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Hepatobiliary elimination of bile acid-modified oligodeoxynucleotides in Wistar and TR$^-$ rats: evidence for mrp2 as carrier for oligodeoxynucleotides

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Abstract

As therapeutic antisense tools, oligonucleotides (ODNs) must enter cells to bind to their target structures. ODNs distribute in nearly each tissue with relatively high concentrations in kidney and liver from where excretion into urine and bile occurs. To investigate mechanisms involved in hepatic ODN transport, normal mixed backbone phosphodiester/phosphorothioate ODNs (*n*-ODN) and two different bile acid-conjugated mixed backbone ODNs (1BA-ODN and 2BA-ODN) were applied to two different rat strains, normal Wistar rats and Wistar TR$^-$ rats. In normal Wistar rats, concentration-dependent hepatobiliary elimination of the ODNs was observed with a remarkable increase of excretion of the cholic acid BA-ODN conjugates. In contrast to normal Wistar rats, *n*-ODN excretion into bile by TR$^-$ rats, a mutant Wistar rat strain lacking a functional multidrug resistance-associated protein 2 (mrp2) at the canalicular membrane, was strongly diminished, whereas these rats excreted an ODN conjugated with two cholic acid molecules (2BA-ODN) into bile. Concomitant application of substrates transported by mrp2 such as bromosulfophthalein (BSP) or the synthetic chlorogenic acid derivative S 3025 significantly reduced the biliary appearance of normal ODN and 2BA-ODN in Wistar rats and also in TR$^-$ rats. To inhibit the expression of cRNA derived from the Na^+-dependent taurocholate cotransporting polypeptide (Ntcp), antisense ODNs were constructed which fully retained the antisense properties when coupled with two bile acid molecules. The results indicate that ODNs are secreted via the mrp2 into bile. In the absence of mrp2, further excretory transport systems with affinity for bile acids seem to be relevant for their excretion. The results further indicate that bile acid tagged ODNs are useful tools for liver specific antisense therapy.

Keywords: Oligodeoxynucleotides; Liver targeting; Liver evasion; mrp2; Bile acids; Bile salt export pump

1. Introduction

Oligonucleotides (ODNs) represent an innovative therapeutic principle by mainly affecting the genetic site of diseases. Fomivirsen (Vitravene^{TM}), the first antisense acting drug, was licensed in 1998 for ocular cytomegalovirus infection. Membrane permeation and sufficient stability in biological material are regarded as the major difficulties for broad application of these mostly polyanionic and hydrophilic substances *in vivo*. The latter problem was sufficiently resolved by introducing various structural modifications [1,2] such as phosphorothioates where a substituted sulphur stabilises ODNs against enzymatic degradation. In order to improve their biological properties, mixed backbone ODNs are used to reduce unspecific binding, rapid degradation and toxicity, e.g. by combination of naturally occuring phosphodiester linkages in the mid and phosphorothioate linkages at the 3′ and 5′ end of the molecule chain [3].

On the basis of therapeutic considerations, the oral route of administration is most suitable for chronic dosing. To reach the systemic circulation from the intestinal tract,

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Abbreviations: TC, taurocholic acid; ODN, oligodeoxynucleotide; *n*-ODN, normal 15 mer mixed backbone phosphodiester/phosphorothioate oligodeoxynucleotide; 2BA-ODN, two bile acid-oligodeoxynucleotide (*n*-ODN tagged at the 3′ and 5′ end with cholic acid); 20 mer AS-*n*-ODN-DE, 20 mer antisense-normal oligodeoxynucleotide-diester.

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ODNs must flow through the liver. *In vivo* distribution studies with phosphorothioate ODNs after intravenous application [4–6] revealed that in the liver tissue a significant proportion of the applied ODN dose is retained. However, these studies did not report on the appearance of ODNs in bile. In an isolated perfused rat liver model, neither phosphorothioate nor methylphosphonate ODNs were found in bile [7]. Recently in a preceding study we observed a small proportion of radiolabeled [^{35}S]-mixed backbone phosphorothioate/phosphorodiester ODNs in rat bile, when the ODNs were applied *in situ* in a mesenterial vein [8]. It was observed that a 15 mer ODN which was coupled to one bile acid molecule per ODN chain (1BA-ODN) eliminated with a slightly greater efficiency into bile than the normal uncoupled 15 mer ODN. We were, therefore, interested to determine the molecular excretion route of ODNs in general and of bile acid-conjugates ODNs in particular into bile. Therefore, we have studied the hepatobiliary elimination of stabile mixed backbone phosphorothioate/phosphodiester ODNs in bile duct-fistulated rats *in situ*.

At the canalicular pole of hepatocytes cholephilic compounds are known to be secreted into bile by means of transport ATPases, which belong to the family of ABC-cassette proteins [9–11]. In particular, organic hydrophilic anions, which are mostly glucuronic acid conjugates, sulfates or glutathione conjugates of endo- and xenobiotics, are substrates of the mrp2 [12] and/or the bile salt export pump (bsep) [13,27]. With respect to mrp2, the *in situ* excretion of ODNs in the naturally occurring mrp2 knock-out rat, the TR$^-$ Wistar rat, was compared with normal Wistar rats. By means of a cholic acid-double conjugate of a 15 mer ODN (2BA-ODN) and of competition experiments with substrates of ABC-cassette carriers the participation of canalicular export pumps such as the mrp2 and bsep was investigated.

2. Materials and methods

2.1. Animals

Male Wistar rats and TR$^-$ rats were inbred and kept in our lab according to governmental rules. These were used at an age of about 3 months (250–300 g b.wt.). The animal experiments were registered and approved from the local administration. *Xenopus laevis* frogs were a gift from Universitätsklinikum Eppendorf, Hamburg.

2.2. Materials

Oligonucleotides were obtained from Tib MolBiol and Biospring. The sequence used was GsGsCs TGCs CAsT GGTs CsCsC where "s" stands for phosphorothioate derivatisation.

Synthesis and purification of bile acid-oligonucleotide conjugates are described in detail elsewhere [14]. In short, amino-modified cholic acid methylester was treated with succinic anhydride to form a bile acid monoamide with a reactive 3′-carboxylic group. After activation, the bile acid derivative was coupled to amino-linked ODNs via amide binding forming a one-bile acid conjugate (1BA-ODN) with cholic acid at the 3′ end or a two-bile acid conjugate (2BA-ODN) with cholic acid at the 3′ and 5′ end of the ODN. As a control, the unconjugated oligonucleotide was used (*n*-ODN). Radioactive labeling of the 5′ end was performed by T4-polynucleotide kinase (MBI Fermentas) with [γ^{35}S]-adenosine triphosphate (ICN). Purification of both unmodified or modified ODNs was performed by polyacrylamide (PAA) gel electrophoresis (20% acrylamide, 2% bisacrylamide, 7 M urea) and analysed by UV-imaging on a TLC-plate (Merck) at 254 nm with subsequent excision and elution of the ODN band.

Sulfobromophthalein (BSP) was from Sigma, S 3025 (1-[2-(4-chloro-phenyl)-cyclopropylmethoxy]-3,4-dihydroxy-5-(3-imidazo-[4,5-*b*]pyridine-1-3-yl-3-[4-carboxy]-phenyl-acryloyloxy)-cyclohexancarboxylic acid) was synthesised according to Ref. [15], 1-chloro-2,4-dinitrobenzene was from ICN and MK 571 was from Alexis. TC-monosodium salt was purchased from Sigma and [^{3}H]TC (74 GBq/mmol) from Amersham. Five hundred microliters injection solutions of [^{3}H]TC in physiological NaCl solution contained 150 MBq/mmol.

