Semisynthetic Bile Acid FXR and TGR5 Agonists: Physicochemical Properties, Pharmacokinetics, and Metabolism in the Rat

Aldo Roda, Roberto Pellicciari, Antimo Gioiello, Flavia Neri, Cecilia Camborata, Daniela Passeri, Francesca De Franco, Silvia Spinozzi, Carolina Colliva, Luciano Adorini, Marco Montagnani, and Rita Aldini

Dipartimento di Chimica “G. Ciamician” Alma Mater Studiorum-University of Bologna, Italy (A.R., C.C.; S.S.); Dipartimento di Chimica e Tecnologia del Farmaco, Università degli Studi di Perugia, Italy (R.P., A.G.); Dipartimento di Scienze Mediche e Chirurgiche, Alma Mater Studiorum-University of Bologna, Italy (F.N., M.M.); TES Pharma S.r.l., Corciano, Italy (R.P., D.P., F.D.F., C.C.); Intercept Pharmaceuticals, Inc., New York, New York; and Dipartimento di Farmacia e Biotecnologie, Alma Mater Studiorum-University of Bologna, Italy (R.A.)

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ABSTRACT

We report on the relationship between the structure-pharmacokinetics, metabolism, and therapeutic activity of semisynthetic bile acid analogs, including 6α-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid (a selective farnesoid X receptor [FXR] receptor agonist), 6α-ethyl-23(S)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (a specific Takeda G protein–coupled receptor 5 [TGR5] receptor agonist), and 6α-ethyl-3α,7α-dihydroxy-24-nor-5β-cholan-23-sulfate (a dual FXR/TGR5 agonist). We measured the main physicochemical properties of these molecules, including ionization constants, water solubility, lipophilicity, detergency, and protein binding. Biliary secretion and metabolism and plasma and ionization constants, water solubility, lipophilicity, detergency, and protein binding. Biliary secretion and metabolism and plasma and hepatic concentrations were evaluated in terms of high-pressure liquid chromatography-electrospray-mass spectrometry/mass spectrometry in bile fistula rat and compared with natural analogs chenodeoxycholic, cholic acid, and taurochenodeoxycholic acid and intestinal bacteria metabolism was evaluated in terms of 7α-dehydroxylation. The hepatic metabolism and biliary secretion were different: 6α-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid, as chenodeoxycholic acid, was efficiently conjugated with taurine in the liver and, only in this form, promptly and efficiently secreted in bile. 6α-Ethyl-23(S)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid was poorly conjugated with taurine because of the steric hindrance of the methyl at 23(S) position metabolized to the C23(F) isomer and partly conjugated with taurine. Conversely, 6α-ethyl-3α,7α-dihydroxy-24-nor-5β-cholan-23-sulfate was secreted in bile unmodified and as 3-glucuronide. Therefore, minor structural modifications profoundly influence the metabolism and biodistribution in the target organs where these analogs exert therapeutic effects by interacting with FXR and/or TGR5 receptors.

Introduction

Bile acids (BAs) have long been known to facilitate digestion and absorption of lipids and to control cholesterol homeostasis. Unexpected new therapeutic opportunities have arisen from the discovery that endogenous BAs, as chenodeoxycholic (CDCA), are potent signaling molecules through activation of the nuclear farnesoid X receptor (FXR) (NR1H4) (Makishima et al., 1999; Parks et al., 1999). FXR controls BA homeostasis through transcription of pivotal biosynthetic enzymes and transport proteins for BAs (Sinal et al., 2000; Tu et al., 2000; Maruyama et al., 2002). Activation of FXR inhibits the synthesis of BAs from cholesterol and protects against their toxic accumulation in the liver. FXR controls lipid (Urizar et al., 2000; Kast et al., 2001; Mak et al., 2002; Houten and Auwerx, 2004; Boyer et al., 2006) and glucose metabolism (Claudel et al., 2002; Duran-Sandoval et al., 2004; Düfer et al., 2012), regulating gluconeogenesis (De Fabiani et al., 2003; Stayrook et al., 2005) and glycogenolysis in the liver and increasing peripheral insulin sensitivity (Maneschi et al., 2013). FXR activation may

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ABBREVIATIONS: AUC, area under the curve of the biliary secretion rate values over the 3 hours of bile collection; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CMC, critical micellar concentration; EC50, half-maximal effective concentration; ES, electrospray; FXR, farnesoid X receptor; GLP, glucagon-like peptide; HPLC, high-pressure liquid chromatography; INT-767, 6α-ethyl-3α,7α-dihydroxy-5β-cholan-23-sulfate; INT-777, 6α-ethyl-23(S)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; LCA, lithocholic acid; MS, mass spectrometry; OCA (obeticholic acid), 6α-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid; SBmax and TSmax, the maximum biliary secretion value and time; SPE, solid phase extraction; TCDC, taurochenodeoxycholic acid; TGR5, Takeda G protein–coupled receptor 5.
play a role in anti-inflammatory effects in atherosclerosis (Porez et al., 2012), inflammatory bowel disease (Gadaleta et al., 2011), and cholestasis (Zollner et al., 2009).

More recently, BAs have proven to be ligands for the membrane G protein–coupled receptor TGR5 (GPBAR1, M-BAR/BG37) (Maruyama et al., 2002), a novel pharmacological target. TGR5 activation reduces body weight (Watanabe et al., 2006) and drives secretion in the gut of the hormone glucagon-like peptide (GLP-1) (Kumar et al., 2012), which promotes insulin release from pancreatic β cells (Schmidt et al., 1985), controlling glucose levels (Thomas et al., 2008) and body weight. TGR5 is highly expressed in monocytes and macrophages (Naiki et al., 2008), where it modulates immune responses (Kawamata et al., 2003; McMahan et al., 2013). It is a target for preventing atherosclerosis and metabolic syndrome. A new synthetic cholic acid (CA) analog, 6α-ethyl-23-(S)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (INT-777), a selective TGR5 agonist, has been synthesized (Pellicciari et al., 2009, 2012; Gioello et al., 2012). Its usefulness for treating diabetes (Thomas et al., 2009) and atherosclerosis has been demonstrated in different animal models (Pols et al., 2011). Its agonist potency (EC50) is more than 50 times higher than that of CA (Pellicciari et al., 2009).

