Oral treatment of unconjugated hyperbilirubinemia
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Orlistat treatment increases fecal bilirubin excretion and decreases plasma bilirubin concentrations in hyperbilirubinemic Gunn rats

CHAPTER 3

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ABSTRACT

We determined whether serum levels of unconjugated bilirubin (UCB) can be decreased by enhancing fecal fat excretion. Gunn rats were fed a high-fat diet (control) or the same diet mixed with the lipase inhibitor orlistat. At regular intervals, plasma UCB concentrations were determined and 72-hour fat balances were performed. Orlistat treatment decreased plasma UCB concentrations (at 3 weeks; 100 mg/kg, -33 ± 8%, p<0.05; 200 mg/kg, -46 ± 10%, p<0.01). Within days of treatment, orlistat treatment increased fecal excretion of UCB (at day 3, +220%, p<0.05). During 24 weeks of orlistat treatment (200 mg/kg chow), plasma UCB concentrations were continuously ~35% lower than in control rats. Plasma UCB concentrations were negatively correlated with the amount of fecal fat excretion (n = 12, r = -0.87, p<0.001). In conclusion, orlistat treatment increases fecal excretion of fat and enhances disposal of UCB in Gunn rats. This approach could lead to novel strategies for prevention and treatment of unconjugated hyperbilirubinemia in patients.
INTRODUCTION

Unconjugated bilirubin (UCB), the major breakdown product of heme, is normally glucuronidated to bilirubin glucuronides in the liver, catalyzed by the hepatic enzyme bilirubin-UDP-glucuronosyltransferase (Ugt1a1, EC 2.4.1.17). Bilirubin glucuronides are significantly more water-soluble than UCB and can be readily excreted via bile into the feces, either as parent compound or, after intestinal metabolism, as UCB or urobilinoids. Unconjugated hyperbilirubinemia develops when the formation of UCB (mainly determined by hemoglobin degradation) is not matched by the hepatic glucuronidation capacity, which occurs for example during increased hemolysis, Gilbert syndrome, Crigler-Najjar disease or neonatal hyperbilirubinemia. UCB can accumulate in several organs, including the central nervous system, where it can lead to toxicity (kernicterus) and death. Treatment of unconjugated hyperbilirubinemia has mainly concentrated on inhibition of UCB production (heme oxygenase inhibitors), stimulation of UCB metabolism (phenobarbital, phototherapy), and, for severe cases of UCB accumulation, on removal of UCB by plasma exchange transfusion.

It has been demonstrated that UCB can diffuse from the blood compartment into the intestinal lumen across the intestinal mucosa, and that UCB can be reabsorbed from the intestinal lumen. Prevention of intestinal reabsorption of UCB, for example by agar, cholestyramine, or calcium phosphate, has been demonstrated to decrease its plasma concentrations. In contrast to enhancing UCB metabolism or to plasma exchange transfusion, however, prevention of intestinal UCB reabsorption is not used on a wide-scale basis in clinical practice because of conflicting results of specific treatments and because of reported side effects.

Recently, we hypothesized that an inverse relation exists between neonatal unconjugated hyperbilirubinemia and the degree of neonatal fat malabsorption. Available data support the hypothesis that the exaggerated neonatal jaundice in breast-fed compared with formula-fed infants coincides with higher fat absorption and, therefore, lower fecal fat excretion in the former. In the present study we investigated in an animal model whether stimulation of fecal fat excretion could function as an interruption of the enterohepatic circulation of UCB, in agreement with the hypothesized hydrophobic association between UCB and unabsorbed fats. As animal model homozygous Gunn rats were used that have an unconjugated hyperbilirubinemia based on the genetic deficiency of Ugt1a1 activity (human homologue: Crigler-Najjar disease type I). Fecal fat excretion was stimulated by feeding Gunn rats high-fat diets supplemented with orlistat. Orlistat, a chemically synthesized derivative of the natural product lipstatin, is a non-absorbable, specific inhibitor of acylglycerollipases, such as pancreatic lipase and gastric lipase.
METHODS

Animals
Homozygous male Gunn rats (RHA/jj, body weight 280-390 g) were obtained from the breeding colony at the Central Animal Facility, Academic Medical Center (Amsterdam, The Netherlands). All animals were housed individually and kept in an environmentally controlled facility with diurnal light cycling (lights on from 7 AM until 7 PM), were fed ad libitum and had free access to water. Experimental protocols were approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences (University of Groningen, The Netherlands).