2.3. In situ liver perfusion

Male Wistar rats and Wistar TR$^-$ rats were anaesthetised by intraperitoneal application of 1.5 g/kg urethane as a 20% aqueous solution. For the whole excretion time, rats were placed in a temperature-controlled hood. After laparotomy, the bile duct was catheterised by a teflon tube size 0.3 mm × 0.6 mm inner/outer diameter (Bohlender) fixed with fibrin adhesive (Histoacryl®, B. Braun). ODNs, cholic acid-ODN conjugates and other named compounds were injected as a bolus into a mesenteric vein if not described otherwise. Excreted bile fluid was collected in fractions over a time period of 120 min as depicted. Radioactive labeled ODNs as well as [^{3}H]taurocholate in bile were quantified by a Wallac 1409 liquid scintillation counter.

As a stability test of the ODNs in bile, 10 nmol 2BA-ODN or 200 nmol *n*-ODN was injected. The bile fluid was extracted with phenol:chloroform:isoamylalcohol 50:49:1 (Rotiphenol, Roth) and the ODN-containing upper aqueous phase was run on a PAA gel and visualised as described above.

Hitherto the specificity of chlorogenic acid derivative S 3025 as a substrate of oatp1/mrp2 is not completely clear. Thus, we investigated the influence of S 3025 on hepatobiliary excretion of oatp1/mrp2 substrate BSP and Ntcp/bsep substrate TC. Application of 5 μmol S 3025, dissolved in 1 mL DMSO/glycerol/PBS buffer 15:15:70 (v/v/v) into a femoral vein was followed by injection of 1 or 5 μmol

BSP, dissolved in 500 μL PBS into the second femoral vein of male Wistar rats and 5 μmol BSP was applied in the same manner to TR^- rats. BSP in bile was detected and quantified by a Beckman DU 640 spectrometer at $\lambda = 580$ nm wavelength. BSP calibration curves were obtained by measuring mixtures of 10 μL of the respective aqueous BSP solution, 20 μL bile (BSP-free) and 470 μL 0.05 M NaOH. The mixture was measured photometrically as described above. For determination of BSP excretion in bile, 3–20 μL of the BSP-containing bile sample were diluted to 500 μL with 0.05 M NaOH and equally measured.

Experiments with TC were carried out as follows: at $t = 0$ min, Wistar rats received 1 resp. 5 μmol and TR^- rats 5 μmol of the [^{3}H]TC solution into a mesenteric vein. Application of 5 μmol S 3025, dissolved in 1 mL DMSO/glycerol/PBS buffer 15:15:70 (v/v/v), in a femoral vein followed at $t = 24$ min. Six minutes later ($t = 30$ min) injection of the above given TC doses into a mesenteric vein was repeated.

In order to elucidate the possible role of mrp2 in hepatobiliary elimination of ODNs, mrp2 substrates BSP and S 3025 were injected into a femoral vein 2 min before application of the respective ODN. Injection solutions consisted of 5 resp. 15 μmol BSP, dissolved in 500 μL PBS buffer, or 5 resp. 15 μmol S 3025 in 1 mL DMSO/glycerol/PBS buffer 15:15:70 (v/v/v). Both dosages of these mrp2 substrates were administered to Wistar rats, whereas TR^- rats received the lower of the given dosages only in order to avoid toxicological symptoms in these rats.

2.4. Antisense properties of bile acid-conjugated oligodeoxynucleotides as tested in expression experiments with X. laevis oocytes

In order to test the antisense properties of 2BA-ODNs, two 20 mer antisense ODNs, namely a pure phosphodiester 20 mer antisense ODN (20 mer AS-*n*-ODN-DE, DE stands for diester) and a mixed backbone phosphodiester/phosphorothioate 20 mer antisense ODN (20 mer AS-*n*-ODN) were each coupled as described with two bile acid molecules to yield the corresponding 2BA-ODN (20 mer AS-2BA-ODN-DE and 20 mer AS-2BA-ODN). The sequence of both 20 mer antisense ODNs was taken from [16], who showed that an antisense 20 mer phosphodiester ODN with bases corresponding to the bp 815–834 of the coding region of the Ntcp-carrier protein (Na^+-dependent taurocholate cotransporting polypeptide) suppressed the expression of full length Ntcp-cRNA in *X. laevis* oocytes. Therefore, full length Ntcp-cRNA was incubated at identical conditions as described [16] with nonbile acid and bile acid conjugates 20 mer antisense ODNs for 1 hr and was microinjected into *X. laevis* oocytes. After 2 days of heterologous expression of the Ntcp protein, taurocholate uptake was measured. Control oocytes were injected (i) with H_2O, (ii) with cRNA hybridised with 20 mer sense oligo corresponding to bp 593–612 (according to Ref. [16]) and (iii) with cRNA hybridised with 15 mer scrambled ODN with and without bile acid tags at their 3′ and 5′ ends (see legend to Fig. 9).

2.5. Mathematical regression model

To evaluate excretion curves of BSP and ODNs, cumulative data were fit to a mathematical model and single parameters were used for variance analysis. The function is described as the sum of a *Mitscherlich* function with exponential saturation and a linear term describing a constant elimination after the initial exponential period.

For mathematical description of the excretion curves of TC, cumulative data were fit by a combined model consisting of two *Mitscherlich* functions without a linear part.

After estimation of the parameters of the model, their values were compared between the different conditions of the experiments by one- and two-way ANOVA. The statistical analysis was carried out with the statistical program package BMDP [17].

3. Results

3.1. ODN excretion in bile by normal Wistar rats

Unmodified (*n*-ODN) and cholate-conjugated ODNs (1BA-ODN or 2BA-ODN) were applied into a mesenteric vein of anaesthetised normal Wistar rats and their appearance in bile was monitored over a period of 2 hr. Samples of bile, collected after 30 and 60 min, contained radioactive ODNs which were detected by autoradiography in a polyacrylamide gel (Fig. 1). By comparison of those bile

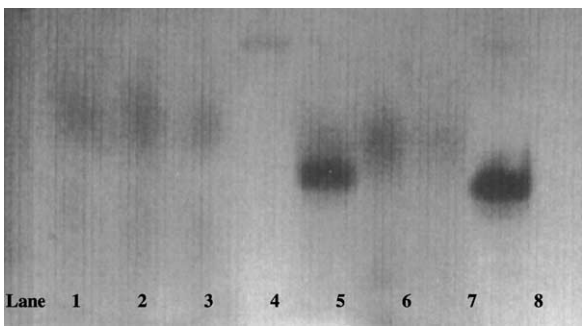

Fig. 1. *In vivo* stability of ODNs in bile. Bile was collected by a teflon catheter from *in situ*-perfused rats which received 200 nmol *n*-ODN (15 mer mixed backbone phosphodiester/phosphorothioate ODN) or 10 nmol 2BA-ODN (*n*-ODN conjugated at 3′ and 5′ ends with one molecule cholic acid each) into a mesenteric vein. Shown is a PAGE-separation of bile sampled over 30 or 60 min. As a control, prebile was spiked with the appropriate ODNs (lanes 1 and 5). Pure ODNs (in solvent) are run on lanes 4 and 8. All samples (including lanes 4 and 8) were subjected to the extraction procedure for bile samples described in Section 2. Lane 1: prebile spiked with 0.375 nmol 2BA-ODN. Lane 2: 30 min bile sample containing 2BA-ODN. Lane 3: 60 min bile sample containing 2BA-ODN. Lane 4: 0.5 nmol 2BA-ODN. Lane 5: prebile spiked with 7.5 nmol *n*-ODN. Lane 6: 30 min bile sample containing *n*-ODN. Lane 7: 60 min bile sample containing *n*-ODN. Lane 8: 5 nmol *n*-ODN.

sample ODNs with the injected ODNs or with a bile sample spiked with the injected ODNs (internal standard probe) identical runs were observed for 2BA-ODN, indicating the appearance of intact 2BA-ODN in bile *in vivo*. In bile samples containing *n*-ODN, the runs were slightly different but may indicate hairpin aggregation of *n*-ODNs (the dark spots running ahead) when applied in high concentration onto the gel. No further chemical analysis was applied due to the small absolute ODN amount in collected bile samples.

