Summary and general discussion
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The research in this thesis focuses on oral treatment of unconjugated hyperbilirubinemia. Permanent unconjugated hyperbilirubinemia is a primary characteristic of Crigler-Najjar disease, due to an inherited deficiency of the hepatic enzyme bilirubin-UDP-glucuronosyltransferase (UGT1A1) necessary for glucuronidation of unconjugated bilirubin (UCB).\(^1\) UCB is the hydrophobic end product of heme catabolism that requires conjugation for its efficient secretion into bile. After biliary secretion, bilirubin is deconjugated in the biliary tree and intestinal lumen, followed by intestinal metabolism, reabsorption and/or fecal excretion. Unconjugated hyperbilirubinemia becomes visible as jaundice when plasma UCB concentration exceeds \(\sim 85 \, \mu\text{mol/l}\). Untreated, plasma UCB levels in Crigler-Najjar patients range between 350-800 \(\mu\text{mol/l}\). UCB accumulation can cause bilirubin-induced neurologic damage and kernicterus, resulting in physical and mental handicaps or even death.\(^2;3\)

Conventional treatment for Crigler-Najjar disease involves life-long daily phototherapy which has considerable disadvantages. Main problems are a decreasing efficacy with age and a profound impact of the intensive phototherapy regimen on the quality of (social) life.\(^4;5;6\)

We aimed to develop an alternative or additional treatment for unconjugated hyperbilirubinemia that is based on oral administration and intestinal capture of UCB. Particularly when plasma UCB levels are high as in Crigler-Najjar disease, a considerable amount of UCB diffuses from the blood into the intestinal lumen across the intestinal mucosa.\(^7;8\) Intestinal capture of UCB followed by enhanced fecal excretion was previously shown to reduce its enterohepatic circulation\(^9;10\) and to decrease plasma UCB concentration. We hypothesized that UCB could associate with fat in the intestine, considering the relatively lipophilic character of UCB.\(^11\) According to our hypothesis, enhancing fecal fat excretion would increase fecal UCB excretion and reduce the enterohepatic circulation of UCB (see figure 1).

**Figure 1.** Intestinal capture of UCB. **A:** In Crigler-Najjar disease, UCB can enter the intestine via biliary secretion and via transepithelial diffusion from the blood into the intestinal lumen, across the intestinal mucosa. UCB and its metabolites are partly excreted with the feces, and partly reabsorbed into the enterohepatic circulation (EHC). **B:** Capture of UCB in the intestine followed by enhanced fecal excretion reduces the enterohepatic circulation of UCB and subsequently decreases plasma UCB concentration.
Orlistat treatment increases fecal UCB concentration and decreases plasma UCB concentration in hyperbilirubinemic Gunn rats

In chapter 3, we investigated in the animal model for unconjugated hyperbilirubinemia, the Gunn rat, whether stimulation of fecal fat excretion increases fecal UCB excretion and decreases plasma UCB concentrations. We increased fecal fat excretion by dietary supplementation with the lipase inhibitor orlistat. Orlistat inhibits hydrolysis of triglycerides and thereby reduces dietary fat absorption. Short-term and long-term effects of orlistat treatment were studied. Within days, orlistat treatment increased fecal excretion of both fat and UCB. After 3 weeks of treatment, plasma UCB concentrations had decreased by approximately 46% at the highest dose of orlistat used (200 mg/kg chow). Plasma UCB concentrations were strongly, negatively correlated with fecal fat excretion. The increase in fecal UCB excretion was transient, in contrast to permanently decreased plasma UCB levels, strongly suggesting that a new steady-state in UCB homeostasis had been reached. Short-term orlistat treatment was thus successful, but, if orlistat was ever to be used for treatment of patients with unconjugated hyperbilirubinemia, its effects should be sustained and side effects should be acceptable. We therefore treated Gunn rats with orlistat during 24 weeks. Plasma UCB concentrations remained significantly lower (~35%) in treated rats compared with control Gunn rats throughout the 24 weeks, indicating that the hypobilirubinemic effects of orlistat were indeed sustained. Side effects were not observed. Orlistat treatment did not affect body weight, growth or plasma concentrations of lipids or fat soluble vitamins. Interestingly, net fat uptake was not affected because the rats compensated the increased fecal fat excretion by increasing their intake.

