Effective oral treatment of unconjugated hyperbilirubinemia in Gunn rats

CHAPTER

4

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ABSTRACT

We aimed to develop an oral treatment for unconjugated hyperbilirubinemia. In the Gunn rat model of unconjugated hyperbilirubinemia, dietary supplementation with the lipase inhibitor orlistat (Orl) or with calcium phosphate (CaP) decreases plasma unconjugated bilirubin (UCB) levels. We determined whether Orl, CaP, or their combination is superior to the conventional treatment, phototherapy (PT), and whether effects of Orl and CaP are influenced by dietary fat content. Gunn rats were treated with Orl (200 mg/kg chow), CaP (20 g/kg chow), Orl + CaP, or continuous PT (19 µW/cm²/nm) during low-fat diet (LF, 13 energy%) or high-fat diet (HF, 35 energy%). Plasma UCB and fecal fat excretion were measured before, during and/or at the end of treatment. Orlistat treatment for 2 weeks (HF diet) reduced plasma UCB concentrations similarly as phototherapy (-34%; -28%, respectively); combination of both was more effective than either treatment alone (-48%, p<0.001). After 3 weeks HF diet, plasma UCB was 46% lower compared with LF diet (p<0.001). Plasma UCB concentrations were negatively correlated with fecal fat excretion (r = -0.96, p<0.001). Irrespective of dietary fat content, 3 weeks of combined treatment (Orl + CaP) decreased plasma UCB by ~50% (p<0.01), and was more effective than phototherapy (p<0.05), at the intensity provided. In conclusion, plasma UCB concentrations in Gunn rats are negatively related to fecal fat excretion and dietary fat content. Orlistat is equally effective as phototherapy for treatment of unconjugated hyperbilirubinemia in Gunn rats, and combined oral treatment with Orl + CaP is more effective than phototherapy. Present results support the feasibility of an efficient oral treatment of unconjugated hyperbilirubinemia.
INTRODUCTION

Crigler-Najjar disease is characterized by a permanent unconjugated hyperbilirubinemia due to absent (type I) or decreased (type II) activity of the hepatic enzyme bilirubin-UDP-glucuronosyltransferase.\textsuperscript{1} Severe unconjugated hyperbilirubinemia can lead to bilirubin encephalopathy, kernicterus, and death.\textsuperscript{2,3} Phenobarbital treatment can usually control unconjugated hyperbilirubinemia in Crigler-Najjar type II patients via residual enzyme induction.\textsuperscript{4,5} Phenobarbital is not effective in Crigler-Najjar disease type I, however, and these patients have to undergo daily phototherapy, which has considerable disadvantages. Phototherapy becomes less effective with age, probably due to skin alterations,\textsuperscript{6,7} to a decrease in the surface area to body mass ratio,\textsuperscript{8} and to a diminishing compliance to the intensive phototherapy regimen, which may take up to 12 hours per day.\textsuperscript{6} To prevent irreversible brain damage due to kernicterus, many patients with Crigler-Najjar disease type I undergo liver transplantation in their second decade.\textsuperscript{9,10}

We aimed to develop an alternative treatment for unconjugated hyperbilirubinemia that is based on oral administration and that has an equal or higher efficacy than phototherapy. The oral treatment strategy used in the present study is based on reducing reabsorption of UCB\textsuperscript{11,12} through intestinal capture. Reabsorption of UCB can contribute substantially to the pathogenesis of unconjugated hyperbilirubinemia, as for instance in neonatal jaundice. Even under conditions of diminished glucuronidation, bilirubin can enter the intestinal lumen via biliary secretion of low amounts of UCB.\textsuperscript{13} In addition, UCB can diffuse from the blood, across the intestinal mucosa, into the intestinal lumen.\textsuperscript{14,15} Particularly when plasma UCB levels are high, as in Crigler-Najjar disease, large amounts of UCB can enter the intestinal lumen via extrabiliary (transintestinal) excretion.\textsuperscript{14,15} In humans, under certain conditions up to 25\% of the total amount of bilirubin that enters the intestine might be reabsorbed as UCB.\textsuperscript{16} Amorphous calcium phosphate was shown to bind to UCB \textit{in vitro},\textsuperscript{17} and intestinal capture of UCB by calcium phosphate decreased plasma UCB concentrations in Gunn rats,\textsuperscript{18} a well-established animal model for Crigler-Najjar disease type I.\textsuperscript{19,20} In Crigler-Najjar patients however, the effects of calcium phosphate treatment were less pronounced.\textsuperscript{7} Other capturing agents like agar,\textsuperscript{21} activated charcoal,\textsuperscript{22} and cholestyramine\textsuperscript{23} are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects.\textsuperscript{24-26} More recently, zinc salts were shown to decrease plasma bilirubin levels in patients with Gilbert’s syndrome, but simultaneously serum zinc levels increased.\textsuperscript{27} Other pharmacological interventions for treatment of neonatal jaundice and Crigler-Najjar disease include metalloporphyrins, which inhibit heme degradation, and modified bilirubin oxidase. Concerns about safety and efficacy have so far limited widespread use.\textsuperscript{28,29}