Distinct biliary excretion profiles occurred. Whereas only a low amount of *n*-ODN was excreted over the 2-hr time period, showing a concentration peak at about 8 min, increasing absolute amounts (1.7-fold) and peak concentration (2.5-fold) were observed when applying the 1BA-ODN in which the ODN was coupled to one cholate molecule. If an ODN was coupled with 2 bile acid molecules (2BA-ODN) and was injected intravenously, the hepatobiliary excretion was markedly increased: total amount of biliary excretion was 5-fold and peak concentration 9-fold of that of *n*-ODN (Fig. 2).

The excreted total amounts of the applied ODNs were constant relative to the applied doses and are summarised in Table 1.

Table 1
Percentage of ODN excretion relative to the applied amount (mean ± SEM) 2 hr after application of three ODN derivatives (*n*-ODN, 1BA-ODN, 2BA-ODN) to normal Wistar rats

Substrate	Excreted amount in percent of applied dose per rat		
	0.4 nmol applied	0.8 nmol applied	4 nmol applied
n-ODN	5.0 ± 0.5	4.7 ± 0.4	4.5 ± 0.7
1BA-ODN	9.8 ± 1.3	7.9 ± 0.6	7.2 ± 1.1
2BA-ODN	23.6 ± 6.7	23.3 ± 1.0	25.3 ± 1.7

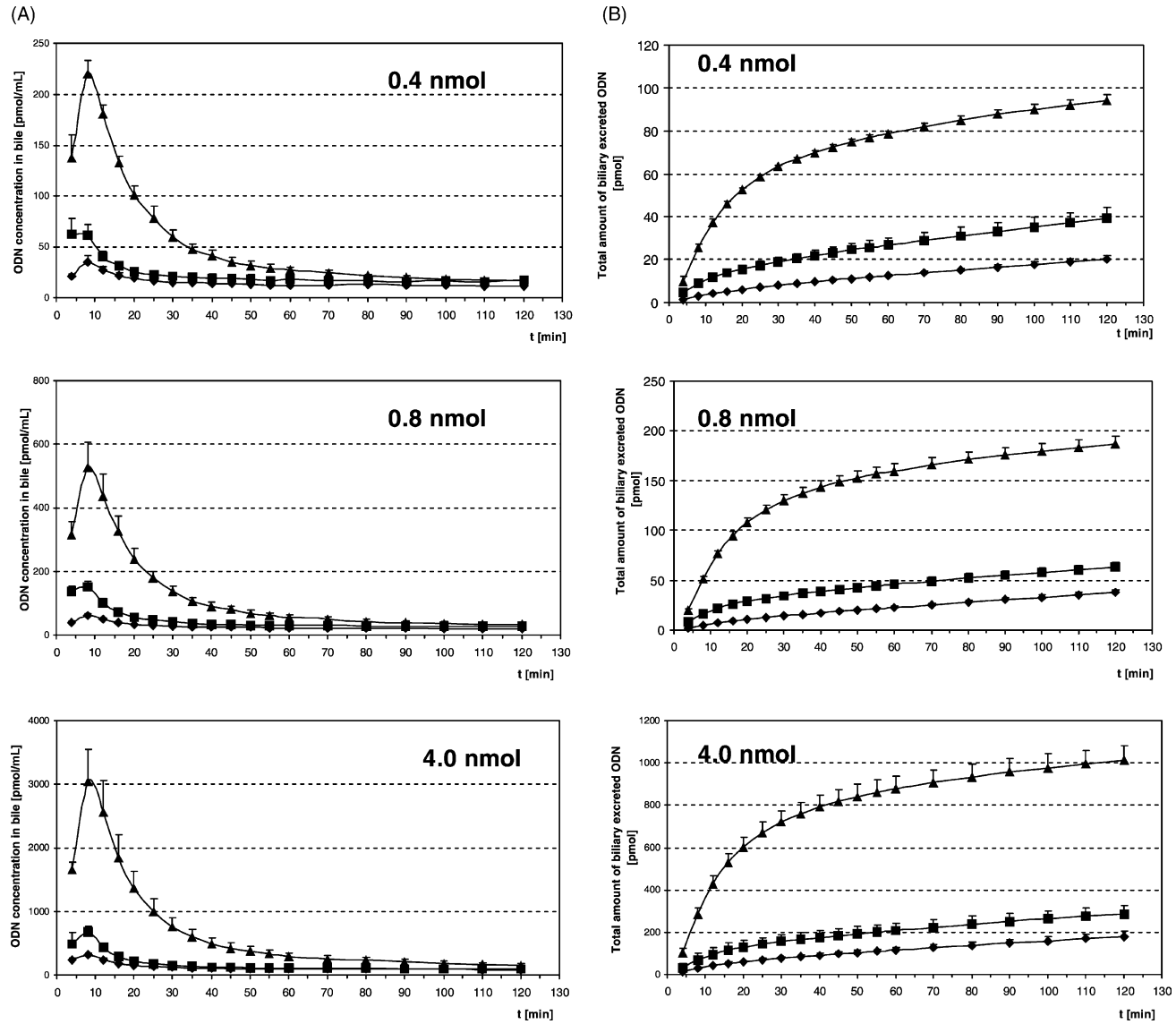

Fig. 2. Dose-dependent excretion of labeled ODNs in bile from *in situ*-perfused rats: enhancement of excretion by coupling of *n*-ODN with cholic acid. 0.4, 0.8, and 4 nmol of *n*-ODN (◆), 1BA-ODN (■), or 2BA-ODN (▲) were administered intravenously to Wistar rats. The graphs depict the ODN concentrations in each bile sample (fractionated curves, A) and the amount (cumulated curves, B) of their excretion into bile.

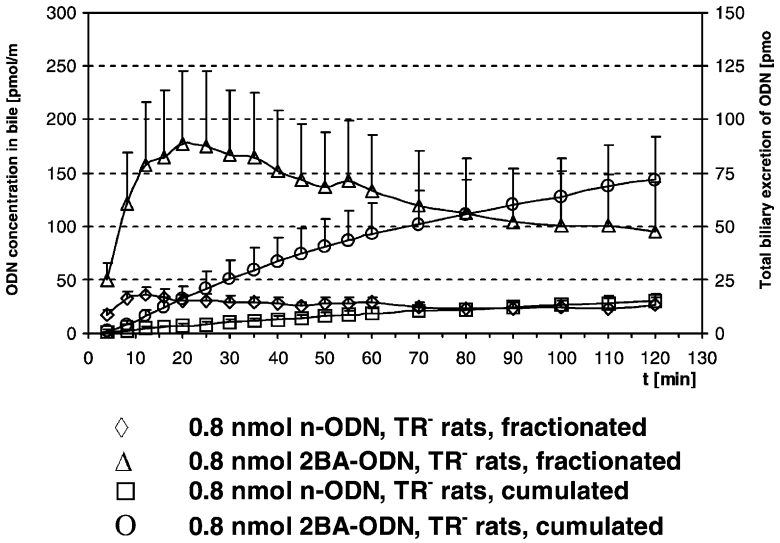

Fig. 3. Alteration of excretion into bile of *n*-ODN and 2BA-ODN by TR$^-$ rats after injection of 0.8 nmol *n*-ODN or 0.8 nmol 2BA-ODN into a mesenterial vein. The graphs with symbols ◇ (*n*-ODN) and △ (2BA-ODN) represent bile concentration curves (fractionated determinations); symbols □ (*n*-ODN) and ○ (2BA-ODN) represent total amount of biliary secreted ODNs (cumulated calculation).