Recently, 6α-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid (OCA), a potent selective FXR agonist, was developed (Pellicciari et al., 2002, 2004, 2012; Gioello et al., 2011). Ongoing clinical trials are demonstrating its hepatoprotective effect in patients with primary biliary cirrhosis (www.clinicaltrials.gov/ct2/show/NCT01473524), type 2 diabetes with nonalcoholic fatty liver disease (Mudaliar et al., 2013), and nonalcoholic steatohepatitis (Adorni et al., 2012; clinicaltrials.gov/ct2/show/NCT01265498).

More recently, 6α-ethyl-3α,7α-dihydroxy-24-nor-5β-cholan-23-sulfate (INT-767) (Rizzo et al., 2010), a dual FXR and TGR5 agonist, has shown potential for treating nonalcoholic fatty liver disease in obese diabetic mice (McMahan et al., 2013) and liver injury in a mouse model of chronic cholangiopathy (Baghdasaryan et al., 2011). Whether selective FXR or TGR5 or dual FXR/TGR5 ligands, these agonists hold promise as a new class of drugs for treating chronic liver disease and extrahepatic inflammatory and metabolic disorders (Schaap et al., 2014).

Previously, we studied a large number of natural (Roda et al., 1987, 1988; Aldini et al., 1996) and semisynthetic BA analogs (Pellicciari et al., 2004, 2009) to elucidate the relationship between BA structure, physicochemical properties, pharmacokinetics, and metabolism. We defined the “critical” structure-related values of their properties, which could predict their pharmacokinetics, metabolism, and biodistribution. Because FXR (Makishima et al., 1999; Parks et al., 1999; Kalaany and Mangelsdorf, 2006; Lefebvre et al., 2009) and TGR5 (Kawamata et al., 2003; Keitel et al., 2007, 2008, 2009; Poole et al., 2010) expression overlaps in some but not all tissues, OCA, INT-777, and INT-767 biodistribution should differ from each other, consistent with their intended target.

We compared three analogs with naturally occurring BAs in terms of their physicochemical properties, including lipophilicity, detergency, ionization properties, and protein binding (Hofmann and Roda, 1984).

We studied their metabolism by intestinal bacteria in vitro, and their pharmacokinetics and metabolism in the bile fistula rat after intravenous and intraduodenal administration. This was done by measuring their recovery and those of their metabolites in bile, bile flow, plasma, and hepatic concentration at the end of the study. New HPLC-ES-MS/MS methods were developed and validated to identify and quantify these BAs and their main metabolites in different biologic fluids. The results are discussed in terms of biodistribution and metabolism in the target organs where they exert their pharmacological activity.

Materials and Methods

Chemicals

**Synthetic BA Analogs.** The studied molecules include 6α-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid (OCA, INT-747), 6α-ethyl-23-(S)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (INT-777), and 6α-ethyl-3α,7α-dihydroxy-24-nor-5β-cholan-23-sulfate (INT-767) (Fig. 1). CDCA, CA, TCDCA, and other BAs were purchased from Sigma-Aldrich (St. Louis, MO). The synthesis of OCA and INT-777 was performed following a previously reported procedure (Pellicciari et al., 2009).

INT-767 was synthesized starting from OCA by initial reaction with phenylmagnesium bromide, followed by oxidative cleavage of the diphenyl methyliden derivative thus obtained. Reduction of the resulting C23-aldehyde derivative, followed by selective C23-O-sulfatation, afforded the desired product. A detailed description of the synthetic pathway leading to INT-767, as well as a description of the structure-activity relationship profile of carboxyl-substituted 6-ECDCA derivatives, will be reported separately.

**Analytical Methods**

All the studied BAs, including the semisynthetic analogs, naturally occurring BAs, and their metabolites, were identified and quantified by high-pressure liquid chromatography-electrospray-mass spectrometry/mass spectrometry (HPLC-ES-MS/MS) by optimized methods suitable for use in pure standard solution in plasma and bile samples after appropriate clean-up preanalytical procedures.

**HPLC-ES-MS/MS Method.**

**Chemicals.** All solvents were of high purity and used without further purification. Acetonitrile for HPLC was from Merck (Darmstadt, Germany); methyl alcohol RPE, ammonia solution 30% RPE, glacial...
acetic acid RPE were from Carlo Erba Reagent (Milan, Italy); ac- tivated charcoal was from Sigma-Aldrich; and ISOLUTE C18 car- tridges (500 mg, 6 ml) for the plasma sample pretreatment were purchased from StepBio (Bologna, Italy).

Plasma BA free rat plasma was treated with activated 50 mg/ml charcoal and stirred at 4°C overnight. After centrifugation at 3000g for 5 minutes the plasma was filtered through Millipore A10 Milli-Q Synthesis (0.45 μm) and stored at -20°C.

Instrumentation. Samples were analyzed using ALLIANCE 2695 HPLC separations module (solvent and sample management platform) (Waters, Milford, MA) combined with a triple quadruple mass spectrometer QUATTRO-LC (Micromass; Waters) using an electro- spray interface [HPLC phenyl-hexyl column (Luna; 4 μm, 150 × 2.1 mm) protected by a Security Guard Phenyl (4 × 2.0 mm i.d.) guard column was used for BA separation, both supplied by Phenomenex (Torrance, CA)] (see Supplemental Data I).

BAs were separated in elution gradient mode using 15 mM ammo- nium acetate buffer (pH 8.00) as mobile phase A and acetonitrile/methanol (75:25 v/v) as mobile phase B. The MS system was set with an electrospray ionization source (ES) in the negative mode with op- timized parameters (see Supplemental Data II).

Chromatograms were acquired using the mass spectrometer in multiple reaction monitoring mode. MassLynx (Waters) software version 4.0 was used for data acquisition and processing. In addition, experiments were performed to identify metabolites using mass spectrometry in both single MS and tandem MS/MS configurations.

Potential or expected metabolites including those conjugated with sulfate, glucuronic acid, or dehydroxylated, decarboxylated, oxo and isomer derivaties (isomers have the same m/z ratio but often different retention times) were investigated, even if the standards necessary for their quantification were not available. These com- pounds were identified according to the m/z values.