To resemble human dietary fat intake more closely, rats were fed a semisynthetic high-fat diet. A previous report indicated that plasma UCB concentrations in Gunn rats increase upon feeding a semisynthetic diet instead of standard lab chow. Possibly this phenomenon occurs because lab chow, in contrast to a semisynthetic diet, may contain naturally occurring indoles extracted from cruciferous vegetables which induce microsomal bilirubin oxidation. In control experiments, we confirmed the transient increase in plasma UCB concentrations (~50% after 2 weeks), returning to control concentrations after 4 weeks. For this reason, rats were fed the semisynthetic high-fat (control) diet for a run-in period of 4 weeks.

Materials
All diets were produced by Hope Farms BV (Woerden, The Netherlands). Orlistat (tetrahydrolipstatin, Ro 18-0647) is a synthetic product and was obtained as capsules containing 120 mg active compound from Roche Nederland B.V. (Mijdrecht, The Netherlands).

Experimental Design
Study with different doses of orlistat
Male Gunn rats (n = 14) were fed a high-fat diet (35 energy%; fatty acid composition measured by gas chromatography analysis, in molar percentages; C8-C12, 4.4; C16:0, 28.5; C18:0, 3.9; C18:1ω9, 33.2; C18:2ω6, 29.3; C18:3ω3, 0.2) for a 4 week run-in period. Rats were then anesthetized with halothane, and baseline blood samples for measuring plasma UCB concentration were obtained by tail bleeding. After collection of baseline plasma samples, rats were randomly assigned to be fed either the high-fat diet (control, identical to that used in the run-in period), or the same diet mixed with the lipase inhibitor orlistat (dose, 100 mg/kg chow or 200 mg/kg chow; n = 4-5 per group). At 1.5-week intervals, plasma samples were obtained and, at the end of the experimental period, a 72-hour fecal fat balance was performed to evaluate the total amount of food (and fat) intake, the amount of fat excreted via the feces, and the difference between the two (i.e. the net fat absorption), and the coefficient of fat malabsorption (100-fold the ratio between amount of fat excreted via the
feces and amount of fat ingested). During the 72-hour period, feces was collected in one fraction and chow intake was determined by weighing the chow container. After 3 weeks of feeding, rats were anesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone) and diazepam and their bile ducts were cannulated for collection of bile for 30 minutes. Bile volumes were determined gravimetrically. At the end of bile collection, a large blood sample was obtained by heart puncture.

**Long-term experiment**
A separate set of experiments was performed to determine whether effects of orlistat on plasma UCB concentration are sustained over time. After a run-in period of 4 weeks on high-fat (control) diet feeding, Gunn rats were kept on control or orlistat-containing (200 mg/kg chow) high-fat diets for 24 weeks (each group n = 4). Body weight was assessed and blood samples were obtained by tail bleeding at regular intervals (0, 2, 4, 8, 12, 16, 20 and 24 weeks). Fat balance studies were performed at 2, 8 and 20 weeks to confirm the sustained effects of orlistat. After 24 weeks of feeding, bile samples were collected as described above.

**Acute experiment**
A separate experiment was performed to determine whether starting orlistat treatment was associated with a change in fecal UCB excretion in parallel to a decrease in plasma UCB concentration. After a run-in period of 4 weeks on high-fat (control) diet feeding, Gunn rats were fed orlistat-containing (200 mg/kg chow) high-fat diets for 3 days (n = 4). Before (T₀) and daily for 3 days after starting orlistat treatment plasma samples were obtained (tail bleeding) and feces was collected in 24h fractions. Fecal UCB excretion, fecal fat excretion and plasma UCB concentrations were determined by use of the methods described below.

**Analytical techniques**
Samples of plasma, bile and feces were submitted to alkaline methanolysis and extracted into chloroform. After evaporation, the residue was redissolved in chloroform and analyzed by reverse-phase high-performance liquid chromatography, as described previously. Total biliary bile salts were assayed by the 3α-hydroxysterol dehydrogenase method. Biliary cholesterol and phospholipid were measured after lipid extraction, according to the methods of Gamble et al. and Bötcher et al., respectively. For the fat balance determination, feces and chow pellets were freeze-dried and mechanically homogenized. Lipids from aliquots of diet and freeze-dried feces were extracted, hydrolyzed and methylated. Resulting fatty acid methyl esters were analyzed by gas chromatography using heptadecanoic acid (C17:0) to measure the amount of the major fatty acids (palmitate, stearate, oleate, linoleate, and arachidonate). Fatty acid contents were expressed in molar amounts as detailed previously, and used to calculate the amounts of fat ingested, amount of fat excreted through the feces, and the net amount of fat uptake, defined as the difference between the two.
Coefficients of fat absorption were calculated as 100-fold the ratio between the net amount of fat uptake and the amount of fat ingested.