3.2. ODN excretion in bile by TR$^-$ rats

For comparison with normal Wistar rats, *n*-ODN and 2BA-ODN were applied at 0.8 nmol dose to TR$^-$ rats and the excretion profiles in bile were determined. The TR$^-$ rat is a mutant Wistar rat strain which lacks the functional mrp2 at the hepatocyte canalicular membrane [18]. Mrp2 is responsible for the active ATP-dependent extrusion of various endogenous and exogenous anionic substrates, in particular glucuronidated, sulfated and glutathione-conjugated xenobiotics and was formerly named cmoat, canalicular multispecific organic anion transporter [12,19]. Compared with normal Wistar rats, the elimination profiles of all ODNs were altered in TR$^-$ rats. With *n*-ODN, the

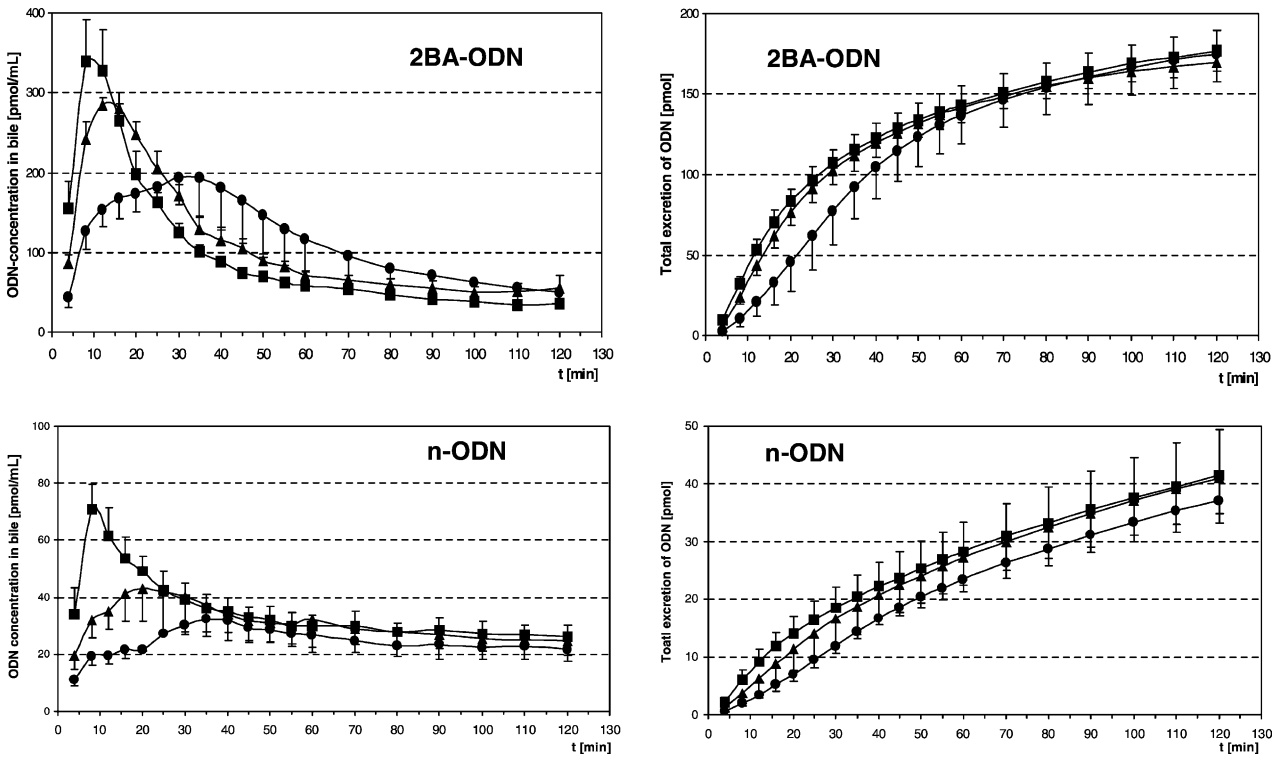

Fig. 4. Inhibition of biliary ODN excretion in rats by bromosulfophthalein (BSP). BSP (5 μmol (▲) and 15 μmol (●) dissolved in 500 μL PBS) was applied to Wistar rats intravenously prior to 0.8 nmol 2BA-ODN (upper panels) or 0.8 nmol *n*-ODN (lower panels) injection. Control rats received 500 μL solvent (PBS-buffer) (■). Left side: ODN concentrations in bile (fractionated determinations); right side: amount of excreted ODN in bile (cumulated calculation).

initial 8-min peak is absent, the concentration level is much lower and the absolute excreted amount over the indicated time period is diminished at about half to one-third (Fig. 3).

3.3. Role of mrp2 for biliary oligonucleotide excretion

The results obtained with TR^- rats indicate a role of mrp2 in biliary ODN excretion. To evaluate ODN excretion by mrp2, substrates of this canalicular transporter such as sulfobromophthalein (BSP) [12], S 3025 [20], 1-chloro-2,4-dinitrobenzene [21,22] and MK 571 [23] were applied concomitantly to normal Wistar rats and TR^- rats in order to show interference of excretion.

In normal Wistar rats, the biliary excretion of 2BA-ODN was markedly delayed during BSP excretion but the total amount of 2BA-ODN excretion was not reduced within 2 hr. This was reflected by much lower bile concentration in the early excretion phase and smaller initial slopes of the 2BA-ODN excretion curves generating a S-shaped excretion profile instead of a hyperbolic curve. It appears that as long as BSP was present at the canalicular membrane ODN excretion was inhibited but the more BSP was proportionally excreted into bile in parallel, the weaker was its blockade. After vanishing from the cytosol the intracellular BSP blockade disappeared, 2BA-ODN excretion recovered and reached identical plateaus as before (Fig. 4). Although it was not within the scope of these experiments to outline the type of inhibition precisely, this transient inhibition more likely indicated a direct transport competition rather than any indirect interaction such as functional modifications (e.g. ATP-depletion or protein destruction). When *n*-ODN excretion was measured in normal Wistar rats, in principle similar BSP effects were observed (Fig. 5). In TR^- rats, albeit ODN excretion was already diminished, BSP (5 μmol) even further reduced the total amount of 2BA-ODN for about one-third and also of *n*-ODN excretion (not shown), indicating that non-mrp2-mediated excretion is also sensitive to BSP.

The most potent inhibition of ODN-excretion into bile was achieved with S 3025. This compound exerted the strongest inhibitory effect on bile excretion of 2BA-ODN and *n*-ODN, particularly in normal Wistar rats. When applied at a dose of 15 μmol per animal (9.72 mg), S 3025 abolished the excretion peaks of both kinds of ODNs