Effective oral treatment of unconjugated hyperbilirubinemia in Gunn rats

Having determined that orlistat treatment effectively decreased plasma UCB concentrations in Gunn rats, we wanted to assess the efficacy of orlistat relative to the efficacy of the conventional treatment for unconjugated hyperbilirubinemia, phototherapy. Since the majority of patients with Crigler-Najjar disease are dependent on phototherapy, we questioned whether orlistat and phototherapy could be used as adjunct treatments. In chapter 4 we describe our attempts to develop an oral treatment for unconjugated hyperbilirubinemia with an equal or higher efficacy than phototherapy. We focused on intestinal capture of UCB by a combination of fat and calcium phosphate. Previously, calcium phosphate treatment had been effective in Gunn rats, but efficacy was less pronounced in patients with Crigler-Najjar disease. We determined the separate and combined effects of orlistat and calcium phosphate on plasma UCB concentrations in Gunn rats, and compared their efficacies with continuous phototherapy. One and two weeks of orlistat treatment were equally effective as continuous phototherapy in decreasing plasma UCB concentrations, and the combination of orlistat with phototherapy was more effective than either treatment alone. Combined oral treatment with orlistat and calcium phosphate for 3 weeks reduced plasma UCB concentration by ~50%, and
was more effective than continuous phototherapy in Gunn rats. To determine whether the effects of orlistat and calcium phosphate were influenced by dietary fat content, the effects were determined during a low-fat (13 energy%) or a high-fat (35 energy%) diet. Dietary fat content had a profound effect on plasma UCB concentration in Gunn rats. Changing from a low-fat to a high-fat diet without any additional treatment decreased plasma UCB levels by 46% after 3 weeks. Further analysis indicated that dietary fat content greatly influenced fecal fat excretion and, correspondingly, plasma UCB concentration. Fecal fat excretion increased from ~0.07 mmol/24 hours during a low-fat diet to ~0.7 mmol/24 hours during a high-fat diet. Irrespective of dietary fat content, orlistat treatment effectively reduced plasma UCB concentrations. Calcium phosphate treatment, however, was only significantly effective during a low-fat diet. We hypothesized that dietary fat content could partly explain the lower efficacy of calcium phosphate treatment in patients with Crigler-Najjar disease compared with Gunn rats, as observed by Van der Veere et al. The human (western) diet is a high-fat diet containing 35-40 energy% fat, whereas the Gunn rats in Van der Veere’s study received a low-fat (13 energy%) diet, identical to our low-fat diet.

As previously demonstrated, phototherapy increased biliary UCB secretion. The observation that phototherapy enhanced the efficacy of orlistat, supported our proposed concept that orlistat treatment decreases the enterohepatic circulation of UCB. The results of this study supported the feasibility of an effective oral treatment of patients with unconjugated hyperbilirubinemia.

**Novel kinetic insights into treatment of unconjugated hyperbilirubinemia: phototherapy and orlistat treatment in Gunn rats**