Recently, we demonstrated that dietary supplementation with the lipase inhibitor orlistat decreased plasma UCB concentrations in Gunn rats, parallel to an increase in fecal fat excretion.\textsuperscript{30} The decrease in plasma UCB concentration was strongly related to the amount of
fat excreted via the feces, supporting the concept of intestinal capture of UCB. This observation raised the question whether dietary fat content influences plasma UCB concentration. It was also unknown whether orlistat treatment, or combined treatment with orlistat and calcium phosphate, is similarly or more effective in reducing plasma UCB levels than the conventional treatment, phototherapy. In the present study, we addressed these issues by first comparing in Gunn rats the efficacy of orlistat with that of phototherapy. Secondly, we studied the influence of dietary fat content on plasma UCB concentration in control Gunn rats and in Gunn rats treated with orlistat, calcium phosphate, or with both. Finally, combined treatment with orlistat and calcium phosphate was compared with continuous phototherapy in Gunn rats.

METHODS

Materials

Animals. Homozygous male Gunn rats (RHA/jj), weighing 210 to 270 g, were obtained from the breeding colony of the Academic Medical Center, (Amsterdam, The Netherlands). All animals were housed in an environmentally controlled facility with a 12-12 hour light/dark cycle, were fed ad libitum and had free access to water. Animals were housed individually or, in case of phototherapy treatment, per experimental group. Experimental protocols were approved by the Ethics Committee for Animal Experiments (Faculty of Medical Sciences, University of Groningen, The Netherlands).

Phototherapy lamps. Two phototherapy devices were developed according to the prototype designed by Ostrow. Each device consisted of two blue phototherapy lamps (Philips, TL 20W/03T) suspended in a reflective canopy 20 cm above the bottom of the cage. Phototherapy (19 µW/cm\(^2\)/nm from 380-480 nm, as measured by an Elvos LM-1010 Lux meter at 20 cm distance) was administered continuously to Gunn rats that were shaven every 7-10 days on their backs and flanks. The light intensity at the level of the rat’s back was therefore higher than 19 µW/cm\(^2\)/nm.

Chemicals. Xanthobilirubin-methyl ester was a generous gift from Dr. J. Fevery (Leuven, Belgium). Heptadecanoic acid (C17:0) was purchased from Sigma Chemical Co. (St Louis, MO). Orlistat (Xenical®) was obtained from Roche Nederland BV (Woerden, The Netherlands). Orlistat is a selective inhibitor of gastrointestinal lipases that dose-dependently inhibits hydrolysis of dietary triglycerides.

Diets. Diets were custom synthesized by Hope Farms BV (Woerden, The Netherlands). The high-fat (HF) control diet (code 4141.07) was a semisynthetic, purified diet containing 35 energy% fat and 16.2 wt% long-chain fatty acids (fatty acid composition (in mol%): C8-C12:0, 1.7; C14:0, 1.3; C16:0, 11.9; C16:1, 1.2; C18:0, 1.1; C18:1, 21.6; C18:2, 53.3; C18:3, 8.0). The low-fat (LF) control diet (code 4063.02) was a semisynthetic, purified diet containing 13 energy% fat and 5.2 wt% long-chain fatty acids (fatty acid composition (in
mol%): C8-C12:0, 6.9; C14:0, 0.7; C16:0, 30.0; C18:0, 3.7; C18:1, 29.9; C18:2, 28.8). Supplemented diets were identical to control diets except for supplementation with orlistat (200 mg/kg chow) and/or calcium phosphate (20 g/kg chow). Codes of these diets were: HF + orlistat: 4141.13; HF + CaP: 4141.15; HF + orlistat + CaP: 4141.16 and LF + CaP: 4063.04. For LF diet studies, orlistat (200 mg/kg chow) was mixed into diets 4063.02 and 4063.04. Similar to previous studies, Gunn rats in all experiments were fed the control diets for a run-in period of at least 4 weeks. All diets were semisynthetic and purified for comparability. The composition of the LF control diet was comparable with standard rat chow (RMH-B; Hope Farms BV, Woerden, The Netherlands). The HF control diet was chosen to contain approximately 35 energy% fat, thus resembling human dietary fat intake in an industrialized country.