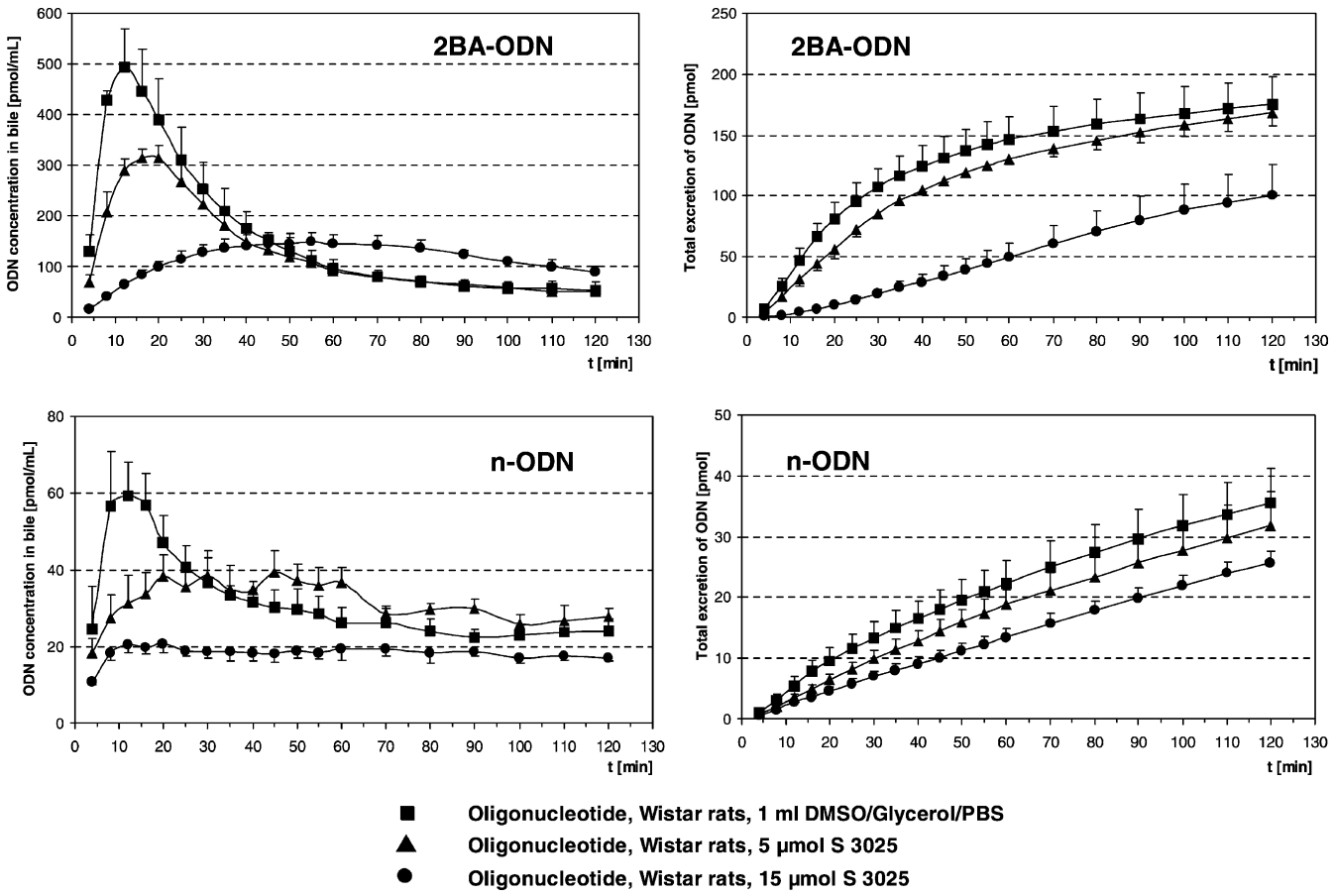

Fig. 5. Inhibition of biliary ODN excretion in rats by S 3025. S 3025 (5 μmol (▲) and 15 μmol (●) in 1 mL DMSO/glycerol/PBS 15:15:70 (v/v/v)) was applied to Wistar rats intravenously prior to 0.8 nmol 2BA-ODN (upper panels) or 0.8 nmol *n*-ODN (lower panels) injection. Control rats received 1 mL DMSO/glycerol/PBS 15:15:70 (v/v/v) (■) prior to ODN injection. Left side: ODN concentrations in bile (fractionated determinations); right side: amount of excreted ODN in bile (cumulated calculation).

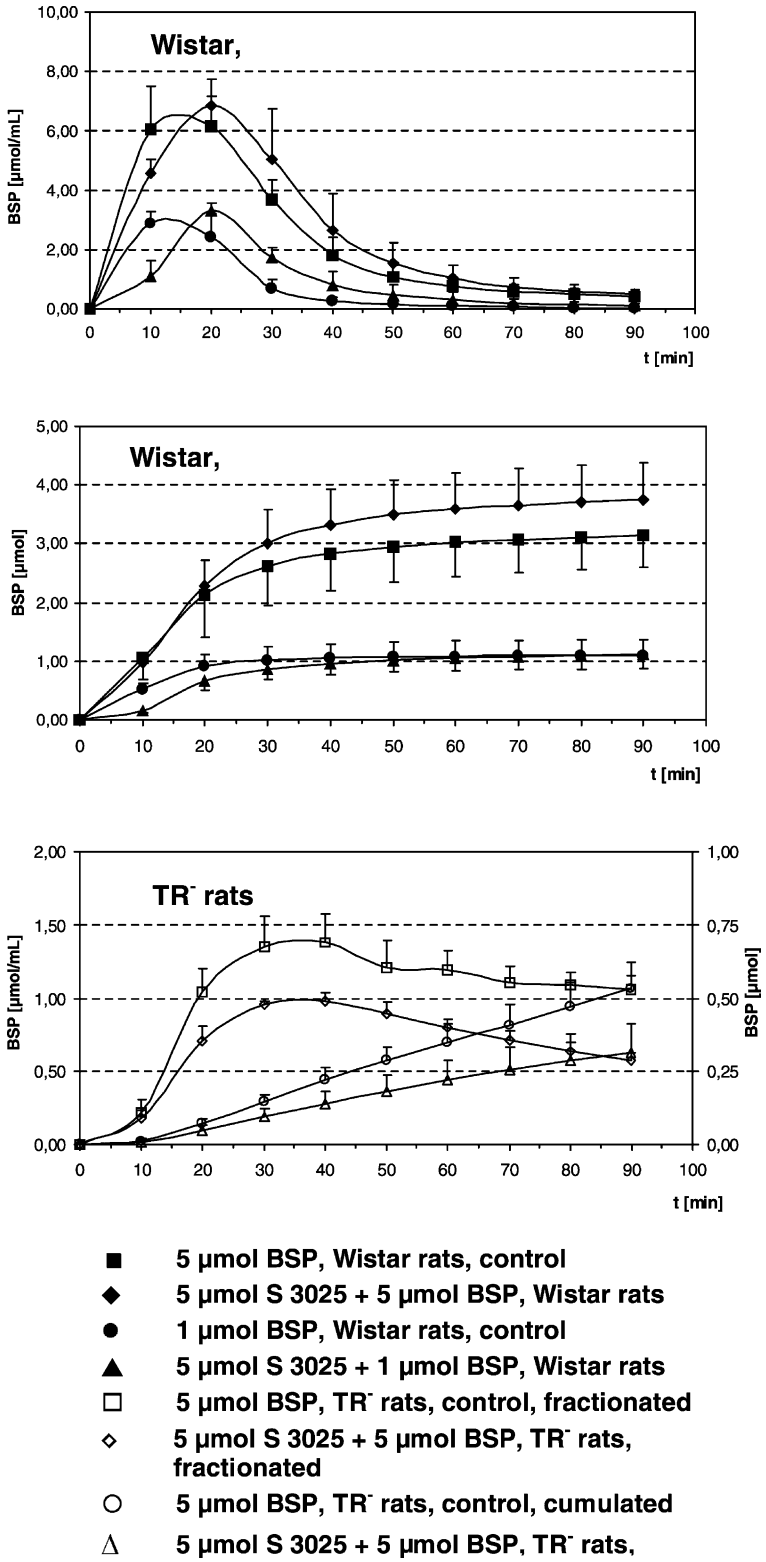

Fig. 6. Inhibition of BSP excretion into bile by S3025. S 3025, 5 µmol dissolved in 1 mL DMSO/glycerol/PBS 15:15:70 (v/v/v), was applied to Wistar rats intravenously by bolus injection prior to bolus injection of 1 µmol (▲) or 5 µmol (◆) BSP; TR$^-$ rats merely obtained the higher BSP dose (□ fractionated excretion, Δ cumulated calculation). Control Wistar rats received 1 (●) or 5 µmol (■) BSP, and 5 µmol BSP was applied to Wistar TR$^-$ control rats (□ fractionated excretion, ○ cumulated calculation). BSP excretion is delayed and the total amount is reduced under S 3025. Upper panel: Wistar rats, bile concentrations (fractionated excretion); middle panel: Wistar rats, cumulated amount of BSP in bile during excretion (cumulated calculation); lower panel: TR$^-$ rats.

