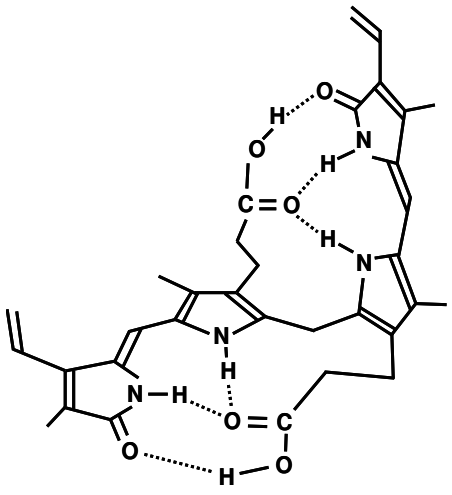
Oral treatment of unconjugated hyperbilirubinemia



Anja M. Hafkamp

Department of Pediatrics
Center for Liver, Digestive and Metabolic Diseases
University Medical Center Groningen

RIJKSUNIVERSITEIT GRONINGEN

Oral treatment of unconjugated hyperbilirubinemia

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Prof. dr. P.J.J. Sauer

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The more I study
the more I know

♦

The more I know
the more I forget

♦

The more I forget
the less I know

♦

So why study?

♦

(Oxford University)

Voor mijn familie
en vrienden

Paranimfen
Anniek Werner
Renate Wachters-Hagedoorn

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Cover: Unconjugated bilirubin (UCB). Design by Anja. Adapted from McDonagh and Lightner *(Pediatrics 1985;75:443-455).*

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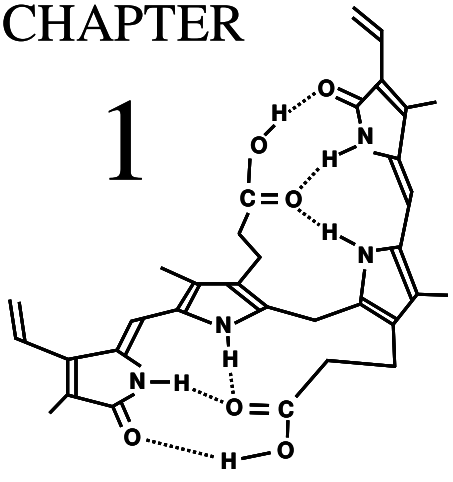
CONTENTS

ABBREVIATIONS

ALT	alanine-aminotransferase activity
a.o.	among others
AST	aspartate-aminotransferase activity
BBBP	bilirubin/BSP binding protein
BDG	bilirubin diglucuronide
BIND	bilirubin-induced neurologic dysfunction
BMG	bilirubin monoglucuronide
BMI	body mass index
Bsep	bile salt efflux pump
BSP	bromosulfophthalein
BTL	bilitranslocase
BW	body weight
CA	cholic acid
CaP	calcium phosphate
CN	Crigler-Najjar
DMSO	dimethylsulfoxide
e.g.	for example
EHC	enterohepatic circulation
et al.	and others
FFA	free fatty acid(s)
Hb	hemoglobin
HF	high-fat
HO	heme oxygenase
HPLC	high-performance liquid chromatography
Ht	hematocrit
Ibabp	ileal bile acid-binding protein
i.e.	in other words
i.v.	intravenous
LF	low-fat
Mrp	multidrug-resistant protein
NS	not significant
Oatp2	organic anion transport protein 2
Orl	orlistat
PT	phototherapy
r	correlation coefficient
RBC	red blood cell
RES	reticuloendothelial system
SD	standard deviation
UCB	unconjugated bilirubin
UDCA	ursodeoxycholic acid
UDPGT	uridine-diphosphoglucuronosyltransferase

General introduction

CHAPTER 1



1. BILIRUBIN

The term *bilirubin* is derived from the Latin words for bile (*bilis*), and red (*ruber*). Städeler[1] first used it in 1864 to describe the orange-red colored bile pigment. When bilirubin accumulates in the body it causes a yellow discoloration of the skin, sclerae and other tissues, referred to as *jaundice* (from the French *jaunisse*) or *icterus* (from the Greek *ikteros*), and high levels of bilirubin in the blood, hyperbilirubinemia. This thesis focuses on oral treatment options for unconjugated hyperbilirubinemia. This general introduction successively describes bilirubin metabolism, unconjugated hyperbilirubinemia, Crigler-Najjar disease, kernicterus, current treatment options, the Gunn rat animal model, and two of our proposed treatment options: orlistat, and bile salts. The outline of this thesis is presented in chapter 2.

2. BILIRUBIN METABOLISM

2.1. Bilirubin production

Bilirubin is the end product of heme catabolism. The major source of heme (75-80%) is hemoglobin, from breakdown of erythrocytes. Other heme sources include cytochromes, peroxidase, catalase, myoglobin, and ineffective erythropoiesis.[2;3] The life span of erythrocytes is approximately 120 days in adults, 90 days in neonates and 50-60 days in rats.[4] Senescent erythrocytes are removed from the circulation and destroyed in the reticuloendothelial system (RES), mainly localized in the spleen, liver and bone marrow. In the RES, heme is phagocytized by macrophages. Macrophages contain microsomal heme oxygenase and cytosolic biliverdin reductase, two essential enzymes for degradation of heme to bilirubin. Heme oxygenase catalyzes the first step in heme degradation: the opening of the porphyrin ring structure at the α-methene bridge (Figure 1).

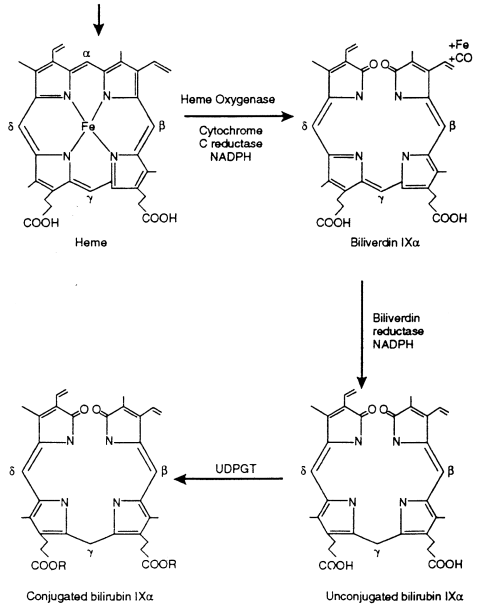


Figure 1. Bilirubin production from heme catabolism.

The intermediate blue-green pigment formed, biliverdin IXα, is water-soluble and nontoxic. The iron (Fe) is recycled and carbon monoxide (CO) is excreted by the lungs.[5] In mammals, biliverdin IXα is reduced by NADPH-dependent biliverdin reductase to produce bilirubin IXα, also known as unconjugated bilirubin **(UCB)**. Why the nontoxic, water-soluble biliverdin is converted to the non-water-soluble and potentially toxic UCB is unclear. One hypothesis involves the need for products of fetal heme degradation to cross the placenta.[6;7] Biliverdin cannot, whereas the more lipophilic UCB can cross the placenta. Another reason could be the antioxidant properties of UCB[8] (see paragraph 3.3).

UCB production can be assessed by measurement of CO formation. Conversion by heme oxygenase of one heme molecule to biliverdin produces one molecule of CO. Production rate of UCB is approximately 6-8 mg/kg per 24 hours in healthy full-term infants, and 3-4 mg/kg per 24 hours in healthy adults.[9;10] Infants produce more UCB per kg body weight because of their higher red blood cell (RBC) count, the relatively larger fraction of hepatic heme proteins, and the shorter life span of fetal RBC's. Fetal hemoglobin (HbF), which has a higher affinity for oxygen than "adult" HbA, is broken down postnatal in the relatively oxygen-rich environment. Apart from CO measurements, UCB production rate can be derived from turnover of radioisotopically labeled bilirubin, under steady-state conditions.