**Study Design**

**Effects of orlistat and/or phototherapy on plasma UCB concentrations**

Three groups of Gunn rats (n = 4-5 per group) on HF diet were randomly assigned to the orlistat-supplemented diet, continuous phototherapy, or to the combination of orlistat-supplemented diet and continuous phototherapy for 2 weeks. Before starting treatment and after 1 and 2 weeks of treatment, blood samples were obtained by tail bleeding under isoflurane anesthesia for determination of plasma UCB concentrations. After 2 weeks of treatment, the enterohepatic circulation was interrupted through surgical cannulation of the common bile duct, after which bile was collected for 20 minutes under light-protected conditions. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. After bile collection, a large blood sample was obtained by vena cava inferior puncture.

**Effects of orlistat and/or calcium phosphate on plasma UCB concentrations and fecal fat excretion during LF or HF diet**

After a run-in period of 7 weeks on LF diet, 4 groups of Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Both diets were either not supplemented (controls), or supplemented with orlistat, calcium phosphate, or with both. Blood samples were obtained every 1.5 weeks by tail bleeding under isoflurane anesthesia. Feces were collected per animal after 2.5 and 5.5 weeks during 72 hours to determine fecal fat and calcium excretion. Plasma UCB, fecal fat, and fecal calcium concentrations were determined by HPLC, gas chromatography, and flame spectrometry, respectively (see Analytical Methods).

**Effects of phototherapy compared with combined oral treatment with orlistat and calcium phosphate**

We compared the efficacy of continuous phototherapy with the efficacy of combined oral treatment with orlistat and calcium phosphate. Three groups of Gunn rats (n = 5 per group)
were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. One group was continuously treated with phototherapy during these 6 weeks. The diets of another group were supplemented with orlistat and calcium phosphate. Blood samples were obtained every 1.5 weeks by tail bleeding under isoflurane anesthesia.

**Analytical Methods**

**Plasma.** For UCB measurements, blood samples were protected from light and processed immediately. Plasma was submitted to alkaline methanolysis and chloroform extraction. Theoretically, it is not necessary to use alkaline methanolysis for determination of plasma UCB concentrations in Gunn rats. This standard method was nevertheless chosen since it is a validated HPLC method for clinical samples of patients with an undetermined type hyperbilirubinemia, and since it had been used in previous studies. After evaporation under nitrogen, the residue was re-dissolved in chloroform and analyzed by reversed-phase HPLC, as previously described, using a Li-Chrosorb 5160-5µm column (VDS optilab, Montabaur, Germany), a detection wavelength of 430 nm, and xanthobilirubin-methyl ester as internal standard. Plasma hemoglobin (Hb) and hematocrit (Ht) were determined on a Sysmex XE-2100 hematology analyzer (Goffin Meyvis, Etten-Leur, The Netherlands). Aspartate-aminotransferase activity (AST), alanine-aminotransferase activity (ALT), triglycerides (TG) and cholesterol were determined with routine clinical chemical procedures on a Mega analyzer (Merck, Darmstadt, Germany).

**Bile.** All analytical procedures were performed in dim light. UCB was extracted from bile according to the method described above for UCB in plasma. Bile salt concentration was determined by the 3α-hydroxysterol dehydrogenase method. Cholesterol and phospholipids were measured after lipid extraction, according to methods of Gamble et al., and Bötcher et al., respectively.