Sample Preparation.

Bile. Rat bile samples were brought to 25°C and diluted 1:1000 or 1:10 (v/v) with ammonium acetate buffer 15 mM, pH 8.00, and acetonitrile/methanol (3:1 v/v) in ratio 65:35 (v/v). The final solution was transferred to an autosampler vial, and 5 μl was injected onto the column.

Plasma. Plasma samples (100 μl) were diluted 1:6 (v/v) with 0.1 N NaOH and heated to 64°C for 30 minutes. The solid phase extraction (SPE) cartridge was conditioned with 5 ml of methanol and 5 ml of water prior to sample loading. Plasma samples were loaded into the conditioned cartridge and then washed with 10 ml of water. The cartridge was then eluted with 5 ml of methanol. The eluate was dried under vacuum and then reconstituted with 200 μl of the mobile phase, and 5 μl was injected into the HPLC-ES-MS/MS instrument.

Liver. Aliquots weighing approximately 1 g were taken from different points of the liver sample. Each aliquot was weighed, and 2 ml of phosphate buffer (0.005 M, pH 7.2) was added. The mixture was homogenized using a potter, which was then washed with methanol (3 × 1 ml). The mixture was sonicated for 5 minutes, vortexed for 2 minutes, heated to 37°C for 20 minutes, and centrifuged at 2100g for 15 minutes. One milliliter of the supernatant was spiked with 10 μl of the internal standard working solution and dried under vacuum. The residue then was resuspended with 2 ml of sodium hydroxide (0.1 N). The solution was sonicated for 10 minutes, heated to 64°C for 30 minutes, and SPE was carried out on C18 extraction cartridges (as shown above). The eluate was dried under vacuum and reconstituted with 200 μl of the mobile phase (ammonium acetate buffer: acetonitrile = 70:30 v/v) and injected into the HPLC-ES-MS system.

Stools. Stool sample was collected and homogenized using a mixer. Aliquots weighing approximately 1 g were taken from the homoge- nate. Each aliquot was weighed, and 3 ml of isopropl alcohol was added. The mixture was vortexed for 2 minutes and centrifuged at 2100g for 10 minutes. The supernatant was then diluted 1:100 v/v with mobile phase, and 190 μl of these final solutions were spiked with 10 μl of internal standard working solution, mixed, and injected into the HPLC-ES-MS system.

Physicochemical properties. The samples from water solubility, lipophilicity (log P), and albumin binding studies were brought to 25°C and diluted 1:100 or 1:10 (v/v) with ammonium acetate buffer 15 mM pH 8 and acetonitrile: methanol (3:1 v/v) in ratio 65:35 (v/v). The final solution was transferred to an autosampler vial, and 5 μl was injected onto the column.

Quantification

Calibration Curves. For each BA, stock solutions were prepared in methanol at 1 mM and stored in screw cap disposable glass tubes at approximately -20°C. Appropriate volumes of these stock solutions were further diluted with methanol to obtain working solutions containing all the BAs studied in the rat administration experiments and in samples from the physicochemical properties.

A six-point calibration curve (0.1 to 20 μM) was prepared by adding the appropriate amount of each corresponding bile acid working solution and suitable volume of internal standard working solution (see Supplemental Data III) to obtain a concentration of 1 μM to each biologic matrix. For bile calibration curve, bile samples collected at time zero (before the analog infusion) and diluted with mobile phase 1:10 (v/v) were used. For plasma calibration curve, plasma free was used to quantify plasma samples with clean-up following SPE procedure (see sample preparation). Samples from physicochemical properties studies were directly diluted with the mobile phase.

Method Validation. Quality control samples were prepared at three concentrations, starting from the working solutions used for the calibration curve: low level, 0.3 μM; medium level, 8.0 μM; and high level, 17.5 μM. The three concentrations of quality control samples were assayed in duplicate for each analysis.

Calibration curves were generated by plotting the peak area ratio of the respective compound to the corresponding internal standard versus the nominal concentration. The line of best fit was determined by linear-weighted (1/x) least-squares regression. The linearity acceptance criterion for the correlation coefficient was 0.99 or better. Each back calculated standard concentration should be within ±15% deviation from the nominal value, except for the low limit of quanti- fication, for which the maximum acceptable deviation was ±20%. In terms of linearity, accuracy, and precision, the bioanalytical method used in this study fulfills the compliance criteria described by the Food and Drug Administration guidance for the industry: bioanalytical method validation (see Supplemental Data III).

Physicochemical Properties

Critical Micellar Concentration. This value was determined by surface tension measurements using a maximum bubble-pressure method as previously reported (Poole et al., 2010) using a Sensadyne 6000 tensiometer (Chem-Dyne Research Corp., Milwaukee, WI). The surface tension of aqueous solutions at various concentrations (range 0.1–100 mM) of the BA sodium salts in 0.1 M NaCl was measured at 25°C. The surface tension values were plotted against the logarithm of the bile salt concentration; the regression lines corresponding to the two parts of the curve (monomeric and micellar phases) were calculated using the method of least squares. The critical micellar concentration (CMC) value (mM) was obtained by the intersection of the two lines.

Water Solubility. OCA and INT-777 were suspended in 100 ml of 0.1 M HCl, pH 1.00, and the saturated solutions were transferred to a thermostat-equipped water bath maintained at 25°C. After incu- bation for 1 week, the solutions were filtered on a Millipore filter (0.22 μm), and the concentration of BA was measured by HPLC-ES- MS/MS as reported above.

Lipophilicity. 1-Octanol/water partition coefficient was eval- uated using a conventional shake-flask procedure as previously described (Roda et al., 1990). The experiments were carried out on 1 mM initial bile salt solution buffered at pH 8.00 with 0.1 M potassium phosphate buffer to ensure complete ionization of the BA.
The log P values refer to the BA in the ionized form \([\log P_{\text{A}^+}]\), not to the protonated species \([\log P_{\text{BA}}]\). The initial concentration of each BA was below its own CMC value. BA concentration in the water phase before and after partition in 1-octanol was measured by HPLC-ES-MS/MS as reported above.