2.2. Bilirubin chemistry

The systemic name of UCB (bilirubin IXα) is 1'8'-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-*a*,*c*-dipropionic acid (4,5).[11;12] Its molecular weight is 584,7 gram. UCB is a nearly symmetrical tetrapyrrole, consisting of two rigid, planar dipyrroles joined by a methylene (-CH_2-) bridge at carbon atom 10 (Figure 2).[13]

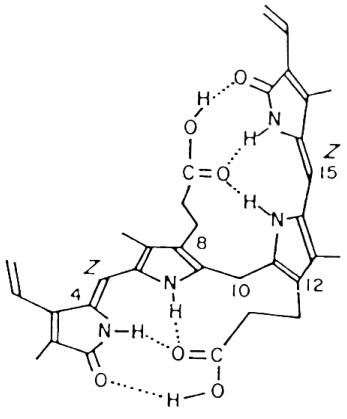


Figure 2. Structure of unconjugated bilirubin. From McDonagh and Lightner.[14]

UCB preferably has a "ridge-tile" conformation, *i.e.* is shaped like a partially open book. UCB structure was identified by analysis of X-ray diffraction.[15] Six internal hydrogen bonds make the molecule insoluble in water because the hydrophilic polar COOH and NH groups

are not available for attachment of H_20 and the hydrophobic hydrocarbon groups are on the outside of the molecule.[16] When the hydrogen bonds are opened at, for example, an alkaline pH or by addition of (m)ethanol, diphyllin or caffeine, UCB becomes more labile, more polar and water-soluble. This allows UCB to react rapidly with the diazo reagent, the basis for measurement of unconjugated or indirect bilirubin by the Van den Bergh reaction.[17] UCB exists as three species with different degrees of ionization (H_2B or diacid, HB^- or monoanion, and B^{2-} or dianion,[13] see paragraph 3.1 for details). The integrity of the hydrogen-bonded structure requires the interpyrrolic bridges at positions C4 and C15 to be in the *trans* or *Z* configuration (*Z* for *zusammen*). During phototherapy this configuration is disrupted (see paragraph 7.1).

2.3. Bilirubin transport

Once the hydrophobic UCB leaves the reticuloendothelial system, over 99.9% is bound in plasma to albumin in a non-covalent fashion and transported to the liver. Albumin has a high affinity binding site for UCB. Beyond a molar ratio of 1:1, which is equivalent to a plasma UCB concentration of approximately 600 µmol/l, UCB can bind to albumin at additional lower affinity binding sites.[18;19] In the absence of albumin, the aqueous solubility of UCB at pH 7.4 is less than 0.1 µmol/l, emphasizing the importance of albumin for preventing unbound (*i.e.* free) UCB, which is considered toxic (see paragraph 3). Recently, high-density lipoprotein (HDL) has been reported to be the principal nonalbumin carrier of UCB in human plasma. The affinity of HDL for UCB is primarily the result of binding to apolipoprotein D.[20]

2.4. Hepatic uptake of bilirubin

Albumin delivers UCB to the liver where fenestrae in the sinusoidal endothelial cells allow albumin-bound substances to reach the subendothelial space of Disse.[21] Hepatocytes have a highly efficient capacity for removing UCB from plasma. The uptake of UCB into the hepatocyte results from dissociation from albumin and transfer across the plasma membrane.[16] This transfer of UCB is carrier mediated, although controversy exists regarding the exact mechanism.[21;22] Several proteins have been suggested as putative UCB transporter, including the organic anion transport protein (Oatp2 / Slc21a6)[23;24] and the bilirubin/BSP binding protein (BBBP) which also transports other organic anions such as bromosulfophthalein (BSP).[25] The role of Oatp2 is disputed; several other Oatp's have been implicated. Bilitranslocase (BTL)[26] has also been implicated but evidence for its existence and structure is questionable.

Once within the hepatocyte, UCB is bound by the major cytosolic binding protein for UCB, glutathione S-transferase, traditionally referred to as ligandin or Y-protein.[27;28] UCB flux across the hepatocyte membrane is bidirectional. Binding to glutathione S-transferase decreases the unbound fraction and thereby the reflux of UCB and conjugated bilirubin back into plasma.[29;30]

2.5. Bilirubin conjugation

Figure 3 shows metabolism of UCB in the hepatocyte. In order to excrete bilirubin efficiently into bile, conjugation is required to convert the non-polar, water-insoluble UCB (at pH 7.4) to a water-soluble conjugate. Glucuronic acid is the major conjugating group.[31] Traces of other conjugates (e.g. glucose and xylose conjugates) have been identified in human bile,[32] and higher proportions of glucose and xylose conjugates are present in rat and dog bile. Bilirubin glucuronides are present as mono- and diglucuronides. The enzyme bilirubin-uridine diphosphoglucuronosyltransferase (UDPGT, UGT1A1, EC 2.4.1.17), primarily located in the endoplasmic reticulum, catalyzes the transfer of one or two glucuronic acid(s) from UDP-glucuronate (UDPGA) to UCB, forming, respectively, bilirubin monoglucuronides (BMG, ~20%) or bilirubin diglucuronides (BDG, ~80%) that are excreted into bile.[33] Absence of UGT1A1 in Crigler-Najjar disease results in unconjugated hyperbilirubinemia (see paragraphs 4 and 5).

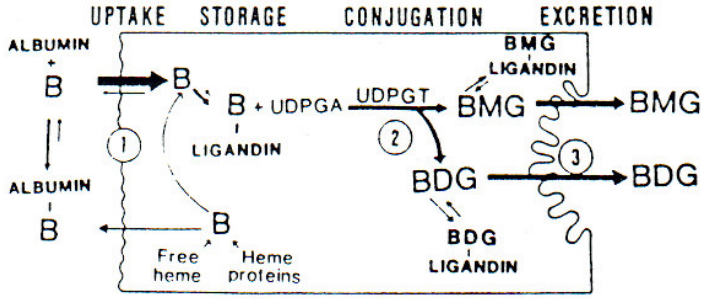


Figure 3. Hepatic metabolism of UCB. (1). UCB (B) is transferred from plasma into the hepatocyte and bound to ligandin. (2). UDP-glucuronosyltransferase (UDPGT, UGT1A1) catalyses the transfer of glucuronic acid from UDP-glucuronate (UDPGA) to bilirubin, forming bilirubin monoglucuronide (BMG) and diglucuronide (BDG) that are excreted into bile, (3) across the canalicular membrane. Ligandin is gluthathion-S-transferase (see paragraph 2.4). From Roy Chowdhury et al.[11]

2.6. Bilirubin excretion

Conjugation is an important step in UCB catabolism. Efficient biliary secretion of bilirubin requires conversion to polar conjugates. A very small amount of UCB is excreted into bile without conjugation, where it rapidly associates with mixed micelles.[11;34] UCB in bile is seldom more than 2% of total bilirubin and is believed to derive in large part from hydrolysis of secreted conjugates in the biliary tree. Conjugated bilirubin leaves the hepatocyte via Mrp2 (multidrug resistant protein 2, Abcc2). Mrp2 is an ATP-dependent transporter that carries conjugated bilirubin across the canalicular membrane into the biliary tree. Absence of Mrp2 in patients with Dubin-Johnson syndrome, and in analogous rat models (the TR- rat and the Eisai hyperbilirubinuria rat), causes conjugated hyperbilirubinemia.[35-37] However, Mrp2 cannot be the only canalicular transporter that is able to excrete conjugated bilirubin, because in the TR- rat organic anion transport was found to be preserved.[38] Mrp3 (multidrug resistant protein 3, Abcc3) is considered an important candidate for basolateral excretion of conjugated

bilirubin.[39] Conjugated bilirubin is retained in hepatocellular and cholestatic disorders. Increased plasma levels of conjugated bilirubin result in formation of a bilirubin-albumin complex called δ-bilirubin,[40] which reacts directly with diazo reagents, as does conjugated (*i.e.* direct) bilirubin.[17]

Multidrug resistant protein 1 (Mrp1, Abcc1) is a proven exporter of UCB that requires glutathione as a co-factor. Mrp1 protects cells against UCB-induced cytotoxicity.[41-44]

2.7. Intestinal metabolism and enterohepatic circulation of bilirubin

Conjugated bilirubin is hydrolyzed in the intestine to UCB, which can be reabsorbed into the enterohepatic circulation (EHC,[45;46] Figure 4). Hydrolysis of conjugated bilirubin to UCB can occur nonenzymatically under the influence of mild alkaline conditions as in the duodenum or jejunum,[47] and enzymatically by β-glucuronidase. Endogenous tissue β-glucuronidase exists in the enteric mucosa and liver,[48;49] but the major part of enzyme activity is of bacterial origin.[50;51] In neonates, a relative lack of bacterial flora and a high mucosal β-glucuronidase activity increase the enterohepatic circulation of UCB. β-glucuronidase is present in human breast milk and was thought to exaggerate jaundice in breastfed infants.[52] However, the small amounts of enzyme in milk relative to the large amounts of mucosal β-glucuronidase would not be expected to add much to the overall activity.[16] UCB in the intestine not only results from deconjugation of conjugated bilirubin. UCB can also diffuse from the blood into the intestinal lumen across the mucosa,[53;54] particularly when plasma UCB levels are high (*e.g.* neonatal jaundice; Crigler-Najjar disease, see paragraph 5). Preventing enterohepatic circulation of UCB is one of the strategies for treatment of unconjugated hyperbilirubinemia (see paragraph 7.5).