**Statistical Analyses**

All values are expressed as mean ± SD. Differences between the treatments were determined by Student t test (2 treatments, long-term experiment) or by 1-way analysis of variance (ANOVA), with post-hoc comparison by Newman-Keuls t test. The level of significance was set at \( P \) values < 0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

**RESULTS**

*Experiments with orlistat in different doses*

*Fat balance study*

Orlistat administration was associated with fat malabsorption. The amount of fat excreted into the feces increased dose-dependently during orlistat treatment (Figure 1). Interestingly, the orlistat-treated animals tended to eat slightly more (Figure 1, fat ingestion). Despite the presence of increased fecal fat excretion (and thus of fat malabsorption), the orlistat treated animals succeeded to reach similar amounts of net fat uptake as control animals (Figure 1). The net amount of fat uptake is defined as the difference between the amount of fat ingested and the amount of fat excreted via the feces. The similar net fat uptake during orlistat treatment was associated with similar growth rates and body weights during and at the end of the experimental periods, respectively (NS, data not shown). The coefficient of fat absorption was significantly decreased in orlistat treated rats compared with that of controls (control, 95.3 ± 1.4 %; Orl-100, 84.9 ± 2.0 %, \( p < 0.05 \); Orl-200, 76.9 ± 1.4 %, \( p < 0.01 \)). The orlistat treatment in the *ad libitum*-fed Gunn rats thus resulted in the peculiar combination of increased fecal fat excretion/fat malabsorption, but nevertheless unaffected net amount of fat uptake.

**Figure 1.** Dietary administration of orlistat to Gunn rats on a high-fat diet induces a dose-dependent increase in fecal fat excretion. Amounts of dietary fat ingestion, fecal fat excretion and net fat uptake in Gunn rats fed a high-fat diet without supplement (control) or supplemented with orlistat (100 mg/kg chow or 200 mg/kg chow). Experimental diets were fed for 3 weeks, at the end of which a 72-hour fat balance was performed. Values represent mean ± SD, of \( n = 4-5 \) per group. Amount of fat is expressed in mmol of fatty acids, as determined by gas chromatography. Coefficients of fat absorption were: control, 95.3 ± 1.4 %; Orl-100, 84.9 ± 2.0 % (\( p < 0.05 \)); and, Orl-200, 76.9 ± 1.4 % (\( p < 0.01 \)), respectively. *\( p < 0.05 \), **\( p < 0.01 \).
Orlistat treatment decreases plasma UCB concentrations in Gunn rats

**Effects of orlistat on plasma UCB concentrations**

Figure 2 shows the effects of the different diets on plasma UCB concentrations at 1.5 and 3 weeks. Plasma UCB concentrations did not significantly change in Gunn rats fed the control diet. In contrast, both orlistat-containing diets led to a decrease in plasma UCB concentration. At 3 weeks of treatment, plasma UCB concentrations were, respectively 26% (orlistat 100, p<0.05) and 40% (orlistat 200, p<0.01) lower than corresponding values in control rats. Compared with initial plasma concentrations of UCB (before starting orlistat treatment), concentrations were 33 ± 8% (orlistat 100, p<0.05) and 46 ± 10% (orlistat 200, p<0.01) lower after 3 weeks of treatment, respectively (difference between the orlistat 100 and the orlistat 200 mg/kg group: p=0.06).

Figure 3 shows the relation between fecal fat excretion and plasma UCB concentration after 3 weeks of control or orlistat-containing diet, based on data from individual animals. A negative, apparently linear relation between the parameters was observed (r = -0.87, p<0.001).
Effects of orlistat on biliary excretion of UCB and biliary lipids

After three weeks of feeding the control or experimental diet, biliary secretion of bilirubin and lipids was determined (Table 1). In either of the groups, virtually all bilirubin detected in Gunn rat bile was UCB as analyzed by high-performance liquid chromatography. In the control group, biliary bilirubin output rate tended to be slightly higher compared with those in the orlistat-treated groups, but the differences did not reach statistical significance. Biliary secretion rates of bile salts, phospholipids, and cholesterol were not significantly different among the three groups.