**Albumin Binding.** Albumin binding was evaluated by equilibrium dialysis at a fixed BA-albumin ratio (Roda et al., 1982). BA was dissolved at a concentration of 100 \(\mu M\) in 5% bovine serum albumin-saline solution and left to stand for 24 hours at 25°C. Two milliliters of this solution was dialyzed in cellulose sacs with a molecular weight cut-off of 12–14 kDa (Spectra/Por; Spectrum Medical Industries Inc., Rancho Dominguez, CA) against 25 ml of saline solution. The system was equilibrated by mechanical shaking for 72 hours at 25°C. BA concentrations in the starting solution and in the dialyzed solution were determined by HPLC-ES-MS/MS as reported above.

**Biologic Properties**

All the experiments were carried out using the sodium salts of OCA and INT-777. The sodium salts were prepared by adding an equimolar amount of NaHCO₃ to the aqueous suspension of the free acid, which was heated to 80°C, mixed in an ultrasonic bath. INT-767 was used without further preparation because it was fully ionized and soluble at all pH values.

**In Vitro FXR and TGR5 Activation.** Activation of the FXR receptor was determined using a recruitment coactivator assay, namely AlphaScreen technology, according to a previously reported protocol (Thomas et al., 2011). In brief, assays were conducted in a white, low-volume 384-well plate. Different BA concentrations for 30 minutes at 25°C. Luminescence was read in an EnVision 2103 microplate analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA) after incubation with the detection mix (acceptor and donor beads) for 4 hours at 25°C in the dark. Dose-response curves were performed in triplicate and \(Z^\prime\) factor was used to validate the robustness of the assay. Activation of TGR5 was assessed by measuring the level of cAMP using an HTR-FRET assay in NCI-H716 enterocytes, because they express TGR5 physiologically (Rizzo et al., 2010).

Thus, NCI-H716 cells were cultured on 96-well plates coated with Matrigel (Corning Life Sciences, Corning, NY) (0.75 mg/ml) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 \(\mu g/ml\) streptomycin sulfate. After 24 hours, cells were stimulated with increasing concentrations of test BA for 60 minutes at 37°C in Opti-MEM (Invitrogen, Carlsbad, CA) containing 1 mM 3-isobutyl-1-methylxanthine. The level of intracellular cAMP was determined with Lance kit according to the previously reported protocol (Rizzo et al., 2010). \(Z^\prime\) factor was used to validate assay systems.

**Intestinal Metabolism.**

**Bacterial 7-dehydroxylation: substrate specificity.** Fresh human stools were homogenized with saline-water 1:1 (v/v) under a nitrogen stream, and 500 ml was transferred into sterile vials to which 5 ml of sterilized chopped meat-glucose medium (Scott Laboratory, Fiskeville, RI) was added. BAs were then added to this medium at a concentration ranging from 0.01 to 0.1 mM. All the experiments were carried out under nitrogen in capped vials. The anaerobic conditions were maintained with a disposable anaerobic indicator (Gas Pac; BD Biosciences, San Jose, CA). The incubations were carried out at 37°C 1, 2, 4, and 8 hours after the addition of the BA, the reaction was stopped with 150 \(\mu l\) of 30% KOH. The samples were centrifuged at 3500 rpm for 10 minutes, and 3 ml of the supernatant was transferred into a tube to which 8 ml of 0.1 N NaOH was added. The solutions were applied to a C-18 Bond-Elut cartridge that was eluted at a flow rate of 1 ml/min and washed with 10 ml of water; the BAs were subsequently collected with 4 ml of methanol. The eluate was dried under a N₂ stream and reconstituted with 0.5 ml of CH₃OH. The qualitative-quantitative compositions were obtained by HPLC-ES-MS/MS method as described above. The three analogs were also very stable toward the removal of the 6-ethyl group.

**In Vivo Study.**

**Hepatic metabolism and biliary secretion in bile fistula rats.** The study was performed on 60 male Wistar-Han rats (220–250 g body weight) (Charles River Laboratories, Calco, Italy). All experiments were conducted following relevant National and International Guidelines according to Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Ethical Committee of the University of Bologna, Italy (PR 22.03.10) (for details of the experimental procedure, see Supplemental Data IV). After animals were anesthetized, the bile duct was cannulated, and the BAs were delivered either intravenously or intraduodenally per gavage. For the intravenous and intraduodenal delivery, each bile acid was infused at a dose of 1 \(\mu mol/min/kg\) body weight over 1 hour at 2.5 ml/hour. Bile was collected at 15-minute time intervals throughout the infusion and over 2 hours after the infusion was over. In the intraduodenal infusion study, the BA plasma levels of the infused BA were also evaluated. Parallel experiments were conducted with CA, CDCA, and TCDCA as controls. Bile flow was evaluated gravimetrically; the concentrations of the administered BA and its main metabolites were measured in bile and plasma samples with the HPLC-ES-MS/MS as reported above. The intravenous and intraduodenal infusion was preferred over the single bolus infusion because in the anesthetized animal, the intestinal motility is hampered; therefore, the infusion allows the progression of the substance. A single bolus would not progress along the intestine, and in this case, the absorption would also be disturbed by the absence of luminal stirring. Moreover, the infusion technique is an inducer of intrinsic motility, allowing a certain motor activity of the intestine. Similarly, in the intravenous infusion, the BAs do not reach the liver as a bolus. Rather, the liver is exposed to a constant BA load, similar to the intestinal input. BA secretion and the following parameters were calculated: mean ± S.D. maximum secretion rate \((SB_{max})\), the time of the maximum value \((T_{SB_{max}})\), and the area under the curve (AUC) of the biliary secretion rate values over the 3 hours of bile collection.

**Results**

**Physicochemical Properties**

The physicochemical properties of the three compounds were compared with those of naturally occurring BAs with the closest steroid structure without the 6a-ethyl group shared by OCA, INT-777, and INT-767 (Table 1). The physicochemical behavior in water of the three analogs is quite different in terms of water solubility, dissociation constant, detergent, and lipophilicity.