The observation that combined treatment with orlistat and phototherapy decreased plasma UCB concentrations more effectively than either treatment alone, suggested that the two treatments operated by different mechanisms. In chapter 5, we investigated the mechanism(s) underlying the effects of orlistat, phototherapy and combined treatment, using tritium (3H) labeled (i.e. radioactive) UCB. Gunn rats were either not treated (controls), or treated for 3 weeks with orlistat, phototherapy, or combined treatment. 3H-UCB kinetic data during steady-state conditions allowed calculation of different metabolic fluxes of UCB in the body, such as UCB production, turnover and pool size, and characterization of the dynamics of the enterohepatic circulation. As shown in our previous study, combined treatment with orlistat and phototherapy reduced plasma UCB concentrations more effectively than either treatment alone. Steady-state plasma UCB concentrations were strongly, negatively correlated with fractional turnover of 3H-UCB, indicating that phototherapy and orlistat treatment both decreased plasma UCB concentration via stimulation of bilirubin turnover. Total bilirubin turnover, which reflects UCB production rate, was not significantly altered by any of the treatments, confirming steady-state conditions. Plasma UCB levels were strongly, positively correlated with bilirubin pool size, indicating that steady-state plasma UCB concentrations...
closely reflected UCB pool sizes during treatment. The fact that combined treatment was most effective in decreasing plasma UCB concentration and pool size supported a patient study design in which orlistat treatment would be included as adjunct treatment to phototherapy.

The experimental design and mathematical modeling of the present study allowed assessment of the turnover and metabolism of labeled derivatives as well as $^3$H-UCB in the enterohepatic circulation. Phototherapy increased biliary UCB secretion, as shown previously. \(^{15}\) A novel finding of the study was that phototherapy also increased biliary secretion of radioactivity derived from injected $^3$H-UCB but no longer associated with or present in the form of UCB, implying the presence of UCB-derivatives/metabolites. We postulated that the increased biliary secretion of derivatives during phototherapy was due to enterohepatic recirculation of urobilinogen and urobilinoids secondary to either enhanced supply of substrate (=UCB) to the intestinal lumen and/or to alteration of the metabolic activity of the bacterial flora by phototherapy. Orlistat treatment induced net transmucosal excretion of UCB into the intestinal lumen, compatible with our proposed concept that orlistat treatment diminishes the enterohepatic circulation of UCB via intestinal capture of UCB. However, the mechanism behind the hypobilirubinemic effect of orlistat appeared also to result from increased metabolism of UCB to UCB-derivatives. As mentioned above for phototherapy, orlistat might have enhanced the supply of substrate for derivative formation, or might have altered the composition of the intestinal microflora, which was shown by others to be very important for metabolism of bilirubin. \(^{17}\) We speculated that manipulation of the metabolizing capacity of the intestinal flora by antibiotics or specific bacteria (“designer probiotics”) can influence the hypobilirubinemic effects of orlistat, phototherapy or combined treatment. Studies on the interactions between bacterial flora and bilirubin homeostasis in Gunn rats are presently carried out.

**Orlistat treatment of unconjugated hyperbilirubinemia in Crigler-Najjar disease; A randomized controlled trial**

The encouraging results of our studies with orlistat treatment of Gunn rats\(^{16;18;19}\) and the lack of serious side effects of orlistat in Gunn rats\(^{16;18;19}\) and in patients with obesity,\(^{20-24}\) led us to conduct a clinical trial with orlistat in patients with Crigler-Najjar disease. Chapter 6 describes the results of a randomized placebo-controlled double-blind cross-over trial evaluating effects of orlistat treatment on plasma UCB concentrations and on fecal excretion of fat and UCB. The trial was conducted in 16 Crigler-Najjar patients, who continued their regular treatment with phototherapy and/or phenobarbital. Orlistat was thus tested as an adjunct treatment. Patients received orlistat or placebo, each for 4-6 weeks with 2 weeks interval. A clinically relevant response to treatment was defined as a decrease in plasma UCB concentration of at least 10%. This definition was based on the variation in intra-individual plasma UCB concentrations in the control period before start of treatment, which on average was below 10%. Orlistat treatment decreased plasma UCB concentrations in the whole group
by 9%. Although this was a statistically significant decrease, it was not considered clinically relevant for the whole group, based on our definition of treatment response. In a subgroup (7 of 16 patients, 44%) of patients, however, orlistat treatment decreased plasma UCB by more than 10% (mean 21%, range 11-32%). A clinically relevant response to orlistat treatment did not correlate with age, sex, Crigler-Najjar type, BMI or co-treatment with phototherapy or phenobarbital. However, a relatively (compared with the group) lower dietary fat intake (<35 energy%) tended to be more frequent in the responding patients compared with the non-responders. In addition, only in patients with a clinically relevant response to treatment, plasma UCB concentrations during orlistat were strongly, negatively correlated with fecal fat excretion (i.e. similar to the results obtained in Gunn rats). Possibly, patients with a high fat intake (and consequently higher fecal fat excretion) have already reached a maximum level of UCB capture by fat and increasing fat intake and/or excretion may therefore not (further) decrease plasma UCB concentrations. In Gunn rats this might be different, however, since orlistat treatment was effective during a low-fat diet as well as a high-fat diet (chapter 3).