(Fig. 5). S 3025 was reported to be secreted into bile only by normal Wistar rats and almost not at all into bile of TR$^-$ rats [20], indicating that this compound is also a substrate of the mrp2 carrier. Because this compound has been introduced only recently but was so effective as an inhibitor of ODN excretion, it seemed necessary to determine whether it interferes specifically with mrp2. For that purpose, S 3025 was infused shortly before application of either radiolabeled taurocholate (as a substrate of the bsep) or bromosulfophthalein (as a substrate of mrp2) to normal Wistar rats from which bile was collected. S 3025 significantly inhibited BSP excretion (Fig. 6) without inhibiting the excretion of [^{3}H]taurocholate (Fig. 7).

Other organic anions regarded to be substrates of the mrp2 carrier, namely 1-chloro-2,4-dinitrobenzene (after glutathione conjugation) and MK 571, were used at com-

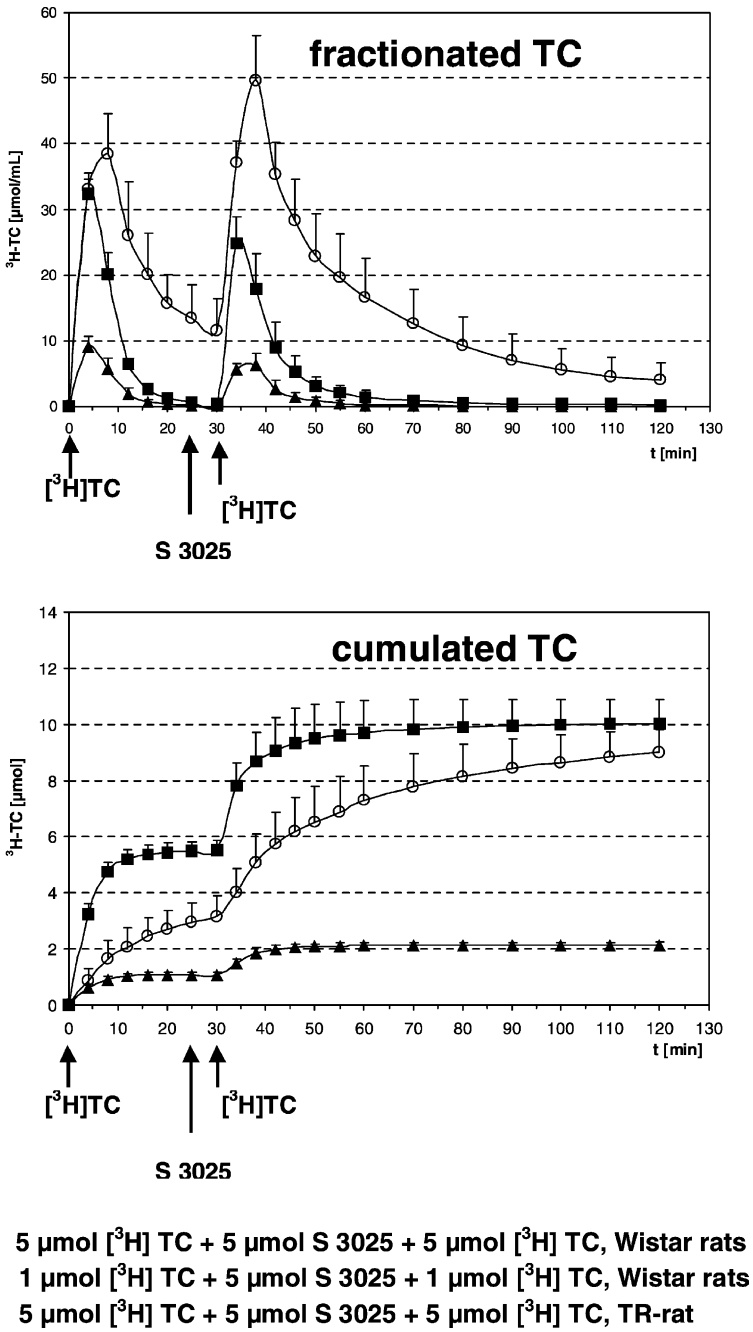

Fig. 7. Lack of inhibition of biliary [^{3}H]taurocholate excretion by S 3025. Normal Wistar rats as well as Wistar TR$^-$ rats were challenged at $t = 0$ min with [^{3}H]TC, 1 (▲) or 5 µmol (■) [^{3}H]TC, which was applied intravenously as a functional test for the hepatobiliary excretion capacity. Wistar TR$^-$ rats merely received the higher [^{3}H]TC dose (○). At $t = 24$ min, when the excretion of the first applied [^{3}H]TC was almost at its end, the same animal received 5 µmol S 3025, dissolved in 1 mL DMSO/glycerol/PBS 15:15:70 (v/v/v), and 6 min later, at $t = 30$ min, a second dose of either 1 (same symbol) or 5 µmol (same symbol) [^{3}H]TC intravenously. Upper panel: taurocholate concentrations in bile of normal Wistar rats and TR$^-$ rats (fractionated excretion); lower panel: cumulated amount of [^{3}H]TC in bile.

parable doses (up to 5 μmol) for inhibition as well, but were not or only weakly effective as inhibitors of biliary ODN excretion (data not shown). Whereas normal Wistar rats tolerated doses up to 5 μmol of MK 571, CDNB was very toxic for TR$^-$ rats and animals died at slightly higher dosage.

3.4. Tissue distribution of n-ODN and 2BA-ODN in normal Wistar and TR$^-$ rats

Because we discovered a deficiency of hepatobiliary ODN excretion in TR$^-$ rat, the hepatic and extrahepatic organ distribution was determined at the end of a perfusion experiment. Fig. 8 shows the distribution pattern between liver and kidney, serum and bile. In TR$^-$ rats, the residual content of *n*-ODN and 2BA-ODN in the liver was higher compared with liver content in normal Wistar rats (2BA-ODN: 11.23% in the liver of TR$^-$ rat vs. 8.3% in the liver normal Wistar rat; *n*-ODN: 7.61% TR$^-$ rat liver vs. 4.05% normal Wistar rat liver) (Fig. 8). The results are in line with the clearance function of the hepatic mrp2 for biliary ODN excretion. They also suggest that hepatic ODN uptake into the livers of TR$^-$ rats was probably not altered since their livers still retained significantly higher amounts of *n*-ODN and 2BA-ODN than livers from normal Wistar rats, even 2 hr after injection. They also indicate that sinusoidal reflux of ODNs back into blood could not (fully) compensate for the disturbed canalicular excretion of ODNs in these rats.

3.5. Antisense properties of 2BA-ODNs

Coupling of oligodeoxynucleotides with cholic acid at the 3′ and 5′ end of the nucleotide chain did not alter specific antisense properties of the ODN. This was investigated with a pure phosphodiester 20 mer antisense ODN which was reported to abolish the expression of the rat sodium-dependent taurocholate cotransporting polypeptide Ntcp in *X. laevis* oocytes [16]. When this antisense 20 mer ODN was coupled with cholic acid in the same way as described above for the 15 mer ODN, and was incubated together with Ntcp-cRNA, the expression of Ntcp-cRNA in *X. laevis* oocytes was completely suppressed (Fig. 9). The same antisense properties were achieved if instead of a pure phosphodiester ODN a 20 mer antisense mixed backbone phosphodiester/phosphorothioate (20 mer AS-2BA-ODN) was applied for specific hybridisation with Ntcp-cRNA (Fig. 9).