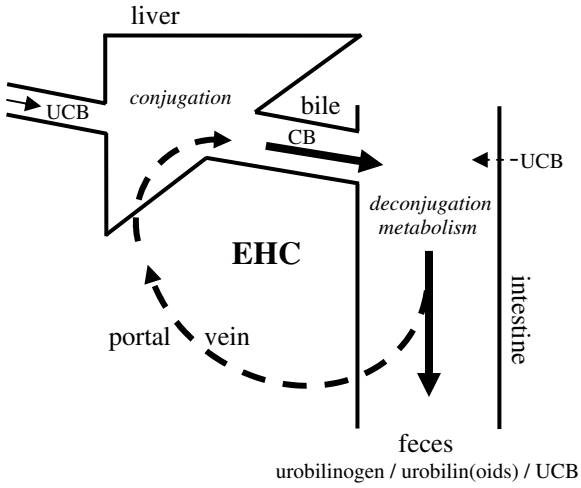


Figure 4. Enterohepatic circulation (EHC) of bilirubin. UCB is conjugated in the liver. Conjugated bilirubin (CB) is excreted into bile. CB is deconjugated to UCB via β-glucuronidase, and partly metabolized in the intestine to urobilinogen and urobilin(oids), which are excreted in the feces. Part of the UCB is reabsorbed into the EHC. Particularly under conditions of severe unconjugated hyperbilirubinemia (*e.g.* Crigler-Najjar disease), UCB can diffuse from the blood into the intestinal lumen across the mucosa (see Figure 1 in Chapter 2).

Conjugated bilirubin must be hydrolyzed to UCB before the tetrapyrrole ring can be reduced to the colorless urobilinogens by intestinal anaerobic bacteria (3 *Clostridia* species and *Bacteroides fragilis*).[55;56] Urobilinogen can be oxidized to the yellow-orange urobilin. The brown color of feces is due to dipyrrolic oxidative derivates of UCB, the mesobilifuscins. Absence of urobilinogen in feces and urine indicates complete obstruction of the bile duct. Oxidation-reduction of the various unsaturated bonds in bilirubin results in a large family of related colorless reduction-oxidation products known as urobilinoids.[57] The formation of urobilinoids is important for the removal of bilirubin from the body because the majority of urobilinoids is excreted via the feces. A small portion is reabsorbed across the intestinal mucosa into the enterohepatic circulation and subsequently excreted by liver and kidney. Urobilinogen can also undergo enterohepatic circulation.[58;59] Conjugated bilirubin cannot be reabsorbed into the portal circulation.

2.8. Bilirubin oxidation

When conjugation of UCB is deficient, as in Crigler-Najjar disease (paragraph 5), or in the animal model for this disease, the Gunn rat (paragraph 8), part of the UCB can be catabolized via an alternative metabolic route: oxidation. Oxidation of UCB leads to more polar metabolites that can be excreted into bile. Hydroxylated products have been identified in bile of Gunn rats.[60;61] Microsomal cytochrome P450 enzymes such as Cyp1a1 and Cyp1a2 catalyze oxidation of UCB. In young Gunn rats, Cyp1a1 and Cyp1a2 are markedly upregulated.[62] Stimulation of P450-1a1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased plasma UCB levels in Gunn rats by approximately 60%.[63] Mitochondrial bilirubin oxidase, a constitutive non-inducible oxidase, was found in liver, intestine and kidney.[64;65] Enzymatic oxidation of bilirubin has also been reported in brain, lung, heart and skeletal muscle.[16;66]

3. BILIRUBIN TOXICITY AND ANTIOXIDANT PROPERTIES

3.1. Bilirubin neurotoxicity

It is generally accepted that UCB bound to albumin or other plasma (lipo)proteins is not toxic. Unbound, *i.e.* free UCB is toxic when the concentration is higher than its aqueous solubility (70 nM)[67] and the free UCB is bound to brain cells. However, free UCB concentrations of 40 nM (*i.e.* below aqueous solubility) also showed toxicity to cultured astrocytes.[41] The diacid (H_2B) is considered the toxic agent because the dianion (B^{2-}) and monoanion (HB^-) do not diffuse readily into cells.[19;68;69] B^{2-} and HB^- are relatively more water-soluble because of, respectively, 2 and 1 open internal hydrogen bond(s). At pH 7.4 in plasma, there is <2% dianion and >80% diacid.[13] Free H_2B can diffuse bidirectionally, passively and rapidly across membranes,[70] including the blood-brain barrier.[71] The mechanism of UCB neurotoxicity is not fully understood. Brain damage probably results from a combination of risk factors. Free

UCB not only enters brain tissue when the UCB binding capacity in plasma is exceeded, or when displacing substances (*e.g.* sulfonamides or free fatty acids) compete for bilirubin-binding sites on albumin.[16] At bilirubin/albumin ratio's below 1.0 toxicity can also occur if free UCB levels increase steeply.[72] Acidosis is considered a risk factor for the development of bilirubin encephalopathy, although the effect of acidosis on UCB-albumin interaction is controversial.[73] The adverse effects of acidosis appear secondary to rapid deposition of insoluble H_2B precipitates in tissues. An increased permeability of the blood-brain barrier via disruption of tight junctions by hyperosmolality, hypercapnia, asphyxia or hypertension can increase entry of free UCB (and even albumin-bound UCB) in the brain.[74] Possibly hyperthermia and septicemia have similar effects.[75] Recently, it has been suggested that transporter molecules in the blood-brain barrier actively pump free UCB out of the central nervous system and maintain a concentration gradient of UCB from cerebrospinal fluid to plasma.[69] Indirect support for this hypothesis can be derived from the observation that UCB induces expression and translocation of multidrug resistance-associated protein 1 (Mrp1, Abcc1) in astrocytes.[41] Intracellular UCB levels may also be diminished by oxidation, conjugation or binding to cytosolic proteins (gluthathione-S-transferases).[69] Regional UCB deposits in the brain are probably mainly explained by regional differences in exporters of UCB,[67;69] but may also relate to differences in lipid composition, blood flow[76] or bilirubin oxidation.[77] Sex-specific regional differences in brain UCB content were demonstrated in Gunn rat pups.[78] Kernicterus, deposition of UCB in brain nuclei, is described in paragraph 6.

The exact mechanism of UCB toxicity at the cellular level is still under debate. In the past, UCB was shown to impair mitochondrial function and to interfere with RNA/DNA synthesis and carbohydrate metabolism in the brain. However, these studies were performed at extremely elevated, *i.e.* not physiologically relevant, free UCB concentrations. More recent papers showed that UCB decreases cell membrane potential and disrupts transport of neurotransmitters.[67;79] UCB also inhibits protein phosphorylation in brain membranes and glycolysis in brain,[76;80] and interferes with intracellular calcium homeostasis[81] and glutamate efflux.[82] Microglia cells and astrocytes damaged by UCB produce cytokines that may contribute to brain toxicity.[83-85] Free UCB induces apoptosis at levels as low as 71-85 nmol/l.[86] Since damage to neurons and astrocytes can occur at free UCB concentrations near or modestly above aqueous saturation,[69] treatment of jaundiced neonates should be intensified if at physical examination early signs of bilirubin encephalopathy are detected, even if plasma UCB levels are only moderately elevated.