**Feces.** Feces were freeze-dried for at least two days and mechanically homogenized. For determination of fatty acids, aliquots of freeze-dried feces were extracted, hydrolyzed and methylated according to the method of Lepage and Roy, with the modification that methanol/hexane was used for methylation and extraction. Resulting fatty acid methyl esters were determined by gas chromatography (HP Ultra-1-column, Hewlett-Packard, Palo Alto, CA) and fatty acid contents were calculated in molar amounts, using C17:0 as internal standard. Determination of calcium concentration was performed in duplicate in plastic tubes as follows. Two aliquots of approximately 10 mg freeze-dried feces were taken from homogenized feces and weighed. One ml of 69% HNO₃ was added and the mixture was heated at 95°C for 5 minutes, after which 5 ml of 0.1% lanthanum chloride (LaCl₃) was added. After mixing, the samples were centrifuged for 10 minutes at 1500g. The supernatant was diluted 20 times with 0.1% LaCl₃ and filtered. Calcium concentration was determined by flame spectrometry (Atomic absorption spectrometer 3300, PerkinElmer BV, The Netherlands).
Statistical Analyses
Analyses were performed in SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). All results are expressed as mean ± SD. Based on a normal distribution of plasma bilirubin levels in large groups of Gunn rats in previous studies, parametric tests were used for statistical analysis. Student t was used to test between two treatment groups. For comparison of more than two treatment groups, analysis of variance (ANOVA) with post-hoc Bonferroni correction was performed. Repeated-measures ANOVA was used for analysis of within-group differences. Linear regression analysis was performed to compare treatment efficacies when LF- and HF diets were used consecutively, and to analyze the relationship between fecal fat excretion and plasma UCB concentration. The level of significance was set at a P value <0.05 (two-tailed).

RESULTS

Effects of orlistat and/or phototherapy on plasma UCB concentrations
Figure 1 shows the effects of orlistat, continuous phototherapy, and combined treatment on plasma UCB levels in Gunn rats fed HF diet. Orlistat treatment decreased plasma UCB concentrations by 34% after 2 weeks of treatment (p<0.01), similarly as continuous phototherapy (-28%, p<0.01). Combined treatment with orlistat and phototherapy induced a more profound decrease in plasma UCB concentrations than either orlistat or phototherapy alone (-48%, p<0.001). Compared with pre-treatment values, one week of treatment decreased plasma UCB concentrations by 16% (orlistat, p=0.06), 15% (phototherapy, p<0.01) and 43% (orlistat + phototherapy, p<0.01), indicating that combined treatment decreased plasma UCB concentrations more rapidly. The three groups did not significantly differ in growth rates during the experiment, in accordance with our previous experience that orlistat treatment does not affect the net amount of energy uptake or growth rate in Gunn rats.

![Figure 1. Effects of orlistat, continuous phototherapy (PT) and combined treatment (orlistat + PT) on plasma UCB concentrations in Gunn rats. Animals (n = 4-5 per group) were fed HF diet for 4 weeks, followed by treatment for 2 weeks with dietary orlistat supplementation, PT, or orlistat + PT. Blood samples were taken before treatment ( ), and after 1 ( ) and 2 weeks of treatment ( ). Plasma UCB values at T0 (µmol/l): orlistat, 159 ± 16; PT, 135 ± 7; orlistat + PT, 145 ± 14. Data represent mean ± SD. *p<0.01, **p<0.001, †p=0.06, compared with before treatment. ^p<0.01.](image)
Table 1. Plasma parameters after two weeks of treatment

<table>
<thead>
<tr>
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<th>orlistat</th>
<th>phototherapy</th>
<th>orlistat + phototherapy</th>
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<tbody>
<tr>
<td>Hb (mmol/l)</td>
<td>8.6 ± 0.3</td>
<td>8.4 ± 0.3</td>
<td>8.6 ± 0.4</td>
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<tr>
<td>Ht (l/l)</td>
<td>0.41 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.41 ± 0.02</td>
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<tr>
<td>AST (U/l)</td>
<td>21.0 ± 4.7</td>
<td>23.6 ± 2.8</td>
<td>24.4 ± 9.4</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>63.0 ± 12.2</td>
<td>57.6 ± 12.1</td>
<td>48.2 ± 11.7</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.2 ± 0.8</td>
<td>1.9 ± 0.6</td>
<td>1.7 ± 0.3</td>
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Table 1 shows that relevant hematological and liver function parameters did not differ among the three treatment groups. Also, bile flow rates and biliary secretion rates of bile salts, cholesterol and phospholipids were similar after 2 weeks of treatment with orlistat, phototherapy, or their combination (Table 2). Biliary secretion rate of UCB was higher in the two groups that received phototherapy, compared with the orlistat treated group (phototherapy, +280%, p<0.01; phototherapy + orlistat, +180%, p<0.01).