**TABLE 1**

<table>
<thead>
<tr>
<th>Property</th>
<th>CDCA</th>
<th>OCA</th>
<th>CA</th>
<th>TCDCA</th>
<th>INT-767</th>
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<tbody>
<tr>
<td>(W_s)</td>
<td>17</td>
<td>29</td>
<td>30</td>
<td>29</td>
<td>19</td>
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<td>48.8</td>
<td>49.1</td>
<td>50.1</td>
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<tr>
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<td>2.5</td>
<td>1.1</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td>(pK_a)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Albumin Binding</td>
<td>96</td>
<td>96</td>
<td>88</td>
<td>62</td>
<td>70</td>
</tr>
</tbody>
</table>

**TABLE 1** Physicochemical properties in aqueous solution of OCA, INT-777, INT-767, and their corresponding natural BA analogs

\(W_s\), water solubility; CMC, critical micellar concentration; \(pK_a\), acid dissociation constant; \(ST_{\text{CMC}}\), surface tension at CMC; \(\log P_{A^+}\), octanol/water partition coefficient; \(pK_a\), acid dissociation constant; \(ST_{\text{CMC}}\), surface tension at CMC; \(W_s\), water solubility.
CDCA and CA (Roda and Fini, 1984). When protonated at a pH 2 unit below their pKₐ, these two compounds are poorly soluble in water (Table 1), whereas at pH values (2 units above their pKₐ) they became very soluble, forming the corresponding ionized species (sodium salt). The pKₐ value of INT-777 is similar to CA, and the C23-methyl group does not modify the acidic properties of the C-24 carboxyl group, lacking electron-withdrawing properties (Roda et al., 1988). These two compounds are usually purified and crystallized as a protonated acid, and the same is true of OCA formulation for oral administration to human studies.

According to the physicochemical properties, these two compounds remain unmodified in the gastric juice acidic contents, and then when released into the duodenum, they undergo a fast and complete dissolution, forming the corresponding ionized species. Under this administration route, the solid state form does not play a major role. This is because the dissolution process is very fast, with a dissolution rate faster than the intestinal transit time.

The behavior of INT-767, with a C23 sulfate head group, is different. It is always ionized at all the pH values and therefore is fully water soluble. This compound exhibits acidic properties similar to taurine-conjugated BAs, such as TCDCA carrying a sulphonate head.

When present in solution as anions, i.e., as a sodium salt, the three compounds self-aggregate to form micelles at concentration values slightly lower than that of the corresponding natural analogs (Table 1) (Roda et al., 1983). This is due to the presence of the 6α-ethyl group, which contributes, according to its orientation toward the β face of the steroid, to increasing the lipophilic area and therefore facilitating the back-to-back aggregation (Roda et al., 1994).

Figure 2 reports the surface tension values versus concentration, as measured by the maximum pressure method in the presence of 0.15 M counter ion Na⁺. Experimental data show a similar behavior to the corresponding naturally occurring BAs. This indicates that, for these molecules too, the micellar formation is a stepwise process, described by a slope of the graph in the premicellar phase similar to natural BAs. The surface tension values at the CMC are relatively low for OCA and INT-777 and slightly higher for INT-767, indicating that, despite these differences, the detergency power is still in the range of naturally occurring BAs such as CA and CDCA, far different from conventional anionic surfactants with much lower CMC and higher detergency.

The lipophilicity values, as measured by the octanol/water partition coefficient log P of the ionized species reported in Table 1, show that the 6α-ethyl group contributes to increasing the log P values for OCA compared with CDCA and for INT-777 compared with CA because of the additional contribution in the latter compound of the C23-(S)-methyl group. For INT-767, the direct comparison with TCDCA is not straightforward, because the latter presents a different side chain structure with an additional amide bond. However, INT-767 is still more lipophilic than TCDCA. The log P values were only calculated for the ionized species, because, when protonated, OCA and INT-777 are poorly soluble and INT-767 is always ionized, making the comparison simpler and more predictive (Roda et al., 1990).

**Biologic Properties**

**In Vitro FXR and TGR5 Activation.** The three BA derivatives OCA, INT-777, and INT-767 were first evaluated for their ability to activate FXR and TGR5 (Fig. 3). The AlphaScreen coactivator recruitment assay was used to determine their capacity to activate FXR, whereas the intracellular level of cAMP was used as a readout for TGR5 activation (Table 2) (Rizzo et al., 2010). The naturally occurring BAs, CDCA and litocholic acid (LCA), were used as reference controls, respectively.

Efficacy was calculated by referencing the same concentration of test compounds with the positive control. As already described (Pellicciari et al., 2009; Rizzo et al., 2010), OCA was...
found to be a selective FXR agonist, INT-777 a selective TGR5 agonist, and INT-767 to possess a dual FXR/TGR5 agonist profile, being the most potent agonist for both receptors. Notably, the semisynthetic BA analogs failed to activate any other BA-responsive receptors, including pregnane X receptor, constitutive androstane receptor, and vitamin D receptor, showing a biologic profile suitable for in vivo preclinical and clinical investigations (Thomas et al., 2008, 2009).

**Intestinal Metabolism.**

**Bacterial 7-dehydroxylase: substrate specificity.** When incubated in human stool anaerobic culture, the three analogs were very stable toward 7-α dehydroxylation. After 24 hours, more than 90% of these compounds were recovered unmodified, as reported in Fig. 4. Conversely, CDCA and CA were promptly metabolized to form lithocholic acid and deoxycholic acid, respectively. The 7-oxo metabolite of both compounds was found but in a limited amount. TCDCA was quickly deconjugated and 7-dehydroxylated to form lithocholic acid. The 6-ethyl group in all the three analogs is very stable and is not removed by intestinal bacteria even after an extensive 24-hour incubation.

**In Vivo Study.**

**Hepatic metabolism and biliary secretion in bile fistula rat.** The bile fistula rat model was mainly designed to evaluate the biliary secretion and the metabolites formed after intravenous and intraduodenal infusion of the semisynthetic BA analogs, providing indirect information about the hepatic first-pass clearance, intestinal absorption efficiency, and kinetics. Moreover, because bile diversion leads to a complete interruption of the enterohepatic circulation, in this short experimental time, it was possible to minimize the effects of anesthesia on the intestinal motility (the so-called “dynamic ileus”). An increased transit time and a delayed delivery of the BAs to the ileum should consequently limit their active carrier-mediated ileal absorption, without affecting the passive transport operating throughout the intestine.