3.2. Bilirubin toxicity to other organs

Apart from the brain, UCB may also have deleterious effects on other organs. Gunn rats develop kidney damage. Bilirubin crystals and necrosis result in impaired urinary concentration with polyuria.[87;88] In patients with Crigler-Najjar disease overt renal dysfunction has not been described. Reduced kidney function has been found in jaundiced

neonates.[89] The liver is relatively resistant to UCB toxicity. This is being re-examined, but may be due to the high conjugating activity or high degree of protein binding in this organ.[80] Dental enamel dysplasia or green discoloration of the teeth may occur.[90] Patterns of bilirubin deposition have also been found in heart, lung, adrenal, pancreas, testes and skin.[91;92] UCB can inhibit cartilage metabolism and growth *in vitro*,[93] and may inhibit cellular immune responses.[94]

3.3. Bilirubin as antioxidant

Bilirubin may not only be a potentially toxic metabolite from heme degradation, it may also be good for you.[95] The first suggestion that UCB might have a physiologic function was made in 1937 when UCB appeared to be part of a protective mechanism designed to overcome (pneumococcal) infection.[96] Subsequently many have demonstrated antioxidant properties of UCB. UCB inhibits auto-oxidation of unsaturated fatty acids,[97] scavenges peroxyl radicals,[8] may prevent oxidative membrane damage, and detoxifies singlet oxygen.[95] Infants with illnesses believed to enhance free-radical production (e.g. sepsis, asphyxia) had a significantly lower daily rise in mean plasma bilirubin levels than control infants, consistent with the hypothesis that bilirubin is consumed as an antioxidant.[16;98] Conjugated bilirubin and biliverdin also have antioxidant properties.[95;99] Exogenous bilirubin had protective effects on ischemia-reperfusion injury in the isolated, perfused rat kidney,[100] and biliverdin administration protected against endotoxin-induced acute lung injury in rats and protected rat livers from ischemia and reperfusion injury.[101;102] Heme oxygenase induction protected human hepatocytes against warm and cold hypoxia. The proposed mechanisms by which heme oxygenase exerts its cytoprotective effects include its abilities to degrade the pro-oxidative heme, to produce biliverdin and subsequently bilirubin, and to generate carbon monoxide, which has antiproliferative and anti-inflammatory as well as vasodilatory properties.[103]

In vitro exposure of neurons and astrocytes to free UCB showed neuroprotection at free UCB levels below aqueous saturation (70 nM).[104] UCB was shown to inhibit oxidation of low density lipoprotein more effectively than a vitamin E analogue,[105] hence it was postulated that UCB may reduce atherogenesis. Elevated bilirubin levels,[106-111] and (inducers of) heme oxygenases[103;112] are associated with a diminished risk of atherosclerosis and appear also negatively related to the risk of cancers and demyelinating neuropathies.[113]

4. UNCONJUGATED HYPERBILIRUBINEMIA

Hyperbilirubinemia can either be unconjugated or conjugated, or involves elevation of both UCB and conjugated bilirubins, as the vast majority of conjugated hyperbilirubinemias. Conjugated hyperbilirubinemia always involves a pathophysiological mechanism located after the level of hepatic conjugation, including secretory defects and bile duct obstructions.

Examples of conjugated hyperbilirubinemia include inherited syndromes with reduced biliary secretion of conjugated bilirubin (Dubin-Johnson syndrome, Rotor syndrome), obstructive jaundice (tumor/stones), and Benign Recurrent Intrahepatic Cholestasis.[11] This introduction will be limited to unconjugated hyperbilirubinemia, which can result from increased UCB production, decreased hepatic uptake, decreased conjugation or increased enterohepatic circulation of UCB.

Unconjugated hyperbilirubinemia becomes clinically apparent with visible jaundice at plasma bilirubin levels of about 85 µmol/l.[16] Normal plasma total bilirubin levels in human adults range from 5 to 17 µmol/l.[114] Neonatal jaundice starts at the head and progresses in a cephalocaudal manner to the trunk, arms, legs, palms and soles.[16] Increased heme catabolism contributes to jaundice in the first days after birth.[115] For the majority of neonates, unconjugated hyperbilirubinemia is a benign transitional phenomenon of no overt clinical significance.[116] However, in some cases and in the presence of risk factors such as prematurity, hemolytic disease or inherited deficiency of UGT1A1, plasma UCB concentration may rise to hazardous levels leading to kernicterus or bilirubin-induced neurologic damage (BIND). These entities and treatment of unconjugated hyperbilirubinemia will be discussed in paragraphs 6 and 7. Although several guidelines for the management of unconjugated hyperbilirubinemia have been published, definitive data on “safe” plasma UCB concentrations have not been established. Controversy remains regarding the toxicity of moderately elevated plasma UCB levels,[117] and regarding pro’s and con’s of too strict versus not strict enough guidelines, both of which may increase the risk of kernicterus. The causes of unconjugated hyperbilirubinemia will now be discussed consecutively in more detail.

4.1. Increased bilirubin production

Neonates have an increased UCB production compared with adults, mainly because of a higher erythrocyte count and a shorter erythrocyte life span (see also paragraph 2.1). Other causes of increased bilirubin production include hemolysis due to blood group incompatibility, due to structural or biochemical erythrocyte defects, or due to sepsis. Extravasation of blood (cephalhematoma, intracranial hemorrhage) and polycythemia contribute to a high bilirubin load.

4.2. Decreased hepatic uptake

A reduced capacity of net hepatic uptake may contribute to the pathogenesis of physiologic jaundice. In newborn monkeys, deficiency of ligandin and reduced clearance of BSP were demonstrated in the first days of life.[118] In humans, this deficiency is of less importance than an absolute deficiency of bilirubin conjugation,[119] or a relative deficiency of conjugation due to a mismatch between increased supply of UCB in the neonatal period and conjugation capacity. In Gilbert syndrome (see paragraph 4.3) some patients have a reduced hepatic uptake of bilirubin.[11;120;121]

4.3. Decreased conjugation

In the first ten days of life, UGT1A1 activity is usually less than 0.1% of adult values.[122] Then UGT1A1 activity increases exponentially to adult values at 6 to 14 weeks of life. The postnatal increase in plasma UCB levels appears to play an important role in the initiation of bilirubin conjugation.[123] Three heritable forms of deficient UGT1A1 activity have been described. Crigler-Najjar disease type I and II will be discussed in paragraph 5. Gilbert syndrome, described in 1901 by Gilbert,[124] is a mild recurrent unconjugated hyperbilirubinemia that usually does not become manifest until after the second decade of life. In Gilbert syndrome UGT1A1 activity is approximately 20-30% of normal and in some patients an additional reduced hepatic uptake of bilirubin has been demonstrated.[11;16;120;121] The prevalence of patients with Gilbert syndrome ranges between 2 and 12%.[125;126] The mode of inheritance is most likely autosomal recessive.[127;128] A polymorphism (an extra TA in the TATAA box) in the promoter region of the UGT1A1 gene appears to be necessary for Gilbert syndrome but not sufficient for the complete manifestation of the syndrome.[128;129] To increase plasma UCB concentration, a concomitant decrease in hepatic uptake and/or increase in UCB production is needed. Some patients have an increased bilirubin turnover rate due to subclinical hemolysis.[125] The majority of patients is anicteric because plasma UCB concentrations are usually less than 50-85 μmol/l. Intercurrent illnesses and fasting may exaggerate the unconjugated hyperbilirubinemia and cause manifest jaundice. Administration of phenobarbital reduces the unconjugated hyperbilirubinemia, but does not enhance UGT1A1 activity in Gilbert patients.[11]

4.4. Increased enterohepatic circulation

Delayed intestinal transit due to starvation, delayed passage of meconium, pyloric stenosis or Hirschsprung's disease increases the enterohepatic circulation of UCB.[130] Increased intestinal motility allows less time for UCB absorption. Frequent feedings[131] and rectal stimulation[132] are associated with lower plasma UCB levels. The absence of anaerobic bacterial flora in the neonatal intestine, with limited conversion of UCB to urobilinogen, greatly enhances the enterohepatic circulation of UCB. In older children and adults a comparable situation occurs during treatment with broad-spectrum antibiotics that suppress the anaerobic flora.[56]