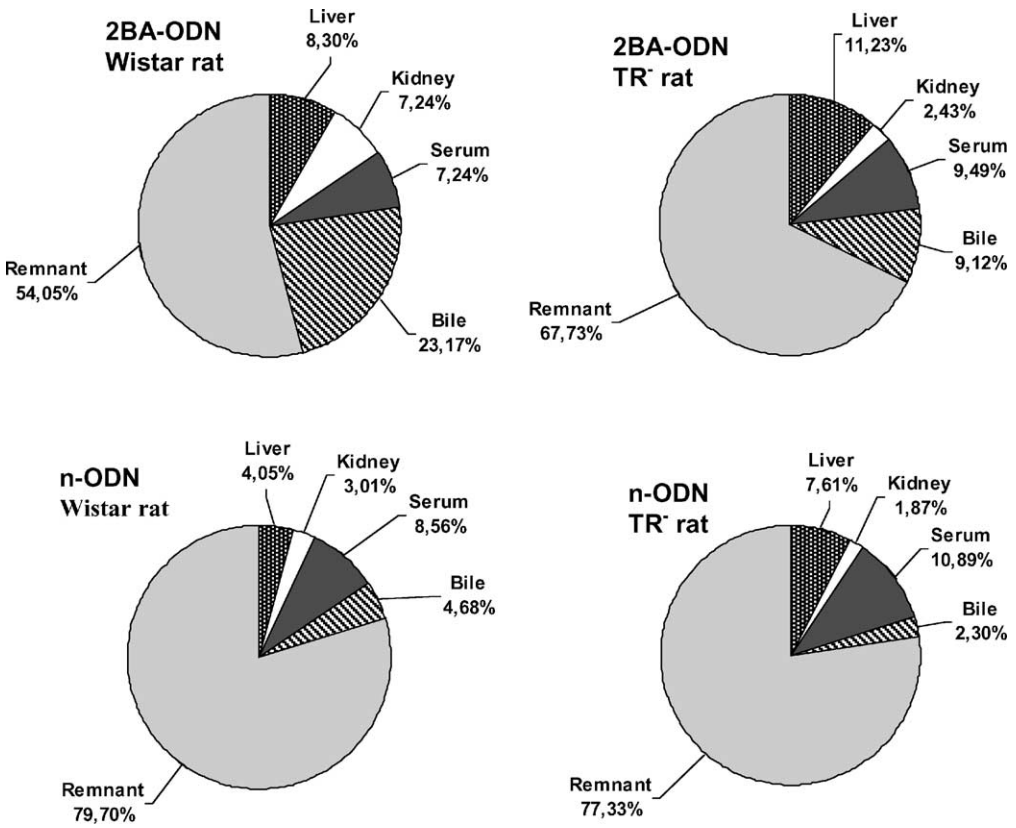

Fig. 8. Liver content of *n*-ODN and 2BA-ODN in Wistar and TR$^-$ rats. 0.8 nmol 2BA-ODN or *n*-ODN were injected into a mesenteric vein of Wistar or TR$^-$ rats. Bile was collected for a period of 120 min. Animals were killed and samples of serum, liver and kidney were analysed for their content of the respective ODN. The distribution of *n*-ODN and 2BA-ODN in liver and kidney, serum and bile of Wistar and TR$^-$ rats 2 hr after oligodeoxynucleotide injection are shown.

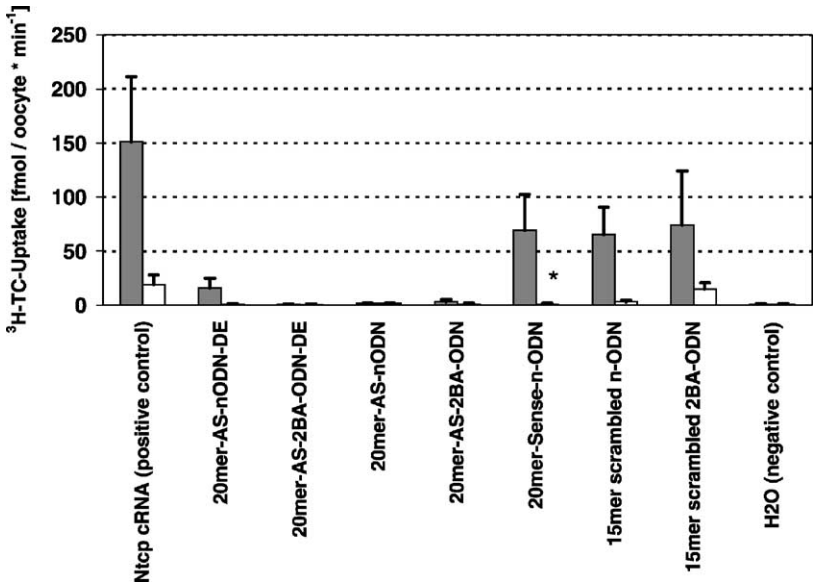

Fig. 9. Antisense properties of a bile acid-conjugated 20 mer phosphodiester ODN. The 20 mer antisense phosphodiester ODN (20 mer-AS-*n*-ODN-DE, DE for diester) corresponding to bp 815–834 of the Ntcp sequence was coupled as described at the 3′ and 5′ end with cholic acid yielding 20 mer-AS-2BA-ODN-DE. The corresponding mixed backbone 20 mer antisense ODN (20 mer-AS-*n*-ODN) was transferred to its 3′,5′-cholic acid conjugate as well (20 mer-AS-2BA-ODN). Full length Ntcp-cRNA was incubated for 1 hr in the presence of the unconjugated 20 mer antisense ODN (20 mer-AS-*n*-ODN-DE), the 2BA-20 mer antisense ODN (20 mer-AS-2BA-ODN-DE) and their corresponding mixed backbone orthologues 20 mer-AS-*n*-ODN and 20 mer-AS-2BA-ODN, and a 2BA-sense 20 mer phosphodiester (20 mer-sense-*n*-ODN), representing the bp 593–612 of the Ntcp sequence according to [16]. Thereafter individual oocytes were injected with 2.5 ng (50 nL)/oocyte of hybridised or nonhybridised full length Ntcp-cRNA and incubated for 2 days. TC uptake in oocytes was measured by incubating groups of 7-17 oocytes with 100 μL of a 10 μM [^{3}H]TC solution (0.37–1.11 MBq/100 μL). As a control, oocytes were injected with full length Ntcp-cRNA (positive control) and with water (H_2O, negative control). Furthermore, the 15 mer scrambled ODN and its corresponding 3′,5′-cholic acid conjugate (15 mer scrambled 2BA-ODN) were hybridised with Ntcp-cRNA as described above. These scrambled ODNs were identical with *n*-ODN resp. 2BA-ODN in the experiments shown in Figs. 1–8. Dark grey columns: uptake in the presence of sodium ions; light grey columns: uptake in the presence of choline chloride. *Not analysable.