Table 1. Bile flow and biliary excretion rate of bilirubin and biliary lipids in Gunn rats on a high-fat diet after 3 weeks of orlistat treatment. Gunn rats were fed a high-fat diet without supplement (control) or supplemented with orlistat (100 mg/kg chow or 200 mg/kg chow) for 3 weeks, at the end of which bile was collected for 30 minutes. Data represent mean ± SD of control and orlistat-fed rats (n = 4-5 per group). NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Orlistat 100</th>
<th>Orlistat 200</th>
<th>P value</th>
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<tbody>
<tr>
<td>Bile flow</td>
<td>183 ± 8</td>
<td>203 ± 40</td>
<td>184 ± 37</td>
<td>NS</td>
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<tr>
<td>(µl/h/100 g)</td>
<td></td>
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<tr>
<td>Bilirubin</td>
<td>8.1 ± 3.3</td>
<td>6.2 ± 2.8</td>
<td>5.4 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>(nmol/h/100 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile salts</td>
<td>6.3 ± 1.0</td>
<td>8.2 ± 1.8</td>
<td>7.2 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>(µmol/h/100 g)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cholesterol</td>
<td>66 ± 19</td>
<td>77 ± 6</td>
<td>56 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>(nmol/h/100 g)</td>
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<tr>
<td>Phospholipids</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>NS</td>
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<tr>
<td>(µmol/h/100 g)</td>
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Long-term effects of orlistat on fat balance, body weight and growth

Feeding Gunn rats the orlistat-containing high-fat diet for a prolonged period did not lead to diarrhea (fecal dry weight as percentage of wet weight was 88.2 ± 2.0% at 2 weeks and 83.8 ± 3.4% at 20 weeks in controls, and 86.4 ± 4.4% at 2 weeks and 86.0 ± 6.0% at 20 weeks in orlistat 200 mg-treated rats, respectively (NS). The daily amount of orlistat ingested over this period was calculated as 6.7 ± 1.6 mg/day (~21 mg/kg body weight). Fat balance studies at 2, 8 and 20 weeks demonstrated a sustained stimulation of fecal fat excretion (Figure 4).

Figure 4. Prolonged dietary administration of orlistat to Gunn rats on a high-fat diet is associated with a sustained increase in fecal fat excretion. Amounts of fecal fat excretion in Gunn rats fed high-fat diet without supplement (control) or supplemented with orlistat (200 mg/kg chow). Experimental diets were fed for 24 weeks, and at 2, 8 and 20 weeks a 72-hour fat balance was performed. Values reflect mean ± SD, n = 4 per group. Amount of fat is expressed as percentage of the amount ingested, as determined by gas chromatography (see Methods section). *p<0.005, **p<0.0005, ***p<0.001.

The coefficient of fat absorption tended to increase over time in the orlistat-treated group (week 2, 78.8 ± 3.3%; week 8, 80.3 ± 6.8%; week 20; 85.8 ± 6.5%), but this difference did not reach statistical significance compared with corresponding control rats (week 2, 95.7 ± 1.0%; week 8, 96.4 ± 1.3%; week 20, 96.1 ± 1.2%, respectively). Similar to the dose-
dependent study (Figures 1-3), food intake in the orlistat group tended to be higher than in the control group (at 2 wk, 28.3 ± 8.1 vs. 22.5 ± 5.7 g/day; at 8 wk, 22.2 ± 12.3 vs. 17.0 ± 7.0 g/day; at 20 wk, 22.9 ± 10.0 vs. 21.5 ± 6.1 g/day, respectively. Similar to the 3-week orlistat-feeding experiment, net amount of fat uptake was similar in the two groups at 2, 8 and 20 weeks. Table 2 shows that neither growth rates, final body weights or plasma lipid concentrations were significantly different between the two dietary groups, nor plasma vitamin E or retinol levels (Table 2).

### Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Orlistat 200</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net amount of fat uptake (after 20 weeks, mmol/day)</td>
<td>9.9 ± 2.9</td>
<td>9.2 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Growth rate (% of initial body weight/24 wk)</td>
<td>13 ± 5</td>
<td>17 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Final body weight (gram)</td>
<td>360 ± 14</td>
<td>365 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Vitamin E (µmol/l)</td>
<td>59 ± 15</td>
<td>67 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Retinol (µmol/l)</td>
<td>3.8 ± 0.8</td>
<td>3.9 ± 0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

### Long-term effects of orlistat on plasma UCB concentrations

Figure 5 shows plasma UCB concentrations in control and orlistat-treated Gunn rats during a 24-week feeding period. Already after 2 weeks of treatment, plasma UCB concentrations were decreased by 38% in orlistat-treated animals compared with controls, and this difference remained relatively constant thereafter. After 24 weeks on the control or experimental diet, the biliary secretion rates were similar for bilirubin (5.6 ± 2.5 vs. 5.6 ± 1.2 nmol/h per 100 g), bile salts (7.0 ± 2.5 vs. 8.6 ± 2.4 µmol/h per 100 g), phospholipids (1.3 ± 0.3 vs. 1.5 ± 0.3 µmol/h per 100 g), and cholesterol (62 ± 12 vs. 63 ± 11 nmol/h per 100 g), respectively (each NS). Bile flow was 214 ± 18 µl/h per 100 g in control rats and 243 ± 69 µl/h per 100 g in orlistat-treated rats (NS).

### Figure 5. The effects of orlistat on plasma UCB concentrations in Gunn rats on a high-fat diet are sustained during treatment for 24 weeks. Plasma UCB concentration, determined by high-performance liquid chromatography, in Gunn rats fed a high-fat diet without supplement (control) or supplemented with orlistat (200 mg/kg chow). Experimental diets were fed for 24 weeks, after a 4 week run-in period on high-fat diet. In orlistat-treated rats, plasma UCB concentrations were continuously lower than in controls. *p<0.05, **p<0.01.
Similar to the study described above, in which orlistat treatment was continued for 3 weeks, an negative relation between fecal fat excretion and plasma UCB concentrations was observed (data pooled from fat balances at 2, 8 and 20 weeks, n = 24; r = -0.57, p<0.005).

**Effects of orlistat on fecal UCB excretion**

It is estimated that fecal UCB excretion only accounts for approximately 50% of total UCB turnover in Gunn rats.\(^\text{17}\) Fecal UCB excretion was similar in orlistat-treated and control rats at 2 weeks (175 ± 83 vs. 149 ± 49 nmol/day per 100 g body weight, NS). The similar amounts of fecal fat excretion suggest the presence of steady-state conditions, in agreement with stable plasma UCB concentrations beyond 2 weeks of treatment (Figure 5). Starting orlistat treatment, however, would then be expected to induce a transient increase in the fecal excretion of UCB and/or UCB-derived metabolites. Figure 6 shows that starting orlistat treatment is associated with a twofold increase in fecal UCB output within 48 hours of treatment (p<0.05). The increase in fecal UCB output was associated with a decrease in plasma UCB concentration, from 173 ± 18 µmol/l before to 138 ± 17 µmol/l at 24 hours of orlistat treatment (p<0.05). Plasma UCB concentrations at 48 hours and 72 hours were 143 ± 14 µmol/l and 138 ± 14 µmol/l, respectively.

**Figure 6.** Effects of starting orlistat treatment on fecal excretion of UCB in Gunn rats on a high-fat diet. Fecal UCB excretion, determined by high-performance liquid chromatography, in Gunn rats that were switched from a high-fat control diet (day –1, run-in period 4 weeks), to the high-fat diet with orlistat (200 mg/kg chow; day 1, 2, and 3). Fecal UCB excretion at day 2 and day 3 after starting treatment was significantly higher compared with fecal UCB excretion before starting orlistat treatment (day -1). Parallel to the increase in fecal UCB excretion, plasma UCB concentration decreased (-20% after 1 day orlistat treatment, p<0.05) and fecal fat excretion increased (+58% at day 3, p<0.05), compared with corresponding day -1 values. \(^*\)p<0.05.

**DISCUSSION**

We recently hypothesized that the neonatal accumulation of UCB is inversely related to the amount of fat excreted via the feces.\(^\text{22}\) In the current study we confirmed the validity of this concept in an animal model. In particular, we determined whether stimulation of fecal fat excretion by the lipase inhibitor orlistat was associated with a decrease in plasma UCB concentrations in Gunn rats, a well-established genetic model for unconjugated hyperbilirubinemia. The results indicate that treatment with orlistat was indeed associated with a sustained decrease in plasma UCB concentrations, without apparent effects on body weight, growth rate, or plasma lipid concentrations. Within one day after starting orlistat
Orlistat treatment decreases plasma UCB concentrations in Gunn rats

treatment in Gunn rats, plasma UCB concentration decreased in parallel with an increased fecal excretion of UCB.