Breast feeding enhances the enterohepatic circulation of UCB via several mechanisms. The first few days, intake is limited, leading to delayed passage of meconium and decreased stool weight.[131;133] Breast milk contains β-glucuronidase which converts conjugated bilirubin to UCB.[52] Breast milk is thought to alter the bacterial colonization of the intestine leading to decreased formation of urobilinogen.[134;135] UGT1A1 polymorphisms or Gilbert syndrome may be an underlying cause of breast milk jaundice.[130;136] Free fatty acids in breast milk have been suggested to contribute to neonatal jaundice through inhibition of UGT1A1.[137;138] However, it is not easy to envision how intestinal free fatty acids would affect the liver, given the physiological post-absorptive transport of intestinal fatty acids in the form of chylomicron

triglycerides. Rather, the association between jaundice and free fatty acids in milk may be based on the presence of lipase activity in breast milk. Lipases in breast milk, in particular bile salt stimulated lipase, may increase the amount of free fatty acids and enhance fat absorption. According to our hypothesis that unabsorbed fat captures UCB in the intestine, a lower fraction of unabsorbed fat in the intestinal lumen will result in less UCB capture, more enterohepatic circulation and subsequently less fecal excretion of UCB.[139] This hypothesis is the basis for the research described in this thesis and is discussed in more detail in chapter 2.

5. CRIGLER-NAJJAR DISEASE

Crigler-Najjar disease type I and II are autosomal recessive inherited diseases characterized by permanent unconjugated hyperbilirubinemia since birth. Crigler-Najjar disease type I was first described in 1952 and is caused by a complete absence of UGT1A1 activity.[140] Untreated, plasma UCB levels would range between 350-800 µmol/l and patients would develop kernicterus and die (see paragraph 6 for kernicterus). Type II Crigler-Najjar disease was defined in 1962 by Arias.[141] In type II patients UGT1A1 activity is usually less than 5% of normal. Plasma UCB concentrations are generally below 350 µmol/l. The diagnosis Crigler-Najjar disease is made using high-performance liquid chromatography (HPLC) analysis of plasma and duodenal bile (Figure 5) and by evaluating the response to phenobarbital.[142] Bile of type I patients contains virtually no conjugated bilirubin, whereas bile of type II patients contains predominantly mono-conjugates and some di-conjugates.[142] Phenobarbital enhances residual enzyme activity and the two other steps in hepatic bilirubin metabolism (see paragraph 7.3). In type II patients, plasma UCB concentration decreases by approximately 30% or more, a few days after phenobarbital is started.[142;143] Type I patients show no response to phenobarbital, or a small response due to induction of ligandin and a partial shift of UCB to the liver, as seen in Gunn rats.[144]

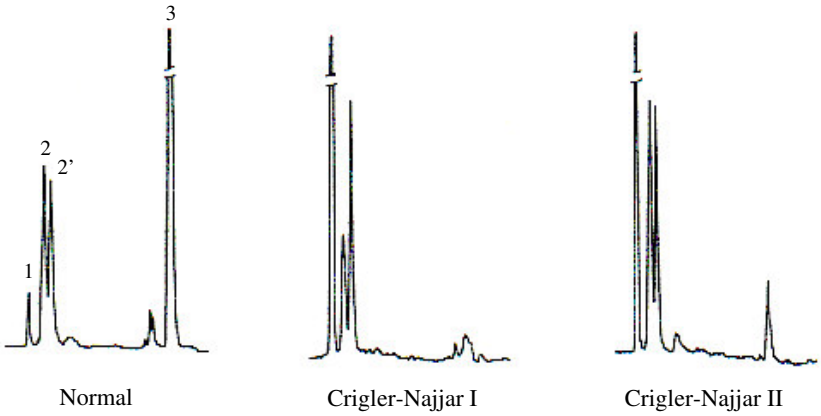


Figure 5. Bilirubin composition in bile, analyzed by HPLC. Normally, bile contains high amounts of conjugated bilirubin. In Crigler-Najjar disease type I, the bile contains virtually no conjugated bilirubin, and in type II disease predominantly mono-conjugates and some di-conjugates. Peak 1, unconjugated bilirubin; peak 2 and 2', bilirubin monoglucuronides (C8,C12 isomer); peak 3, bilirubin diglucuronide. Adapted from Sinaasappel et al.[142]

The prevalence of Crigler-Najjar disease is estimated at 1:1,000,000.[145] In the Netherlands there are approximately 20 patients. The gene encoding for UGT1A1 lies on chromosome 2. Mutations in any of 5 exons (or rarely in introns or promoter region) can cause Crigler-Najjar disease type I or II. Approximately 60 mutations (point mutations, deletions, insertions) in the UGT1A1 gene have been identified,[146-150] indicating that Crigler-Najjar disease is genetically heterogeneous, while there is a homogeneity of its clinical presentation.

Phototherapy is the preferred long-term treatment for Crigler-Najjar disease type I, but has considerable disadvantages (see paragraph 7.1). If plasma UCB levels cannot be kept below 450-500 µmol/l, liver transplantation may be necessary to prevent irreversible brain damage due to kernicterus (see paragraph 7 for treatment options for Crigler-Najjar disease). During exacerbations of jaundice, several measures in addition to continuous high-intensity phototherapy are taken to manage the disease safely, including albumin infusion if the bilirubin-albumin molar ratio is above 0.7, and avoidance of drugs that displace bilirubin from albumin.[151] Before the introduction of phototherapy, all patients with Crigler-Najjar disease died from kernicterus.[140] In recent years, neurological outcome of Crigler-Najjar disease is good if treatment is started early and adequately. Combined data from recent surveys suggest that 23-47% of patients with Crigler-Najjar disease have neurologic damage ranging from mild to severe, 28-50% of patients will need one or multiple exchange transfusions, and 9-38% die of complications related to the disease.[151-155]

6. KERNICTERUS AND BIND

In 1847, Hervieux was the first to report yellow staining of brain nuclei in a severely jaundiced baby.[156] In 1875, Orth observed bilirubin pigment at autopsy in the brains of severely jaundiced infants.[157] The term *kernikterus* (from the German *kern*, nucleus, and the Greek *ikterus*, jaundice), was first used in 1903 by Schmorl, who described similar yellow staining of brain nuclei in infants who died with severe neonatal jaundice.[158] The regions commonly affected are the basal ganglia (globus pallidus, nucleus subthalamicus), the hippocampus, various nuclei in the brain stem (a.o. oculomotor, cochlear, vestibular and olivary nuclei) and cerebellum (nucleus dentatus).[159-161] Paragraph 3.1 discusses how bilirubin enters the brain and what determines bilirubin neurotoxicity.

Originally kernicterus was a pathologic diagnosis, later the term was also used for the acute and chronic neurological syndrome. Classic acute kernicterus in neonates is characterized by three phases.[16;81;162;163] In the first few days the infant becomes lethargic, hypotonic and sucks poorly. In the second phase, the infant becomes hypertonic with retrocollis and opisthotonus, frequently develops a fever and high-pitched cry, and may develop seizures. In the third phase, usually after one week, hypertonia gradually becomes less pronounced and is replaced by hypotonia. Chronic signs of kernicterus, so called long-term sequelae, include choreoathetosis, vertical gaze paralysis, sensorineural deafness and

dental dysplasia (the 'tetrad of Perlstein'),[90] asymmetric spasticity, motor delay and mental retardation. Subtle encephalopathy is referred to as bilirubin-induced neurologic dysfunction (BIND).[81;164] BIND can present with hearing loss, lowered IQ[165;166] and abnormal cognitive function.[167-170] Recently, plasma UCB levels up to ~510 µmol/l treated with phototherapy or exchange transfusion were not associated with adverse neurodevelopmental outcomes in infants born at or near term.[171]

Patients with Crigler-Najjar disease have a life-long risk of developing kernicterus. The risk increases especially during adolescence when phototherapy becomes less effective and compliance gets worse, and during intercurrent infectious illnesses. In some children with Crigler-Najjar disease type I there may be a late clinical presentation of bilirubin encephalopathy with cerebellar symptoms as presenting feature.[172]

