesterol (mg/dl)	121 ± 13	112 ± 11	150 ± 15
HDLc (mg/dl)	81 ± 10	81 ± 8	93 ± 10
VLDLc + LDLc (mg/dl)	40 ± 4	30 ± 3	57 ± 7
Triglycerides (mg/dl)	51 ± 11	68 ± 7	84 ± 6*

Values are mean ± S.D. from 5 to 7 mice per group. The animals received intragastric administration of a trace amount (less than 0.1% of the total pool) of radiolabeled taurocholate to be used as internal Standard. After 24 h, 20 μmol BAPA-3 or BAPA-6 was given orally. The animals were fasted from 48 to 72 h, when serum samples, the liver, the gallbladder and the intestine were collected. GOT-ASAT, glutamic-oxalacetic transaminase-aspartate aminotransferase; GPT-ALAT, glutamic-pyruvic transaminase-alanine aminotransferase.
* $P < 0.05$, as compared with controls by the Bonferroni method of multiple range testing.

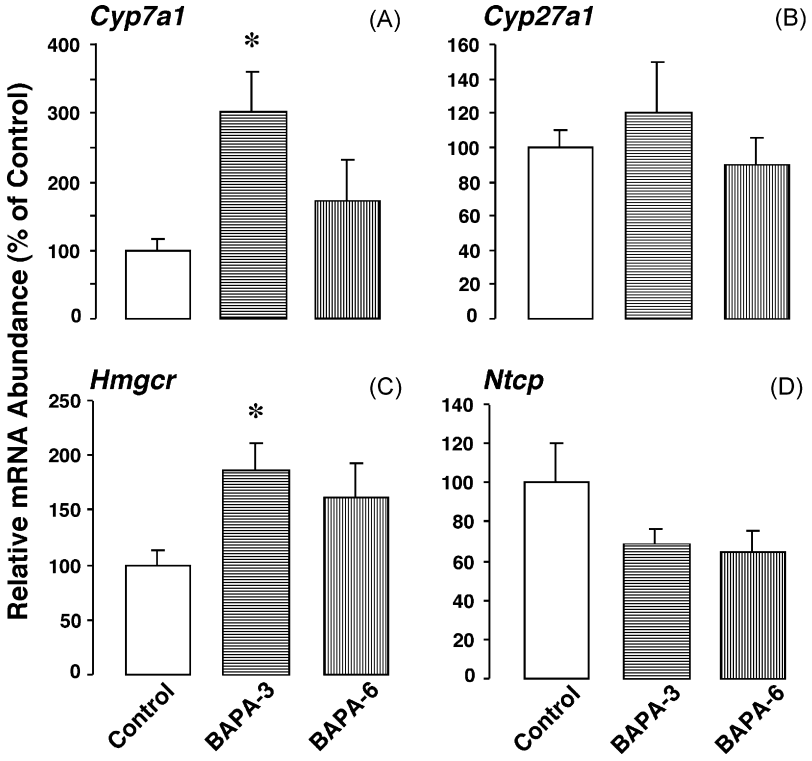

Fig. 6 – Steady-state levels of mRNA as determined by real-time quantitative RT-PCR in the liver of mice that received intragastric administration of the vehicle (saline) alone or containing 20 μmol BAPA-3 or BAPA-6, 48 h before liver collection from anaestethized animals. The animals were fasted for the last 24 h. The abundances of mRNA of Na^+-dependent taurocholate cotransporting polypeptide (Ntcp), key enzymes involved in bile acid biosynthesis, cholesterol 7α-hydroxylase (*Cyp7a1*) and sterol 27-hydroxylase (*Cyp27a1*) and in cholesterol synthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*) were measured. Inter-reaction variability was corrected using total RNA from an adult mouse liver as a calibrator, and the levels of 18S rRNA in each sample were used to normalize the results. Values are mean ± S.D. from six mice per group analyzed in triplicate for each data point. *$P < 0.05$ on comparing with controls using the Bonferroni method of multiple range testing.