Apart from dietary fat intake, probably other parameters determine the responsiveness of Crigler-Najjar patients to orlistat treatment. It is tempting to speculate that gastrointestinal lipase activity levels of the patient play a role. The dose of orlistat should perhaps be individualized to generate a certain degree of fat malabsorption with a certain optimal distribution of fatty acids or partially hydrolyzed triglycerides in the intestinal lumen. The optimal intestinal environment for capture of UCB may also be dependent on the bacterial flora of the patient.

Orlistat treatment did not cause serious side effects in our trial. The generally mild, temporary and tolerable side effects that did occur were similar to those reported in trials where orlistat was used for treatment of obesity. Side effects were almost exclusively related to the gastrointestinal tract (e.g. flatulence, oily leakage, diarrhea, stomach cramps). Orlistat treatment did decrease plasma vitamin E levels (-18%). However, low plasma vitamin levels due to prolonged orlistat treatment could be overcome by dietary vitamin supplementation. The efficacy of orlistat treatment was limited and individual response to treatment unpredictable. Before orlistat could be considered as an adjunct to conventional treatment of Crigler-Najjar disease, we feel that it is necessary to elucidate the possible relationship between treatment response and dietary fat intake, and to identify relevant parameters or patient characteristics that determine or predict the responsiveness to orlistat treatment. Future research might learn us whether orlistat treatment might also be useful for other patients with unconjugated hyperbilirubinemia, e.g. for treatment of neonatal jaundice.

**Effects of bile salts on unconjugated hyperbilirubinemia in Gunn rats**

In the previous chapters, we determined that an increased fecal fat excretion decreased plasma UCB concentrations in Gunn rats. Ursodeoxycholic acid (UDCA) had been suggested to impair fat absorption. UDCA is a hydrophilic, non-toxic bile salt that is used in the
management of conjugated hyperbilirubinemia. In chapter 7 we determined in Gunn rats the effects of dietary supplementation with UDCA or with the more hydrophobic cholic acid (CA) on plasma UCB concentrations, and on fecal excretion of fat and UCB. UDCA and CA treatment both decreased plasma UCB concentration by approximately 30% after 1-3 weeks. However, UDCA treatment did not affect fecal fat excretion, and CA treatment even decreased fecal fat excretion (-67%). Nevertheless, fecal UCB concentration was higher in UDCA treated Gunn rats compared with CA treated or control Gunn rats. Neither treatment increased biliary UCB secretion or fecal urobilinoid excretion. The mechanism(s) behind the hypobilirubinemic effects of these bile salts are unclear at present. It is unlikely that bile salt treatment affected UCB production. It is also unlikely that bile salts induced microsomal oxidation of UCB because liver weights were not increased and Cyp1a2 expression was not increased. However, neither decreased UCB production nor increased bilirubin oxidation can be excluded at present. A possible mechanism of UDCA might be that it interferes with the solubilization of UCB in the intestine and reduces the enterohepatic circulation of UCB. Another explanation might be a change in bacterial flora due to bile salts, leading to an increased metabolism to derivatives. We concluded that dietary supplementation with UDCA or CA decreases plasma UCB concentrations in Gunn rats by an as yet unresolved mechanism that is independent from enhancing biliary UCB excretion or stimulation of fecal fat excretion. It is likely that UDCA and CA decrease plasma UCB levels via a different mechanism, considering the difference in hydrophilicity and the more rapid response to UDCA treatment. Future experiments with $^3$H-UCB are planned to determine whether indeed fractional turnover of UCB is different in UDCA and CA treated animals. Detailed data about UCB production, pool size and the enterohepatic circulation of UCB and its derivatives may reveal the mechanism underlying the hypobilirubinemic effect of bile salts.