In patients with Crigler-Najjar disease type I and in Gunn rats, UCB is not glucuronidated due to the genetic absence of the enzyme UGT1A1. The phenotype of Gunn rats is characterized by an unconjugated hyperbilirubinemia at a high, relatively stable concentration. Since steady-state UCB production and total turnover rates in untreated Gunn rats are the same as in normal rats, the elevated plasma and organ concentrations of UCB must be accompanied by a similar net disposal of UCB from the body by metabolism or excretion as observed under physiologic circumstances. It has been demonstrated that UCB is secreted, to a limited extent, into the bile of Gunn rats, and that UCB can also reach the intestinal lumen via a transintestinal route. The efficacy of UCB disposal into the intestinal lumen in terms of UCB excretion, however, is counteracted by reabsorption of UCB. Previously, it had been demonstrated that the intraluminal presence of calcium phosphate, which binds UCB in vitro, decreases plasma UCB concentrations in Gunn rats. Capture of UCB in the intestine decreases unbound UCB, increasing the plasma-to-lumen gradient and thereby increasing passive diffusion of UCB from plasma to intestinal lumen.

As a model for controlled stimulation of fecal fat excretion we applied supplementation of orlistat to a semisynthetic high-fat diet. We recently demonstrated that orlistat supplementation to high-fat diets allowed for regulated manipulation of the coefficient of fat absorption in rats. Orlistat is a chemically synthesized derivative of the natural product lipstatin, and specifically inhibits lipases at their catalytic triad serine153-histidine264-aspartate177 by covalent binding to the serine residue. The catalytic triad is a highly conserved feature of many biological lipases; the lipases in the gastrointestinal tract which are dose-dependently inhibited by orlistat are gastric lipase, pancreatic lipase, and carboxyl ester lipase. Orlistat treatment has been applied for up to two years in weight reduction programs for persons with obesity, without serious side effects. The observed clinical effects with respect to weight reduction or control, however, were limited. Our present data in Gunn rats also do not indicate the presence of serious side effects during long-term treatment. The present application of orlistat in Gunn rats, however, is clearly distinct from that in the clinical anti-obesity studies. In the Gunn rat study, orlistat was successfully used to increase fecal fat excretion. Interestingly, the orlistat-treated rats had similar growth rates and final body weights after 3 or 24 weeks of orlistat treatment. From fat balance measurements it appeared that net amount of fat uptake was similar in orlistat-treated and control rats, associated with the observed tendency to increase food intake during orlistat treatment. The mechanism underlying this compensatory phenomenon is presently unclear, but may bear resemblance to the rather limited clinical effects of orlistat during weight reduction regimens. Another potential side effect of long-term orlistat treatment, namely deficiency of fat-soluble vitamins, did not occur during the course of our study, probably attributable to
the relatively mild malabsorption induced and to the similar net uptake of fat and, by inference, probably also of fat-soluble vitamins in these ad libitum-fed animals.

Strong, negative correlations were observed between fecal fat excretion on one hand and plasma UCB concentration on the other hand. The observed association is compatible with the hypothesis that UCB associates with (subfractions of) unabsorbed fat in the intestinal lumen. McDonagh demonstrated in an elegant fashion that unconjugated bilirubin admixed in buffer completely partitions into an (olive) oil phase upon vigorous shaking.\(^53\) The physicochemical nature of the lipophilic phase of unabsorbed dietary fat in the intestinal lumen is unexplored. The present data do not directly demonstrate that UCB enters the core of the intraluminal lipophilic phase (although available data indicate the feasibility\(^53\)). It cannot be excluded that UCB associates with polar lipids or soluble amphiphiles at the surface of the lipophilic phase, for example with partially hydrolyzed triacylglycerols, fatty acids or phospholipids.\(^54;55\) Thin layer chromatography of fecal lipids during orlistat treatment indicated that a major fraction of unabsorbed fatty acids was present as fatty acids (~60-70%), and the remainder as intact triacylglycerols, mono- and diacylglycerides and phospholipids (data not shown). Association with unabsorbed phospholipids would be in accordance with the previously demonstrated affinity of UCB for phospholipid membranes.\(^56\)