Table 2. Bile flow and biliary excretion rate of UCB and biliary lipids after two weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>orlistat</th>
<th>phototherapy</th>
<th>orlistat + phototherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (µl/min/100g BW)</td>
<td>3.19 ± 0.49</td>
<td>3.39 ± 1.08</td>
<td>3.73 ± 0.83</td>
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<tr>
<td>Bilirubin (nmol/min/100g BW)</td>
<td>0.09 ± 0.01</td>
<td>0.34 ± 0.12*</td>
<td>0.25 ± 0.08*</td>
</tr>
<tr>
<td>Bile salts (nmol/min/100g BW)</td>
<td>153.9 ± 49.2</td>
<td>159.5 ± 105.6</td>
<td>162.4 ± 57.0</td>
</tr>
<tr>
<td>Cholesterol (nmol/min/100g BW)</td>
<td>0.77 ± 0.31</td>
<td>0.74 ± 0.23</td>
<td>1.02 ± 0.29</td>
</tr>
<tr>
<td>Phospholipids (nmol/min/100g BW)</td>
<td>18.6 ± 6.9</td>
<td>18.6 ± 7.5</td>
<td>26.9 ± 8.9</td>
</tr>
</tbody>
</table>

Table 2. Gunn rats were fed HF diet for 4 weeks, followed by treatment for 2 weeks with dietary orlistat supplementation, continuous phototherapy, or orlistat + continuous phototherapy. After 2 weeks bile was collected during 20 minutes. Data represent mean ± SD from n = 4-5 animals per group. *p<0.01, compared with orlistat.

Effect of dietary fat content on plasma UCB concentrations and fecal fat excretion

Figure 2 shows that dietary fat content has a profound effect on plasma UCB concentration in Gunn rats. Changing from LF to HF diet decreased plasma UCB concentrations by 46% after 3 weeks (p<0.01). Fecal fat excretion increased from 0.07 ± 0.03 mmol/24h on LF diet to 0.74 ± 0.12 mmol/24h on HF diet. Consistent with our previous observation that an increased fecal fat excretion is associated with an increased fecal UCB excretion, plasma UCB concentrations were negatively correlated with fecal fat excretion (r = -0.96, p<0.001).

Effects of orlistat and/or calcium phosphate on plasma UCB concentrations, fecal fat excretion and fecal calcium excretion during LF or HF diet

Figure 3 shows the efficacies of orlistat and/or calcium phosphate treatment during LF- and HF diet. During LF diet, treatment with either orlistat or calcium phosphate decreased plasma UCB concentrations compared with controls by 30% (p<0.05) and 40% (p<0.001),
respectively. During HF diet, plasma UCB concentrations in orlistat treated animals were 28% lower compared with untreated controls (p<0.01), whereas calcium phosphate treatment did not significantly decrease plasma UCB levels (-21%, NS). Combined treatment with orlistat and calcium phosphate decreased plasma UCB concentrations by 54% on LF diet (p<0.01) and by 44% on HF diet (p<0.01). During both LF and HF diet, combined oral treatment was more effective in reducing plasma UCB concentrations than calcium phosphate alone (p<0.05). When compared with orlistat, combined treatment was only more effective during LF diet (p<0.05).

**Figure 2.** Effect of dietary fat content on plasma UCB concentrations in Gunn rats (left panel), and relationship between fecal fat excretion and plasma UCB concentrations (right panel). Gunn rats (n=5) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Data after 3 and 6 weeks of diet are shown and represent mean ± SD. *p<0.01. Plasma UCB values (µmol/l): LF diet, 248 ± 31; HF diet, 135 ± 10. Fecal fat excretion (72h) was determined after 2.5 and 5.5 weeks. Each symbol represents data obtained in an individual animal. r = -0.96, p<0.001.