**Conclusions and future perspectives**

Conventional treatment for unconjugated hyperbilirubinemia involves phototherapy. We aimed to develop an alternative or adjunct treatment for unconjugated hyperbilirubinemia based on oral administration. Our treatment strategy was based on intestinal capture of UCB, which decreases the enterohepatic circulation of UCB and subsequently plasma UCB concentrations. We hypothesized that fat could be used to capture UCB in the intestine. In this thesis we show that stimulation of fecal fat excretion decreases plasma UCB concentrations in Gunn rats, and in a subgroup of patients with Crigler-Najjar disease. In Gunn rats, orlistat treatment, especially when combined with phototherapy, effectively decreased plasma UCB levels and bilirubin pool size. As determined with $^3$H-UCB kinetics, orlistat treatment enhanced fractional turnover and fecal excretion of UCB, induces net transmucosal excretion of UCB into the intestinal lumen and increases metabolism of UCB to derivatives. Our present data do not actually demonstrate that UCB physically associates with (fractions of) unabsorbed fat in the intestine. Theoretically, UCB could associate with partially hydrolyzed
triacylglycerols, fatty acids or phospholipids. Besides influencing fat absorption, orlistat may have an (in)direct effect on a so far unknown parameter that subsequently influences UCB excretion. Solubilization of UCB by bile salts could be affected by the altered lipophilic environment. In vitro experiments will be needed to elucidate the exact mechanism.

As stated above, orlistat treatment decreased plasma UCB concentrations in Crigler-Najjar patients, but the effects were relatively modest in the group of 16 patients as a whole. However, a subgroup of patients had a clinically relevant response to orlistat treatment. A relatively low dietary fat intake correlated with the responsiveness of Crigler-Najjar patients to orlistat treatment. At present, orlistat cannot be recommended as a general treatment for Crigler-Najjar disease. First we would to like to identify patient characteristics or other relevant parameters that reliably predict which patients might benefit from orlistat treatment.

Dietary supplementation with UDCA or CA decreased plasma UCB concentrations in Gunn rats by an as yet unresolved mechanism that is independent from enhancing biliary UCB excretion or stimulation of fecal fat excretion. UDCA is widely used in the management of cholestatic liver diseases, without serious side effects. Resolving the mechanism behind the UDCA effect could become an important step towards a new treatment option for patients with unconjugated hyperbilirubinemia.

In this thesis we investigated several strategies for oral treatment of unconjugated hyperbilirubinemia. Developing an alternative or adjunct treatment could reduce the need for phototherapy or exchange transfusions. This is not only important for patient with Crigler-Najjar disease, for whom daily phototherapy is necessary. Under well-defined conditions, infants with neonatal jaundice could theoretically be eligible for oral (home) treatment instead of phototherapy. An oral treatment may also be more implementable than phototherapy in parts of the world where phototherapy is not yet readily available. The present studies have indicated that oral treatment of unconjugated hyperbilirubinemia seems feasible, and that other strategies than intestinal capture also hold promise for the future. We feel that the next steps towards further identification of clinical usefulness would need to involve in vitro studies into the association of UCB with fat and bile salts, kinetic studies to demonstrate the in vivo mechanisms, studies investigating the effect of orlistat or UDCA on the composition of the intestinal microflora, and a clinical trial with orlistat or UDCA in jaundiced neonates.
REFERENCES