4. Discussion

In a previous paper, we described the appearance of mixed backbone phosphorothioate/phosphodiester ODNs in rat bile after their intravenous application for the first time [8]. The aim of the present study was to determine the excretory hepatobiliary pathway of oligodeoxynucleotides on a molecular level in live anaesthetised rats. We, therefore, compared ODN contents in bile samples collected from two different rat strains, i.e. normal Wistar rats vs. TR^- rats, the latter of which lack the mrp2 transporter. This study showed that: (i) Mixed backbone phosphorothioate/phosphodiester ODNs are rapidly excreted into bile after intravenous bolus injection, and significant degradation did not occur. About 5–25% oft the injected ODNs are excreted into bile, depending on the kind of ODN applied. (ii) The 2BA-ODN is excreted 5 times more effectively into bile than the normal ODN with doses of 0.4–4 nmol/animal, rendering 2BA-ODNs a promising approach for liver drug-targeting. (iii) The major excretory pathway for normal and 2BA-ODNs is the mrp2 system at the canalicular membrane of hepatocytes. Excretion of ODNs is inhibited by other mrp2 substrates. (iiii) Since TR^- rats excrete normal ODN and bile acid-conjugated ODN into bile, the additional participation of transport ATPases other than mrp2 is required for their hepatobiliary elimination, however at a much lower level. Finally, although tagging the nucleotide chain with cholic acid significantly modifies the hepatobiliary elimination pathway of ODNs, it does not alter their antisense properties.

Current knowledge of bile formation and in particular of the excretion of endo- and xenobiotics into bile suggests the canalicular pole of hepatocytes to be the rate limiting step of excretion. At the canalicular membrane, several primary active ATP-consuming pumps (ATPases) belonging to the ABC-cassette carrier proteins maintain hepatobiliary transport into bile [42]. There is some overlap in the substrate pattern of these carriers, among which the best characterised are mrp2, bsep and multidrug resistance protein 1 (mdr1). Mrp2 represents the most important pump for the canalicular excretion of organic anions. Other mrp-ATPases such as mrp 3, 4, 5, and 6 and hitherto undiscovered pumps contribute to the excretion properties of hepatocytes [41]. These ATPases mainly compensate for impaired excretion under cholestatic conditions, e.g. in the absence or dysfunction of mrp2. Taking into consideration that said mrps are located at the lateral or sinusoidal membranes of hepatocytes, excreting their substrates back into blood [22], the present interpretation of our results focuses on mrp2 and bsep: both of these transport anionic organic compounds across the canalicular membrane [24], and ODNs carry negative charges due to the phosphate residues. The mdr1

was not considered to participate in ODN excretion since this carrier is not deficient in TR$^-$ rats and preferentially transports cationic or neutral compounds.

Bile collected from TR$^-$ rats was not ODN deficient. Since dysfunctions of other canalicular ABC-carrier proteins besides mrp2 have not been reported for this rat strain [25,26], we assume that under these conditions bsep could become an alternative secretory pathway. Bsep most specifically transports bile acids [27,28], but also transports taxol [29] and vinblastine [30]. The significantly more efficient excretion of bile acid-conjugated ODNs in bile of normal as well as of TR$^-$ rats gives further credit to a participation of bsep. In TR$^-$ rats, the excretion of the former was even more efficient than the excretion of normal ODNs in normal Wistar rats. These findings represent another important argument for an additional pump with specific affinity for bile acids and which is capable of functioning in the absence of mrp2. The excretion kinetics of this pump are quite different from those of mrp2-dependent ODN excretion. The TR$^-$ rat model lacked a sharp secretory peak maximum which so typically appeared a few minutes after application in normal Wistar rats. In addition, overall excretion was much lower.

ODN excretion into bile was inhibited *in vivo* by several but not all tested mrp2 substrates. In transport studies with membrane vesicles, MK 571 [18,23] in micromolar and CDNB [22] in nanomolar concentrations clearly inhibit mrp2 *in vitro*. To our knowledge, there is no data available concerning the properties of MK 571 *in vivo*. *In vivo*, CDNB was highly toxic expecially in TR$^-$ rats and, as far as we know, no other report has been published demonstrating CDNB-induced mrp2 inhibition *in vivo*. Even at the highest tolerated doses of 20 μmol/kg b.wt., no inhibition of ODN excretion was observed. On the other hand, mrp2 substrates BSP and S 3025 markedly reduced ODN excretion under comparable dosage (20 and 60 μmol/kg b.wt.). ODN concentrations in bile were lower and the excretion curve was shifted to the right, indicating transient inhibition lasting as long as the inhibitor was present at the canalicular membrane. Hyperbolic cumulative excretion curves—calculated in the absence of an inhibitor—changed into S-shaped excretion curves under these conditions.

There is considerable debate regarding the question of how ODNs enter cells, and in particular by what process they cross phospholipid cell membranes. Vlassow *et al.* [31] excluded a diffusion process for all kind of ODNs and suggested fluid phase endocytosis. The influence of asialoglycoprotein-targeted DNA [32] or glycosylated poly-L-lysine complexed ODNs [33] on liver cells receptor-mediated endocytosis have been reported as well [31]. It was suggested that unmodified phosphorothioate ODNs at high doses are endocytosed by means of scavenger receptor internalisation, mainly by endothelial liver cells [34]. This uptake was inhibited by dextran sulphate. Unlike the results supporting the aforementioned suggestion, no change in the distribution kinetics of ODNs was observed in a scavenger receptor knock-out mice model [35]. In our perfusion experiments, dextran sulphate did not change the hepatobiliary excretion of *n*-ODNs (data not shown). Thus, an uptake of ODNs into hepatocytes via scavenger receptor-mediated internalisation appears unlikely. On the other hand, we could not characterise a liver transporter in the isolated rat hepatocyte model because only minor amounts (less than 0.5%) of normal as well as bile acid-conjugated ODNs were taken up from a suspension of isolated hepatocytes within 90 min. In this experimental model, the low level uptake of ODNs was not inhibited by other organic anions. All results considered, the mechanisms and pathways involved in the entry of these polyanions into isolated hepatocytes remain unclear. Our *in situ* animal experiments, however, showed that significant amounts of all ODNs tested appeared in bile within a few minutes, this rapid excretion following transcellular passage of ODNs through hepatocytes in the intact liver. The apparent lack of uptake into isolated hepatocytes as well as into nonliver cells which we and others have observed [36–40] might indicate a very rapid excretion back into the suspension medium, mediated by ABC-cassette pumps. Such an interpretation would mimic the excretion of chemotherapeuticals via P-glycoproteins resulting in a multidrug resistance of tumour cells against these substances.

Bile acid conjugation of ODNs at both the 3′ and the 5′ end resulted in a higher cellular clearance by the liver in comparison to the unconjugated ODN derivatives. The calculated amounts of this head-and-tail tagged ODN in bile of TR$^-$ rats was even higher than the amount of normal ODNs in bile of normal Wistar rats. Whether these evasion kinetics are disadvantageous for therapeutic purposes or not awaits further proof. Because of its nucleotide sequence [14] the 15 mer ODN chosen in the present study was not expected to hybridise significantly with rat RNAs. Nevertheless, small amounts of all ODNs tested were detected in the liver after 2 hr, with higher cell concentrations of 2BA-ODNs than of normal ODNs. Despite their much better hepatobiliary elimination, the 2BA-ODNs reach higher intracellular concentrations and are presumably more suitable tools for liver targeted ODN antisense therapy than normal ODNs. Such an approach requires that neither bile acid tags nor backbone modifications disturb hybridisation of antisense ODNs with their target mRNA. We clearly demonstrated that neither a 3′,5′-bile acid conjugation nor a backbone modification (i.e. mixed phosphorothioate-phosphodiester backbone) altered the inhibitory effect of a phosphodiester antisense ODN on the expression of Ntcp-cRNA in *X. laevis* oocytes *in vitro* in comparison to the unconjugated 20 mer antisense ODN (see Fig. 9). Reports concerning cholesterol tagged ODNs [43] are in agreement with our findings. Under certain circumstances, i.e. length of the linker molecules, length of the nucleotide chain, or position of the attached compound, interference by the tags is avoidable. In summary, it does not seem unrealistic to expect therapeutic progress with

bile acid tagged ODNs *in vivo*. This therapeutic approach is of particular interest in viral liver infections (e.g. hepatitis B or C) in which ODNs are considered to improve the therapeutic regimen [44–46].

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