**Figure 3.** Effects of orlistat (Orl), calcium phosphate (CaP), and their combination (Orl + CaP) on plasma UCB concentrations in Gunn rats, during LF diet and HF diet. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), or supplemented with orlistat, calcium phosphate, or both. Data after 3 and 6 weeks of treatment are shown and represent mean ± SD. Plasma UCB values (µmol/l): LF diet: controls, 248 ± 31; Orl, 173 ± 26; CaP, 150 ± 31; Orl + CaP, 114 ± 14; HF diet: controls, 135 ± 10; Orl, 97 ± 6; CaP, 106 ± 8; Orl + CaP, 76 ± 7. *p<0.05, **p<0.01, ***p<0.001, compared with controls. #p<0.05; NS, not significant.

Figure 4 shows the relationship between fecal fat excretion and plasma UCB concentration of individual Gunn rats from the different groups (controls, orlistat, calcium phosphate, and orlistat + calcium phosphate) after 3 weeks of LF or HF diet. The two parameters were negatively correlated (r = -0.87; p<0.001). When the relationship between fecal fat excretion
and plasma UCB concentration was analyzed separately for controls and calcium phosphate treated Gunn rats (Figure 5), it appeared from the two different data sets that the amount of fat in the diet influenced the efficacy by which calcium phosphate decreased plasma UCB concentrations. The higher efficacy of calcium phosphate on LF diet (UCB -40%), compared with HF diet (UCB -21%), corresponded with a relatively larger increase in fecal fat excretion on LF diet (+199%) versus HF diet (+95%) upon calcium phosphate supplementation. On LF diet, there is likely less fat-bound calcium and UCB and thus more unbound calcium available to trap more unbound UCB in the intestine.

Figure 4. Relationship between fecal fat excretion and plasma UCB concentrations in Gunn rats. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), or supplemented with orlistat, calcium phosphate, or both. Feces were collected per animal after 2.5 and 5.5 weeks during 72h to determine fecal fat excretion. Each symbol represents data obtained in an individual animal. r = -0.87, p<0.001.

Figure 5. Relationship between fecal fat excretion and plasma UCB concentrations in Gunn rats, analyzed separately for controls and calcium phosphate treated animals during LF and HF diet (see Figure 4).

Figure 6 shows that a positive correlation existed between fecal calcium excretion and fecal fat excretion, on LF diet with or without calcium and/or orlistat supplementation (r = 0.96, p<0.001), as well as on HF diet with or without supplementation (r = 0.93, p<0.001). Orlistat treatment alone increased fecal calcium excretion (mmol/24h) on LF diet (LF: 1.09 ± 0.19; LF + orlistat: 1.54 ± 0.28; p<0.05) but not on a HF diet (HF: 2.34 ± 0.38; HF + orlistat: 2.03 ± 0.09; NS; data not shown).

Effects of phototherapy compared with combined oral treatment with orlistat and calcium phosphate
We compared the efficacy of combined oral treatment with orlistat and calcium phosphate with the efficacy of continuous phototherapy. Figure 7 shows that phototherapy alone
Effective oral treatment of Gunn rats

decreased plasma UCB concentrations by 45% on LF diet (p<0.001) and by 29% on HF diet (p<0.001), compared with controls. On LF diet (-54%, p<0.05), as well as HF diet (-44%, p<0.01), combined oral treatment with orlistat and calcium phosphate was more effective in reducing plasma UCB concentrations than continuous phototherapy.

**Figure 6.** Relationship between fecal fat excretion and fecal calcium excretion in Gunn rats. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), or supplemented with calcium phosphate, or calcium phosphate + orlistat. Feces were collected per animal after 2.5 and 5.5 weeks during 72 hours to determine fecal fat excretion and fecal calcium excretion. Each symbol represents data obtained in an individual animal. LF diet (upper panel), r = 0.96, p<0.001; HF diet (lower panel), r = 0.93, p<0.001.