7. TREATMENT OF UNCONJUGATED HYPERBILIRUBINEMIA / CRIGLER-NAJJAR DISEASE

7.1. Phototherapy

Phototherapy was discovered in 1956 when a nurse in England noticed that when jaundiced infants were exposed to sunlight they became less yellow.[173] Pediatric resident Cremer *et al.* subsequently demonstrated the efficacy of phototherapy by exposing preterm infants to blue fluorescent lights, which dropped plasma bilirubin levels.[173] In the mid 1960's other therapeutic trials followed[174] and since then phototherapy has been used extensively for treatment of unconjugated hyperbilirubinemia. The mechanism of phototherapy was studied in Gunn rats.[175-178] Phototherapy detoxifies bilirubin by converting UCB to photoisomers that are less hydrophobic than UCB. The photoisomers are a better substrate for Mrp2 and therefore can be excreted into bile without being conjugated first.[14;179] Phototherapy increases the amount of UCB in bile.[60;175;180]

When bilirubin molecules in the skin absorb (phototherapy)light, 3 photochemical reactions can occur: configurational and structural photoisomerization, (Figure 6) and photo-oxidation. In *configurational photoisomerization*, one (or both) of the double bonds at carbon atoms C4 and/or C15 in the bilirubin molecule is (are) opened, converting it from the ZZ configuration to a ZE, EZ or EE configuration (*Z* for *zusammen*, *E* for *entgegen*). When this occurs, the polar N and O groups are exposed, making the UCB-photoisomer less hydrophobic than UCB and therefore a better substrate for transport into the bile via Mrp2. The predominantly formed 4Z, 15E isomer is an unstable molecule that readily reverts back. This reverse reaction is relatively slow when the isomer is bound to albumin, but occurs rapidly in bile and intestinal lumen.[16] In s*tructural photoisomerization*, intramolecular cyclization of bilirubin occurs to form the non-reversible photoisomer lumirubin.[181;182] Lumirubin is cleared much more rapidly from plasma than the 4Z, 15E isomer, and is therefore considered mainly responsible for the decline in plasma UCB levels during

phototherapy.[183;184] *Photo-oxidation* of UCB involves hydroxylation and cleavage of –CH= bridges yielding mono- and dipyrroles that are small, polar and can be excreted in the urine.[185] Photo-oxidation is a slow process and appears to play a minor role in the photocatabolism of UCB *in vivo*.[16;185]

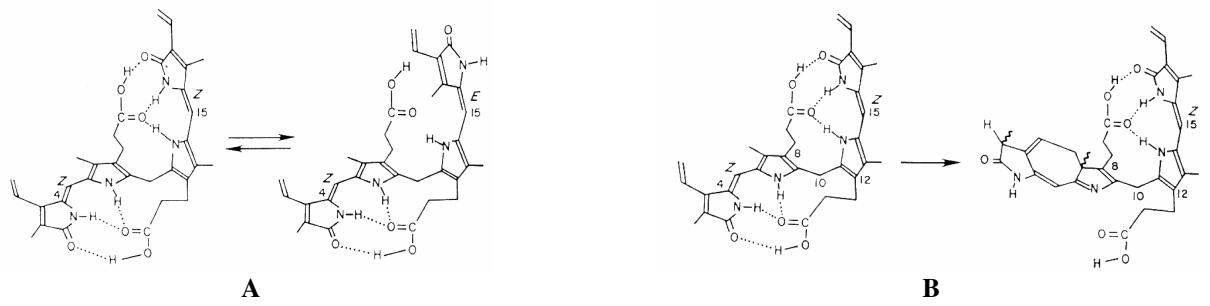


Figure 6. Phototherapy-induced photoisomerization of bilirubin. **A**: configurational photoisomerization. **B**: structural photoisomerization to lumirubin. From: McDonagh and Lightner.[14]

Several types of fluorescent lights have been used for phototherapy, including daylight, broad-spectrum, white, green, (special) blue and violet.[179;186] Efficacy results have been contradictory, but special (narrow spectrum) blue lights are generally considered superior because bilirubin absorbs light maximally in the blue range (from 420-500 nm) with a peak absorption at about 440-460 nm. White light is preferred by some clinicians because blue light distorts skin color, which makes it difficult to assess cynosis and jaundice in the neonate. Phototherapy is most efficient in the first 24-48 hours of treatment. The declining efficacy after 48 hours is probably related to configurational photoisomers that have been reverted to UCB, undergo enterohepatic circulation and increase the UCB load to be cleared by the liver.[16;187] Furthermore, phototherapy is less effective at lower plasma UCB concentrations due to depletion of the bilirubin pool in the skin, which is the main target for phototherapy.[188;189] The efficacy of phototherapy also depends on the body surface area exposed to the light. Therefore, double-sided phototherapy with a conventional overhead lamp plus a "biliblanket" reduces plasma UCB levels more rapidly.[16] Whether phototherapy should be given continuously or intermittently is not quite clear. Some studies reported continuous phototherapy to be more effective, but this was not confirmed by others. Since migration of bilirubin to the skin takes one to three hours[190] and is probably the rate-limiting step, intermittent phototherapy should be effective.[16] Intermittent phototherapy to *in vitro* human cells in tissue culture, however, caused more damage to DNA than continuous phototherapy.[191]

Since phototherapy was introduced almost 50 years ago, serious long-term side effects such as skin cancers have not been observed. However, phototherapy-induced DNA damage to human cell lines *in vitro* does occur and bilirubin was found to enhance this damage.[192;193] Short-term phototherapy has relatively minor side effects and is considered safe.[194]

Phenomena that have been attributed to or associated with phototherapy include retinal damage if the eyes are not shielded from light by eye patches,[195] diarrhea and decreased gut transit time,[196;197] increased insensible water loss,[198] temperature instability, patent ductus arteriosus[199;200] and the "bronze baby syndrome", which appears to be due to accumulation of photodegradation products (bilifuscins) when their biliary excretion is impaired by concomitant cholestasis.[119;201]

Patients with Crigler-Najjar disease type I have to undergo daily phototherapy up to 12 hours per day. Type II patients usually only need a few hours of phototherapy per day, if any. Long-term phototherapy has considerable disadvantages. Phototherapy becomes less effective with age, due to a decrease in surface area to body mass ratio,[151;202] due to a large tissue reservoir of UCB,[151] due to skin alterations,[145;155] and due to a diminishing compliance to the intensive phototherapy regimen which has a profound impact on the quality of (social) life.[155]

7.2. Exchange transfusion

Phototherapy has greatly reduced the need for exchange transfusion. With this technique, approximately 85% of circulating red blood cells will be replaced (when replacing 160 ml/kg BW), and plasma UCB levels will generally be reduced by 50%.[119] The exchange transfusion physically removes defective red bloods cell and UCB, which diffuses from the extravascular space (*i.e.* tissue pool) into plasma. Indications for exchange transfusion include symptoms and signs characteristic of acute bilirubin encephalopathy (kernicterus; see paragraph 5), dangerously high or rapidly rising plasma UCB concentrations despite phototherapy, and progressive anemia due to hemolysis.[203;204] The mortality rate from the procedure is around 0.3%. Significant morbidity is associated with ~5% of exchange transfusions.[205;206] Complications include cardiac and vascular complications such as cardiac arrest and thrombosis of the portal vein in case the exchange transfusion was done via a catheter in the umbilical vein, metabolic and coagulation disturbances, transmission of infectious diseases, graft versus host disease and necrotizing enterocolitis.

In the management of Crigler-Najjar disease, generally exchange transfusions are not required. Sometimes exchange transfusions are used in the neonatal period when the diagnosis is not yet clear. Incidentally, exchange transfusions are performed when plasma UCB concentration is dangerously increased and/or albumin concentration decreased, for example during intercurrent (febrile) illnesses or around surgery.[151;155]

7.3. Phenobarbital

Phenobarbital is an anti-epileptic drug that enhances the three steps in hepatic bilirubin metabolism independently: uptake and storage of UCB by the hepatocyte, conjugation, and biliary secretion.[30;176;207-209] Net uptake and storage is enhanced via an increased concentration of ligandin. Conjugation is enhanced via induction of UGT1A1. Biliary secretion is most likely enhanced due to induction of Mrp2. Phenobarbital is a CAR (constitutive androstane

receptor) agonist. Wagner et al.[210] showed that phenobarbital, and other CAR agonists, induce Mrp2.