The bile fistula model used is the same previously used for a large number of naturally occurring BAs and other semisynthetic analogs. This helps to facilitate the prediction of the expected pathways responsible for their biodistribution according also to their physicochemical properties.

The biochemical parameters quantified were the maximum secretion rate in bile after intravenous and intraduodenal administration and area under the curve (AUC) for the period of the study. This allowed us to predict the biodistribution of the analogs and their main hepatic and intestinal metabolites. Although the interruption of the enterohepatic circulation is a limitation of the study, it did allow us to obtain the above information with the smallest number of animals, providing useful indications for further selected studies closer to human administration.

**Bile Flow.** The model used allows us to properly quantify the bile flow, which is an important parameter for excluding any cholestatic effect for cytotoxicity, allowing us to also evaluate the possible choleretic effect as observed for INT-777 (see below). The effect on bile flow was evaluated in comparison with untreated bile fistula rats by measuring the bile volume secreted at different time intervals after intraduodenal and intravenous administration of the analogs and compared with administration of physiologic solution (Fig. 5). As further controls, the effect of the analogs was also compared with that produced by the administration of CDCA, CA, and TCDCA at equimolar doses, both intravenously and intraduodenally. The mean (n = 6) bile maximum secretion rates (mean ± S.D., microliter per kilogram per minute) after intravenous and intraduodenal administration are reported in Table 3.

After intraduodenal administration, OCA increased bile flow, reaching maximum levels of approximately 70 μl/min/kg. The induced bile flow after intravenous administration was higher. In both cases, the effect was higher than in saline-treated control animals. With respect to the control rats, the infusion of CDCA slightly increased bile flow during
intraduodenal and intravenous administration, with an effect slightly lower than OCA.

The intraduodenal and intravenous infusions of INT-777 significantly increased the bile flow compared with control animals, reaching the highest values found for all the studied analogs, particularly after intravenous administration, as reported in Table 3. The effect was also much higher than that obtained after infusion of CA at the same dose, which slightly increased the bile flow after both intraduodenal and intravenous administration. As reported below, the choleretic observed during INT-777 infusion was not associated with a parallel increase of BA concentration and therefore could be considered a BA-independent bile flow effect. The intraduodenal infusion of INT-767 did not affect the bile flow rate compared with control rats. After intravenous infusion, a significant increase was observed. The effect was higher than that obtained after intravenous administration of TCDCA.

**Biliary Bile Acid Secretion and Metabolism.** The SBmax, TSBmax, and AUC for the 3-hour period of the study after intraduodenal and intravenous infusion are reported in Table 4. When infused, both intraduodenally and intravenously, at the dose of 1 μmol/min/kg over one 1, the three analogs presented different biliary secretion rates as a result of different hepatic uptake, intestinal absorption, and metabolism.

OCA was secreted into bile almost completely conjugated with taurine, with a similar recovery after intraduodenal and intravenous administration. The kinetics of its biliary secretion were similar to that of CDCA with a slight delay of taurine conjugate secretion rate (Fig. 6). Similar to CDCA, the taurine conjugate was the main hepatic metabolite and it was secreted into bile only in this form. At the end of the study, i.e., after 3 hours, OCA was present almost entirely as taurine conjugate, both after intraduodenal and intravenous administration. The biliary AUC of the 3-hour bile collection showed an efficient recovery of OCA after both intravenous and intraduodenal administration similar to CDCA, and almost 100% of the administered dose was recovered in bile as a taurine conjugate.

INT-777 presents a completely different behavior from both OCA and INT-767. The compound was secreted into bile in

---

### Table 2

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>AlphaScreen FXR Agonist Potency EC50 μM</th>
<th>Efficacy versus CDCA 50 μM %</th>
<th>HTR-FRET TGR5 NCI-H716 Cells Agonist Potency EC50 μM</th>
<th>Efficacy versus LCA 10 μM %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural bile acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA</td>
<td>15 ± 5</td>
<td>100</td>
<td>33 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>TCDCA</td>
<td>26 ± 1</td>
<td>74 ± 2</td>
<td>25 ± 5</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>LCA</td>
<td>&gt;100</td>
<td>—</td>
<td>6 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>CA</td>
<td>45 ± 5</td>
<td>12 ± 3</td>
<td>50 ± 10</td>
<td>10 ± 5</td>
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<tr>
<td>Synthetic bile acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCA</td>
<td>0.15 ± 0.05</td>
<td>160 ± 15</td>
<td>15 ± 5</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>INT-776</td>
<td>0.030 ± 0.005</td>
<td>180 ± 5</td>
<td>0.63 ± 0.10</td>
<td>120 ± 5</td>
</tr>
<tr>
<td>INT-767</td>
<td>&gt;100</td>
<td>—</td>
<td>0.9 ± 0.1</td>
<td>110 ± 10</td>
</tr>
</tbody>
</table>

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Fig. 4. In vitro metabolic stability in human stool cultures (intestinal content) toward bacteria 7-dehydroxylation of the three analogs compared with naturally occurring BAs.

Fig. 5. Bile flow after intravenous administration (A) and intraduodenal administration (B) of the semisynthetic BAs and naturally occurring BAs.
part unmodified and in part as a C23(R) isomer, almost entirely as taurine conjugate. The maximum secretion rate after intravenous infusion was much higher than after intraduodenal infusion. The recovery expressed as AUC (Table 4) was much higher after intravenous administration compared with intraduodenal administration (Fig. 7). As a consequence of the presence of the C23(S)-methyl group, INT-777 biliary secretion rate and hepatic metabolism were very different from CA, which was completely conjugated with taurine and was only secreted into bile unmodified in a low-dose-related percentage (Roda et al., 1988). The recovery in bile of the unmodified molecule was incomplete after both intraduodenal and intravenous infusion, with the latter being much higher (Table 4). Despite the possibility of being conjugated with taurine once isomerized from S to R, the contribution to biliary secretion of the taurine-conjugated C23(R) isomer of INT-777 was much lower when compared with taurine-conjugated CA (Pellicciari et al., 2009), which ensures its almost complete recovery in bile.