**Figure 7.** Efficacy of combined oral treatment with orlistat and calcium phosphate compared with continuous phototherapy in Gunn rats. Gunn rats (n = 5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Animals were either not treated (controls), or treated with continuous phototherapy (PT), or with orlistat + calcium phosphate (Orl + CaP). Data after 3 and 6 weeks of treatment are shown and represent mean ± SD. Plasma UCB values (µmol/l): LF diet: controls, 248 ± 31; PT, 137 ± 14; Orl + CaP, 114 ± 14; HF diet: controls, 135 ± 10; PT, 96 ± 9; Orl + CaP, 76 ± 7. *p<0.001, compared with controls. *p<0.05, #p<0.01.
DISCUSSION

We aimed to develop an efficient treatment for unconjugated hyperbilirubinemia that is based on oral administration and that has an equal or higher efficacy than phototherapy. Previously, we reported that treatment with the lipase inhibitor orlistat decreased plasma UCB concentrations in Gunn rats, a well-established model for unconjugated hyperbilirubinemia. In the current study, we show that orlistat treatment is equally effective as continuous phototherapy in Gunn rats, and that combined oral treatment with orlistat and calcium phosphate is more effective than continuous phototherapy, at the intensity of phototherapy provided. The dose of phototherapy used (19 µW/cm²/nm) was comparable with doses used for (single) phototherapy in hyperbilirubinemic human neonates. In the clinical setting sometimes intensive (double-sided) phototherapy is used with doses above 30 µW/cm²/nm.\(^{40}\) Understandably, our results can only refer to the use of phototherapy at the specific dose provided. As demonstrated previously,\(^{31}\) phototherapy increased the amount of UCB secreted into bile. The observation that phototherapy enhanced the efficacy of orlistat supports the proposed concept that orlistat treatment reduces reabsorption of UCB.

Rather than by intestinal capture of UCB by unabsorbed fat, orlistat might theoretically exert its hypobilirubinemic effect via other mechanisms, such as by influencing intestinal transit time, bile salt metabolism or intestinal microflora. The effects of orlistat on gastric emptying and intestinal transit time are equivocal. Guerciolini reported no significant effects of orlistat on intestinal transit time or gastric emptying,\(^{41}\) whereas others have reported accelerated gastric emptying, particularly after consumption of a fatty meal.\(^{42,43}\) In a previous study in Gunn rats,\(^{30}\) we showed that the decrease in plasma UCB levels preceded the increase in fecal bilirubin excretion during orlistat treatment. This observation does not support a significant role for an increased intestinal transit time to explain our present results. Orlistat treatment increases fecal fat excretion and might therefore increase fecal bile salt excretion. However, bile flow rates and biliary secretion rates of bile salts were similar after treatment with orlistat, phototherapy, or their combination (present study), and not different between controls and orlistat-treated Gunn rats.\(^{30}\) Similar biliary excretion rates of bile salts between controls and orlistat-treated Gunn rats are not compatible with major differences in the intestinal concentration of bile salts. Vitek \textit{et al.} recently showed that the intestinal microflora can substantially affect metabolism of bilirubin.\(^{44}\) Effects of orlistat on the composition of the intestinal microflora or on intestinal bilirubin metabolizing activity are not known. Previously, we found similar fecal UCB excretion rates in orlistat treated and control Gunn rats under steady-state conditions.\(^{30}\)

Fecal fat excretion was again negatively associated with plasma UCB concentration in Gunn rats, similar to our previous report.\(^{30}\) Present data indicate that plasma UCB levels are almost twice as high in Gunn rats fed LF diet compared with HF diet. Gollan \textit{et al.} showed that a fat-free diet increased plasma bilirubin concentrations threefold in Gunn rats.\(^{45}\) They
reported that dietary supplementation with a variety of fats largely reversed the increased hyperbilirubinemia, regardless of their fatty acid chain length or degree of saturation. Present results allow to put these observations into perspective. Plasma UCB concentration is strongly determined by the amount of fecal fat excretion, which in turn, is determined strongly by dietary fat content. Therefore, it seems justified to conclude that, under conditions of absent bilirubin conjugation, dietary fat content negatively determines plasma UCB concentration through affecting fecal fat, and probably UCB, excretion. Present data do not determine whether UCB actually associates with unabsorbed fat (partially hydrolyzed triacylglycerol, fatty acids, phospholipids). In vitro experiments will be needed to characterize the exact mechanism.