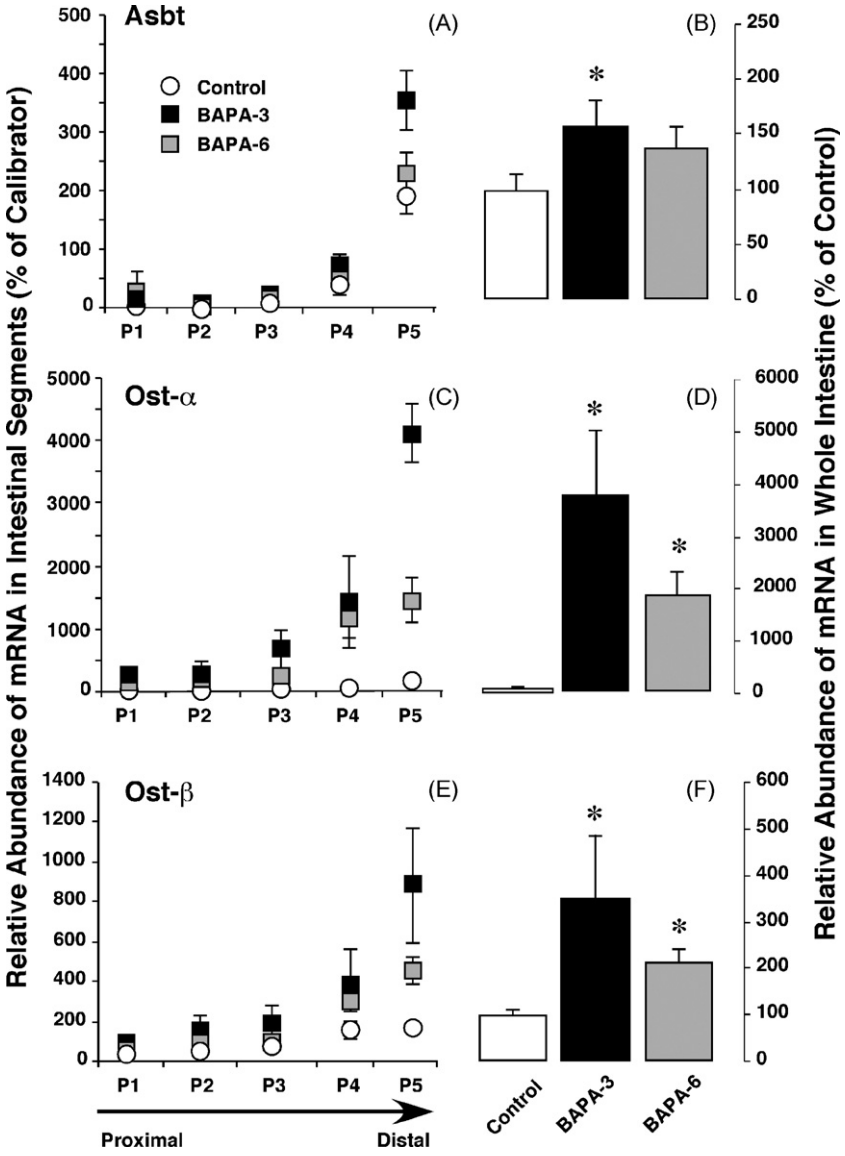

Fig. 7 – Steady-state levels of mRNA as determined by real-time quantitative RT-PCR in the small intestine of mice that received intragastric administration of the vehicle (saline) alone (control, open bars) or containing 20 μmol BAPA-3 (closed bars) or BAPA-6 (grey bars), 48 h before small intestine collection from anaestethized animals. The animals were fasted for the last 24 h. The abundances of mRNA of apical sodium-dependent bile acid transporter (Asbt) and the two components of the heterodimeric organic solute transporter (Ostα/Ostβ) were measured. Inter-reaction variability was corrected using total RNA of whole small intestine from an adult mouse as a calibrator, and the levels of 18S rRNA in each sample were used to normalize the results. Values are mean ± S.D. from six mice per group analyzed in triplicate for each data point. $^{*}P < 0.05$ on comparing with controls using the Bonferroni method of multiple range testing.

minor contribution of MRP3 [34,35], but in which the involvement of anion exchange transport systems [36], probably belonging to the family of the multispecific organic anion transporting polypeptides (OATPs) [37], has been also suggested. (iii) Disruption of the dynamics of bile acid intracellular transit and binding to cytosolic proteins, such as the bile acid-binding protein (BABP) [32,38,39], may also play a role. Whether BAPA-3 may interact with more than one of these mechanisms in polarized *in vivo* epithelial cells, which may have an influence on the net loss of bile acid from the enterohepatic circulation and affect the regulation of enzymes and transporters, cannot be ruled out.

In summary, the results of the present study suggest that these compounds, in particular BAPA-3, owing to its strong ability to inhibit ASBT-mediated bile acid transport and their low absorption by the intestine, are potentially useful tools to carry out pharmacological manipulations of the enterohepatic circulation of endogenous bile acids.

Conflict of interest

In the period of research leading up to this publication we have not received any financial support that may affect in any way

the conclusions of our article. Moreover, the authors have no direct or indirect commercial interest in any company that might be financially affected by the conclusions of the present article.

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