Phenobarbital is used to distinguish between type I and II Crigler-Najjar disease. In type I patients, phenobarbital is not effective because there is no residual enzyme activity that can be enhanced. In the animal model of type I Crigler-Najjar disease, phenobarbital decreases plasma UCB levels, despite the absence of residual enzyme activity, but this has been demonstrated to be due to a shift of the bilirubin pool to the liver.[144] Phenobarbital is effective in type II Crigler-Najjar disease. It usually decreases plasma UCB concentration by 30% or more.[142] Side effects include sedation, and induction of cytochrome P450 enzymes which accelerate the metabolism of many drugs, vitamins, clotting factors and estrogenic and androgenic hormones.[176]

Apart from its use for treatment of type II Crigler-Najjar disease, phenobarbital is not used anymore for treatment of unconjugated hyperbilirubinemia. Originally, it was given to pregnant mothers before delivery or to the infant within 24 hours after birth to limit the severity of unconjugated hyperbilirubinemia and the need for exchange transfusions.[211-214] However, phototherapy is more effective than phenobarbital and combining phototherapy with phenobarbital did not reduce plasma UCB levels more rapidly than phototherapy alone.[215] Furthermore, the effect of phenobarbital does not start until a few days after administration.[211]

7.4. Decreasing UCB production

UCB production can be decreased via inhibition of heme oxygenase (HO), the rate-limiting enzyme in the catabolism of heme to UCB. In theory, inhibition of biliverdin reductase could also be used to decrease UCB production. However, inhibitors of biliverdin reductase have not been explored, probably because their use would cause green babies. HO inhibitors such as tin (Sn)- and zinc (Zn)-protoporphyrin and -mesoporphyrin are synthetic heme analogues.[216] A single dose inhibits HO for several days. The inhibition of heme degradation does not result in accumulation of heme because heme is excreted into bile.[217] Sn-mesoporphyrin is the preferred HO inhibitor for treatment of neonatal jaundice,[218-221] but is currently not recommended for routine treatment because of insufficient evidence,[222] and unknown long-term safety.[223] Recent trials focused on treatment of mice.[224;225] Results of new trials in neonates are awaited. In neonates with glucose-6-phosphate dehydrogenase deficiency (G6PD), Sn-mesoporphyrin supplants the need for phototherapy to control hyperbilirubinemia.[226] Side effects of Sn-protoporphyrin include photosensitization, which can accelerate the destruction of UCB by light but can also cause cutaneous erythema.[219;227] Phototoxicity involves production of free radicals and other reactive oxygen species which can cause cell damage, presumably via accelerated lipid peroxidation.[228]

Sn-mesoporphyrin and Sn-protoporphyrin have been used in the management of Crigler-Najjar disease,[229;230] but results were temporary and disappointing. In Crigler-Najjar patients,

early administration of heme oxygenase inhibitors is expected to be more effective than initiation in adolescence, because in the latter case, total-body amount of UCB is many times greater than the amount of UCB in the intravascular space.[16]

7.5. Intestinal capture of UCB

UCB gets into the intestinal lumen via one of three routes: 1) biliary secretion of conjugated bilirubin, subsequently deconjugated to UCB; 2) biliary secretion of UCB: a very small amount of UCB can be excreted into bile (see paragraph 2.6);[11;34] 3) transepithelial diffusion: UCB can diffuse from the blood into the intestinal lumen across the intestinal mucosa along concentration gradients, particularly when plasma UCB concentrations are high as in Crigler-Najjar disease (see Figure 1 in Chapter 2).

Intestinal capture of UCB followed by fecal excretion reduces the enterohepatic circulation of UCB and subsequently decreases plasma UCB concentration. Several orally administered non-absorbable binders of UCB have been applied for intestinal capture. Agar,[231] activated charcoal[232] and cholestyramine[233] are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects.[234-236] Zinc sulphate was shown to decrease plasma UCB levels in patients with Gilbert syndrome, but serum zinc levels increased simultaneously.[237] Zinc methacrylate did not increase serum zinc levels, but was less effective in Gunn rats than zinc sulphate.[238] Intestinal capture of UCB by calcium phosphate was very effective in Gunn rats,[239] but efficacy was less pronounced in patients with Crigler-Najjar disease.[145]

We hypothesized that fat could be used to capture UCB in the intestine, considering the relatively lipophilic character of UCB.[139] In this thesis we investigated whether stimulation of fecal fat excretion by orlistat (see paragraph 9) decreases plasma UCB concentrations in Gunn rats and patients with Crigler-Najjar disease (see chapter 2).

7.6. Bilirubin oxidase

Bilirubin oxidase was used in several experimental ways for treatment of unconjugated hyperbilirubinemia. UCB was removed from rat- or human blood by passage through a filter containing bilirubin oxidase,[240] bilirubin oxidase was fed to Gunn rats,[241] and PEG-bilirubin oxidase was injected i.v. in Gunn rats.[242] As mentioned in paragraph 2.8, induction of cytochrome P450-1a1 (Cyp1a1) by TCDD decreases plasma UCB concentration in Gunn rats. Treatment of Gunn rats with indole-3-carbinol also induces the oxidative pathway of UCB metabolism.[243] Retention of UCB itself induces this pathway as well.[62] Several naturally occurring indoles extracted from cruciferous vegetables, such as cabbage, cauliflower and sprouts induce P4501a1 and 1a2 in rat liver and intestine.[16;244] Bilirubin oxidase administration or induction of bilirubin oxidation is currently not applied as therapeutic strategy for neonatal jaundice or Crigler-Najjar disease.

7.7. Hepatocyte transplantation

Since liver architecture and function, except for deficiency of UGT1A1 activity, are normal in Crigler-Najjar disease type I, hepatocyte transplantation might be safer and less invasive than liver transplantation. Correction of Crigler-Najjar disease requires only partial replacement of UGT1A1 activity.[245] In Gunn rats, several techniques have been investigated, such as infusion of (unaffected) hepatocytes into the portal vein, or via intraperitoneal injection.[246-249] Hepatocyte transplantation temporarily decreased plasma UCB concentration in Gunn rats.[247] So far, hepatocyte transplantation has been performed in two patients with Crigler-Najjar disease type I. The first patient was a 10 year old girl in whom UGT1A1 activity was restored to 5.5% of normal after hepatocyte transplantation via percutaneous infusion through the portal vein. Afterwards, maximum plasma UCB levels dropped from 455 to 239 µmol/l, and she required 6-7 hours of phototherapy instead of 10-12 hours.[250] Long-term results are awaited. More recently, the second patient, a 9 year old boy, received an allogenic hepatocyte transplantation.[251] Initially, plasma UCB levels decreased from 530 to 359 µmol/l. However, he was treated for cellular rejection and later he received a liver transplantation because of poor compliance to phototherapy. Although hepatocyte transplantation was safe and partially effective in these two patients, problems with long-term efficacy, rejection and immune suppression may prevent future use in Crigler-Najjar disease.

7.8. Liver transplantation

Several patients with Crigler-Najjar syndrome type I have undergone liver transplantation.[252-257] Successful liver transplantation effectively restores UGT1A1 activity which results in low or normal plasma UCB levels and eliminates the need for phototherapy. However, these benefits have to be weighed against the risks and complications of liver transplantation. The one year survival after liver transplantation is between 85 and 90%,[258] although over the past years survival has improved.[259;260] Possible complications include rejection, infection, bleeding, thrombosis and biliary complications.[258] To reduce the risk of rejection, patients receive life-long immunosuppressive medication, which increases the risk of lymfoproliferative disease and late infections, and has side effects as nephrotoxicity and hyperlipidemia.[260] Two types of liver transplantation are used. In orthotopic liver transplantation the patient's own liver is removed and a donor liver is inserted in its place. In auxiliary liver transplantation, (part of) the patient's own liver is left in situ, but supported by the transplantation of a non-affected donor graft.[261;262] The theoretical advantage of the latter procedure is that, if gene therapy would become available in the future, this could still be applied to the native liver, allowing possible withdrawal of immunosuppression.

7.9. Gene therapy

Since Crigler-Najjar disease is caused by molecular lesions of a single gene and partial enzyme replacement would be enough to significantly lower plasma UCB concentrations,

gene therapy would be an elegant potential therapeutic option. However, vector toxicity and concerns about long-term safety have so far prevented the use of gene therapy in patients.