Orlistat treatment effectively reduced plasma UCB concentrations during both HF and LF diet. Calcium phosphate treatment, however, was only significantly effective during LF diet. Previously, van der Veere et al. showed that, in Gunn rats on LF diet, plasma UCB concentrations decreased by approximately 40%, similar to our current LF diet results. Calcium phosphate treatment in Crigler-Najjar type I patients, however, decreased plasma UCB levels only by 18%. In type II patients, calcium phosphate treatment was not effective, possibly because these patients did not receive phototherapy, which enhances biliary excretion of UCB. We hypothesize that dietary fat content could partly explain the lower efficacy of calcium phosphate treatment in Crigler-Najjar patients compared with Gunn rats. The human, Western type diet, is a HF diet containing 35-40 energy% fat, compared with the LF diet (13 energy% fat) of the Gunn rats in Van der Veere’s study. We used the identical LF diet in our studies. Furthermore, we have observed in Gunn rats that combined treatment with calcium phosphate and continuous phototherapy for 3 weeks decreases plasma UCB concentrations more effectively on LF diet (-70%) than on HF diet (-39%; Hafkamp, Verkade 2004; unpublished). An explanation for the low efficacy of calcium phosphate treatment during HF diet (compared with LF diet), could be that UCB capture (by fat) has reached a certain maximum and therefore calcium phosphate cannot act properly as capturing agent.

In our studies, fecal fat excretion was positively associated with fecal calcium excretion. Dietary supplementation with calcium phosphate has been shown to increase fecal fat excretion in rats and humans, probably by formation of calcium soaps. We cannot exclude that part of the effect of orlistat and of calcium phosphate is based on the formation of calcium-fatty acid soaps and subsequent capture of UCB by these soaps. The low efficacy of calcium phosphate treatment during HF diet, however, suggests that other mechanisms must be involved.

In summary, oral treatment of unconjugated hyperbilirubinemia with orlistat and calcium phosphate is effective in Gunn rats. Both treatments seem to induce intestinal capture of UCB, but apparently via different capture mechanisms. Whether orlistat, alone or in combination with calcium phosphate or phototherapy, could prevent unconjugated hyperbilirubinemia in humans is yet unknown. However, with respect to patient applicability, our results in Gunn
rats are encouraging. Calcium phosphate is presently being used in a number of Dutch Crigler-Najjar patients as an adjunct to phototherapy when plasma UCB concentrations reach dangerously high levels, most often during winter time (Sinaasappel 2004; unpublished). Since calcium phosphate is relatively more effective on LF diet, and a LF diet is healthier for other reasons, one would recommend a LF diet in combination with calcium phosphate. On the other hand, absolute plasma UCB concentrations are lower on HF fat diet than LF diet, therefore recommendations about LF or HF diet should be individualized for each patient. Calcium phosphate treatment had no side effects in Gunn rats treated for 30 weeks\textsuperscript{18} and has had up till now no apparent side effects in Crigler-Najjar patients. However, there are some concerns that prolonged treatment with high doses of calcium phosphate might cause calcium depositions in the kidneys. Orlistat treatment had no side effects in Gunn rats treated up to six months. Especially body weight, growth rate and plasma concentrations of fat soluble vitamins were not affected, despite the presence of mild fat malabsorption.\textsuperscript{30} In humans, orlistat acts locally in the gastrointestinal tract and systemic absorption is minimal (~1\%).\textsuperscript{41} Orlistat is widely applied for treatment of obesity. Clinical trials in adults lasting up to two years have not reported serious side effects.\textsuperscript{48} Recent studies in obese adolescents\textsuperscript{49} and prepubertal children\textsuperscript{50} indicate that short-term orlistat treatment is well-tolerated by children and has a side effect profile similar to that observed in adults. Side effects are generally mild, limited to gastrointestinal effects such as flatulence and oily leakage, and decrease with time. Obviously, growth and development are key issues in children and should be very carefully monitored. Nonetheless, our present and previous results with orlistat treatment of Gunn rats, and the absence of serious side effects in human obese adults and children so far, support the potential clinical applicability of orlistat for treatment of unconjugated hyperbilirubinemia, in particular Crigler-Najjar disease.

In conclusion, plasma UCB concentrations in Gunn rats are negatively related to fecal fat excretion and to dietary fat content. In Gunn rats, orlistat treatment is equally effective as phototherapy, and the combination of orlistat and calcium phosphate is more effective than phototherapy, at the intensity of phototherapy provided. Present results support the feasibility of an effective oral treatment of patients with unconjugated hyperbilirubinemia.
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