INT-767 was secreted into bile both unmodified and as a 3-glucuronide metabolite, with an efficient recovery of the sum of INT-767 and its glucuronide after both intravenous and intraduodenal administration (Fig. 8). The kinetics of the secretion rate was delayed after intraduodenal infusion, with a maximum secretion time after 180 minutes compared with 120 minutes after intravenous infusion. As a comparison, TCDCA was secreted in bile unmodified and its recovery was higher when administered intravenously compared with intraduodenally.

**Plasma and Liver Concentration.** The plasma concentration of the administered analogs and their metabolites was measured only during the intraduodenal infusion. The plasma concentration of OCA after intraduodenal infusion was relatively low, as reported in Fig. 9. The compound was present almost entirely unmodified and to a lesser extent conjugated with taurine.

After infusion of INT-777, the compound was present in plasma at a relatively higher concentration than OCA unmodified and to a much lower extent as a C23(R) isomer and its taurine conjugate. After infusion of INT-767, a very small amount was present in plasma unmodified, with only a trace amount of its glucuronide (Table 5).

The concentration in the liver of the studied analogs and their main metabolites was measured at the end of the experiment when the liver was explanted (Table 6). OCA was present in the liver almost entirely as a taurine conjugate, INT-777 was present unmodified and to a lesser extent as a C23(R) isomer, free and taurine-conjugated. INT-767 was present in liver almost entirely unmodified.

### Discussion

**Physicochemical Properties**

Minor structural modifications in the three semisynthetic BA analogs modify their physicochemical properties and, in turn, their pharmacokinetics, metabolism, and specificity as FXR and TGR5 agonists. OCA shares physicochemical properties with CDCA, including detergency, still lower than common detergents, and excluding a direct toxicity for biologic membranes. As a protonated acid, OCA is poorly soluble,
but at pH ≥ 7 it becomes a fully soluble anion. In the duodenum after oral administration, it is promptly solubilized, reaching the intestinal absorption site where, after protonation in the enterocyte, it is passively absorbed like natural BAs.

INT-777 presents differences from the parent CA, having not only the 6α-ethyl but also the C23(S)-methyl group, which increases the lipophilicity and slightly reduces the CMC. INT-777 pK_a value of 5 is similar to OCA and to all natural C-24 unconjugated BAs showing similar pH dependency solubility.

Conversely, substitution of the terminal carboxylic head of OCA with a sulfate group modifies the behavior in aqueous solution, being ionized at any pH. The sulfate group confers acidity properties similar to TCDCA. Moreover, it is more lipophilic because of the absence of the side chain amide bound, having a log P more similar to an unconjugated carboxylated BA and higher than TCDCA. The CMC value is slightly lower than TCDCA, but the behavior of surface tension versus concentration is similar to the other BAs, with an ST value at the CMC of the same order of magnitude.

The complete ionization at any physiologic pH should impair its passive absorption allowing only a possible carrier-mediated transport by organic anion transporters, thus explaining the moderate secretion in bile after id infusion. The percentage of albumin binding (Table 1) is similar among the studied analogs, suggesting the presence of a similar bound fraction in the plasma compartment.

**In Vitro Study**

The three analogs were highly stable in human stools content, and they were not 7α-dehydroxylated or metabolized, while CDCA, CA, and TCDCA were promptly 7α-dehydroxylated. Indeed, the steric effect of the ethyl group toward 7α-dehydroxylase prevents the enzymatic cleavage of the 7α-hydroxy group, as previously shown by the presence of a 6-methyl group in other BAs (Une et al., 1984; Roda et al., 1994), increasing the metabolic stability and preventing the formation of potentially toxic 6α-ethyl monohydroxy analogs. The 6-ethyl group in particular is very stable in all three analogs.

**In Vivo Studies**

**Biliary Secretion and Hepatic Metabolism.** OCA presents pharmacokinetics and metabolism similar to CDCA showing efficient intestinal absorption as indicated by the high maximum secretion rate in bile after intraduodenal infusion and a fast hepatic uptake when infused intravenously. Similar to CDCA, OCA is secreted in bile almost completely taurine-conjugated. This behavior, as with CDCA or other endogenous BAs, is related to lipophilic properties. Usually, BAs with log P values ≥2 need a hepatic metabolism to more polar molecules to facilitate their hepatic excretion.

OCA is also conjugated with glycine (unpublished data from hamster and humans) and secreted in this form. Rodents and

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Fig. 6. Biliary secretion and pharmacokinetics of CDCA (A) and OCA (B) and their respective metabolites after intravenous (Δ) and intraduodenal (○) administration.

Fig. 7. Bile secretion and pharmacokinetics of CA (A) and INT-777 (B) and their respective metabolites after intravenous (Δ) and intraduodenal (○) administration.
dog preferentially conjugate BAs with taurine, whereas other animals such as hamster, pig, and human preferentially conjugate BAs with glycine. This process is not an excretory pathway, and glycine and taurine conjugates are actively absorbed by the ileum by intestinal bile acid transporter. After long-term feeding (unpublished data) in mice, OCA taurine conjugate is the main metabolite present in the enterohepatic circulation.

The metabolic stability of OCA toward intestinal bacteria 7α-dehydroxylation is much higher than CDCA, preventing the formation of potentially toxic monohydroxy BA. The recovery in bile of the taurine conjugate is almost complete after intravenous and intraduodenal infusion, showing that the molecule has pharmacokinetics similar to CDCA. After long-term feeding (unpublished data), the taurine-conjugated OCA, similar to TCDDA, undergoes an efficient ileal carrier-mediated transport. It accumulates in the enterohepatic circulation and can be an optimal agonist for FXR in the liver and intestine, controlling endocrine and metabolic functions (Inagaki et al., 2006; Stroeve et al., 2010). Notably, the taurine- and glycine-conjugated metabolites that accumulate in the body still have a potent FXR agonist activity (unpublished data).

INT-777 presents pharmacokinetics and metabolism very different from OCA and CA. The metabolites recovered in bile indicate that INT-777, because of lower lipophility, can in part be secreted in bile, unlike OCA. Moreover, the additional amidation, which facilitates biliary secretion, is partially inhibited by the steric hindrance of the C23(S)-methyl group on the conjugating enzyme, as with other side chain modified analogs (Roda et al., 1988).