The stable plasma UCB concentrations after 2 weeks of orlistat treatment (Figure 5) indicate that a (new) steady-state condition is apparent after 2 weeks of treatment. After 2 weeks, but not after 3 days (Figure 6), fecal UCB excretion rate was similar in orlistat treated and control rats, also similar to values obtained before starting orlistat treatment (approximately 150-200 nmol/day per 100 g body weight), and, finally, also similar to those described previously in this specific strain of Gunn rats (RHA/jj).\(^7;17;46\) Fecal UCB only contributes ~50% to total bilirubin turnover, the remainder being predominantly urobilinoids.\(^7;17\) The quantitative determination of fecal urobilinoids is notoriously difficult, partly due to instability. Theoretically, orlistat treatment could mediate its effects on plasma bilirubin concentrations by interrupting the enterohepatic cycling of UCB through increasing intestinal UCB metabolism, for example, by alteration of the bacterial flora. Our present results on fecal UCB excretion during short-term (0-4 days) or long-term (> 2 weeks) orlistat treatment are almost identical to those obtained by van der Veere et al., who fed Gunn rats a control or a high calcium phosphate diet. The latter transiently increased fecal excretion of UCB.\(^17\) The observation that the enhanced fecal UCB excretion during orlistat treatment disappears after 2-3 weeks may seem counterintuitive, since the beneficial effects on plasma remain. However, the similar fecal disposal in the new steady-state during orlistat treatment occurs at significantly lower plasma UCB concentrations. The lowering of the plasma UCB level balances the lowering of the unbound UCB in the intestinal lumen due to binding to fat, reverting the plasma-to-lumen gradient to its initial value, though with lower plasma and tissue UCB pools. Apparently, orlistat treatment allows a more efficient extraction (retention) of UCB in the intestinal lumen. Also, fecal disposal of UCB and of its derived metabolites
Orlistat treatment decreases plasma UCB concentrations in Gunn rats

would only alter (in steady-state conditions) if the production rate of UCB would be affected by orlistat. A kinetic turnover study using radiolabeled UCB would be helpful to confirm the present concept.

Increased intraluminal concentrations of bile salts have been hypothesized to enhance reabsorption of UCB in patients with Crohn’s disease. In the present study we did not control for coprophagy, and theoretically, differences between the groups in coprophagy could relate to differences in the availability of intestinal bile salts. Yet, the similar biliary secretion rates of bile salts are not compatible with major differences in the intestinal concentrations of bile salts. Finally, the results from the acute experiment do not support the theoretical possibility that the effects may be related to increased intestinal motility by orlistat.

Introduction of orlistat treatment induced a decrease in plasma bilirubin concentrations within 24 hours, which appeared to precede the increase in fecal bilirubin excretion (~48 hours, Figure 6). This observation strongly suggests that UCB is shifted from the plasma into another compartment, most likely the intestine, but is not rapidly disposed from the body. The one day lag in increase in fecal UCB excretion after orlistat is started probably reflects the transit time from duodenum to feces.

At present we cannot conclude whether the current successful approach in Gunn rats can be applied for clinical conditions with unconjugated hyperbilirubinemia (increased hemolysis, Crigler-Najjar disease, neonatal hyperbilirubinemia). The fecal excretion of unabsorbed dietary fat may play a role in the pathophysiology of (human) neonatal jaundice. Some optimism seems justified, based on several present observations: orlistat treatment does not necessarily have to affect the net amount of energy uptake and thus growth; orlistat is able to exert its effect on plasma UCB concentrations within days; in the dosages used, no major clinical symptoms of steatorrhoea are to be expected; orlistat has been applied in studies in adults for two years without serious side effects; and finally, the potentially decreased absorption of fat-soluble vitamins (which we did not observe after 24 weeks of treatment) could be overcome by supplementation. For neonatal jaundice, one would probably only need to stimulate fecal fat excretion for 1-2 weeks, since after that the bilirubin production profoundly decreases. No studies with orlistat used in neonates have been reported, however, and it cannot be excluded that the absence of serious side effects may be age-related. Nevertheless, it seems justified to investigate further whether the current concept could lead to a new preventive and therapeutic approach for unconjugated hyperbilirubinemia.
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