Interestingly, as an additional metabolic pathway, the partial isomerization at the C23 position of INT-777, leading to the parent C23(R)-isomer, is more prone to conjugation. INT-777 biliary recovery is almost complete after intravenous and partial after intraduodenal administration, suggesting a less efficient passive intestinal absorption due to the relatively low log P value. The pharmacological properties of INT-777 show that it is an optimal molecule for TGR5 targeting in the colon L-cells, triggering GLP-1 secretion. INT-777 has a potent choleretic effect, inducing a very high increase in bile flow after both intravenous and intraduodenal administration due
to the potential presence of a chole-hepatic shunt pathway shown by an increased bicarbonate secretion (see Supplemental Data IV), as previously documented for nor-BAs (Gurantz and Hofmann, 1984; Yoshii et al., 1991), with a moderate lipophilicity including nor-CDCA but not nor-CA, which is more hydrophilic. INT-777 is more lipophilic than nor-CA due to the presence of alkyl substituents and has a longer side chain. It therefore is more prone to being absorbed and undergoing this pathway. The taurine and glycine conjugates of INT-777 are still active on TGR5, whereas the conjugates of the C23(R)-methyl isomer partially lose the agonistic activity (unpublished data). Under long-term feeding we expect a large dose and species dependency in the liver, resulting in a moderate lipophilicity including nor-CDCA but not nor-CA, which is more lipophilic. INT-777 is present in plasma almost completely unmodified and, to a lesser extent, as a glucuronide. In the intestine, it is present mostly unmodified and, to a lesser extent, as a C23(S) isomer, free and taurine-conjugated.

After intraduodenal infusion, INT-767 plasma concentration is much lower than the other analogs, with a very low percentage as glucuronide. Conversely, the liver concentration of unmodified INT-767 and, to a lesser extent, its glucuronide, is relatively high. This suggests a relatively high hepatic residence time as a consequence of a slow intestinal absorption and biliary secretion delayed by the glucuronidation rate.

OCA presents a preferential compartmentalization in bile as a taurine conjugate, with relatively low concentration in liver and systemic circulation (see Supplemental Data V). After the first-pass hepatic extraction, OCA is fully taurine-conjugated and accumulates in this form. Conversely, INT-777 presents lower concentration in bile and higher concentrations in the liver and blood. Its metabolism is much more complex because of the presence of the C23-methyl. This hinders the amidation process, and the molecule will be partly secreted in bile unmodified and partly as taurine-conjugated C23(R) isomer. The delayed appearance of the molecule in bile, both unmodified and as a glucuronide, is intriguing. Because UDP-glucuronosyltransferases are present not only in the liver but also in the intestine and are upregulated by FXR, it is possible that INT-767 undergoes intestinal glucuronon conjugation with delayed and low biliary appearance of the parent form.

OCA biodistribution in the enterohepatic circulation and the potent agonist effect on FXR contribute to making OCA a promising treatment in cholestatic liver disease, nonalcoholic steatohepatitis, and other liver diseases. INT-777 is a potent TGR5 agonist, and because of the relatively low intestinal absorption it can effectively activate TGR5 in colonic L-cells, triggering GLP-1 secretion. In addition, because of its systemic biodistribution, INT-777 can activate TGR5 in different districts, potentially an interesting candidate for diabetes, weight control, and metabolic syndrome.

INT-767 intestinal absorption is efficient. However, 3-glucuronidation is necessary to facilitate its biliary excretion. Thus, INT-767 possesses a pharmacokinetic profile suitable for targeting both FXR and TGR5. The repression of the hepatic BA synthesis together with increased insulin sensitivity and energy expenditure could provide synergistic effects in the treatment of liver and metabolic diseases such as type 2 diabetes and obesity.

Authorship Contributions

*Participated in research design:* Roda, Adorini.

*Conducted experiments:* Gioiello, Neri, Camborata, Passeri, De Franco, Spinozzi, Colliva, Montagnani, Aldini.

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**TABLE 5**

<table>
<thead>
<tr>
<th>BA</th>
<th>Plasma Concentration</th>
<th>Time</th>
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<tbody>
<tr>
<td></td>
<td>µM</td>
<td>min</td>
</tr>
<tr>
<td>INT 747 OCA</td>
<td>0.098 ± 0.005</td>
<td>195</td>
</tr>
<tr>
<td>T OCA</td>
<td>0.037 ± 0.005</td>
<td>135</td>
</tr>
<tr>
<td>INT 777</td>
<td>0.14 ± 0.04</td>
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</tr>
<tr>
<td>T INT(R)-777</td>
<td>0.005 ± 0.001</td>
<td>195</td>
</tr>
<tr>
<td>INT(R)-777</td>
<td>0.008 ± 0.001</td>
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</tr>
<tr>
<td>INT 767</td>
<td>0.009 ± 0.003</td>
<td>225</td>
</tr>
<tr>
<td>Glu-INT-767</td>
<td>0.0008 ± 0.0001</td>
<td>105</td>
</tr>
</tbody>
</table>

**TABLE 6**

<table>
<thead>
<tr>
<th>BA</th>
<th>Liver Concentrations</th>
<th>µmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT 747 OCA</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>T OCA</td>
<td>0.56 ± 0.06</td>
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<tr>
<td>INT 777</td>
<td>0.44 ± 0.05</td>
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<tr>
<td>T INT(R)-777</td>
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</tr>
<tr>
<td>INT(R)-777</td>
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</tr>
<tr>
<td>INT 767</td>
<td>0.24 ± 0.06</td>
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</tr>
<tr>
<td>Glu-INT-767</td>
<td>0.003 ± 0.001</td>
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INT(R)-777, 6a-ethyl-23(R)-methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; T, taurine.
Contributed new reagents or analytic tools: Roda, Pellicciari, Gioiello.

Performed data analysis: Camborata, Passeri, De Franco, Spinozzi.

Wrote or contributed to the writing of the manuscript: Roda, Pellicciari, Adorini, Aldini.

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Address correspondence to: Aldo Roda, Laboratory of Analytical and Bioanalytical Chemistry, Department of Chemistry “G. Ciamician,” Alma Mater Studiorum-University of Bologna, Via Selmi 2, 40126 Bologna, Italy. E-mail: aldo.roda@unibo.it