The structure of the UGT1A1 gene has been elucidated and the gene was successfully cloned in 1991.[263-265] Since then many gene transfer strategies have been evaluated in Gunn rats. Early generation adenoviral vectors effectively corrected UGT1A1 activity,[266;267] but the effects were transient. Several strategies to prolong the duration of transgene expression have been explored, including induction of tolerance[268] and expression of immunomodulatory molecules.[269] However, acute toxicity and immunogenicity of viral proteins were a major disadvantage of these vectors. Subsequently, helper-dependent, or "gutless" adenovectors were developed that have negligible chronic hepatic toxicity.[270] A single i.v. injection successfully corrected unconjugated hyperbilirubinemia in Gunn rats for more than 2 years. One of the side effects was transient thrombocytopenia. Other viruses that have been used as vectors include retrovirus,[271;272] lentivirus[273;274] and recombinant simian virus.[275] Non-viral strategies such as chimeraplasty,[276] liposomes,[277] and plasmids[278-280] have been evaluated. *Ex-vivo* gene therapy with transplantation of manipulated fibroblasts corrected the gene defect but resulted in animals developing tumors.[281] Before any of the above strategies can be used for gene therapy in patients with Crigler-Najjar disease, long-term safety will need to be confirmed.

8. GUNN RAT

In this thesis we used the Gunn rat for our animal studies. In 1938, Gunn first described a spontaneously mutant rat strain with recessively inherited hyperbilirubinemia within a colony of Wistar rats.[282;283] A colony of these rats was maintained for over 15 years. It was not until 1957, when defective bilirubin glucuronidation was reported, that the Gunn rat was recognized as an animal model of Crigler-Najjar disease type I[284;285] (Figure 7).

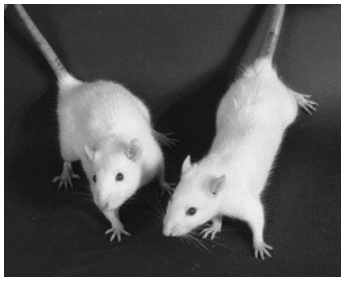


Figure 7. Gunn rats.

The Gunn rat is now a well-established and frequently used model of inherited unconjugated hyperbilirubinemia. Advantages of the model include the ability to use heterozygous littermates (Jj) of affected homozygous Gunn rats (jj) as matched controls.[69] Different strains of Gunn rats exist.[286;287] In our experiments we used albino Gunn rats (RHA/jj).

Although all Gunn rats have degenerative lesions of the brain, not all develop gross disturbances of gait or other signs of kernicterus.[288] The neuropathological lesions in Gunn rat pups are similar to those in humans, with cell loss and gliosis in auditory and oculomotor nuclei, cerebellum, hippocampus and basal ganglia.[69] As in humans, brainstem auditory evoked potentials (BAEPs) are a sensitive indicator of bilirubin neurotoxicity in Gunn rats.[289] In contrast to human kernicterus, cerebellar hypoplasia is a prominent feature of UCB damage in Gunn rats, and the cause of ataxia.[290;291] Besides brain damage, high UCB concentrations can cause renal papillary necrosis. High UCB levels in the medulla interfere with sodium and water transport, resulting in impaired urinary concentration with polyuria.[87;88]

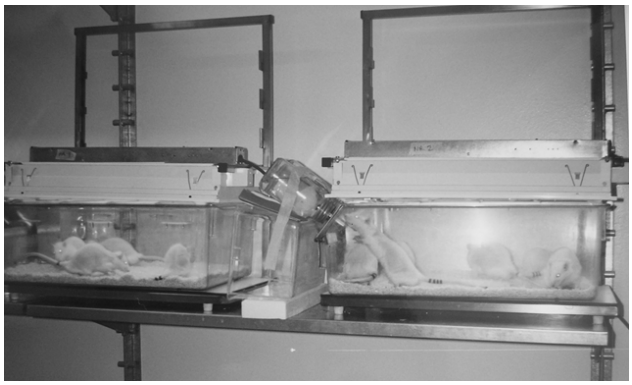


Figure 8. Gunn rats receiving phototherapy in one of our experiments. Blue phototherapy lamps were suspended in a reflective canopy 20 cm above the bottom of the cage. The Gunn rats were shaven on their backs and flanks.

9. ORLISTAT

This paragraph provides background information on orlistat which was investigated in this thesis as option for oral treatment of unconjugated hyperbilirubinemia. We used orlistat to increase fecal fat excretion, hypothesizing that fat could be used to capture UCB in the intestinal lumen (see paragraph 7.5 and chapter 2).

Orlistat (Xenical®; Figure 9) is a selective inhibitor of gastrointestinal lipases that dose-dependently inhibits hydrolysis of dietary triglycerides.[292] It is a chemically synthesized derivative of the natural product lipstatin and specifically inhibits lipases at their catalytic triad by covalent binding to the serine residue.[293] Orlistat has little or no activity against amylase, trypsin and phospholipases.[292] At the recommended dose of 3 times daily 120 mg for adults, dietary fat absorption is reduced by approximately 30%. Orlistat acts locally in the gastrointestinal tract and systemic absorption is minimal (~1%).[294] Orlistat is applied for treatment of obesity and obesity-related co-morbid conditions. In combination with dietary intervention and exercise, orlistat is used for management of weight loss and weight maintenance. Orlistat treatment is associated with beneficial effects on cardiovascular risk

factors including dyslipidemia, decreased insulin sensitivity and hypertension.[294-296] Numerous clinical trials in adults have not reported serious side effects.[294] Rather, side effects are generally mild to moderate, temporary and limited to gastrointestinal effects such as fatty/oily stool, flatulence, and abdominal pain. Recently, orlistat has been introduced in the European Union for treatment of obese adolescents.[297] Clinical trials in obese adolescents[298-301] and prepubertal children[302] indicate that orlistat treatment is well-tolerated by children and has a side effect profile similar to that observed in adults. Orlistat had no significant effect on the balance of six selected minerals in obese adolescents.[303] Besides being an inhibitor of gastric and pancreatic lipases, orlistat was recently reported to be an inhibitor of fatty acid synthase, thereby halting tumor cell progression.[304]

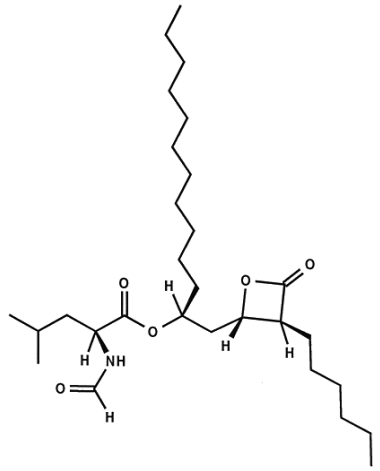


Figure 9. Structure of orlistat.

10. BILE SALTS

This paragraph provides background information on bile salts which were investigated in this thesis as option for oral treatment of unconjugated hyperbilirubinemia.

Bile salts are quantitatively the major organic constituents of bile. Bile formation is crucial for hepatobiliary secretion of bilirubin. The liver synthesizes bile salts from cholesterol. In addition to their role in enhancing bile flow and the biliary secretion of exogenous and endogenous organic compounds, including phospholipids and cholesterol, bile salts are important for intestinal absorption of dietary fats and fat-soluble vitamins (A, D, E, K).[305] Bile salts are secreted into bile by the canalicular bile salt export pump (Bsep, Abcb11).[306] In the intestine, more than 95% of bile salts are efficiently reabsorbed into the enterohepatic circulation.[307] Intestinal absorption is principally mediated by the apical sodium-dependent bile salt transporter (Asbt, Slc10a2) in the terminal ileum.[308] Uptake of bile salts by the liver is mainly mediated by the Na^+ taurocholate cotransporting polypeptide (Ntcp, Slc10a1).[309]

In humans and rats, the major newly synthesized (primary) bile salts are cholic acid (CA) and chenodeoxycholic acid (CDCA) After synthesis, more than 99% of primary bile salts are conjugated with either the amino acid taurine or glycine, which increases hydrophilicity. In the intestine, conjugated CA and CDCA can undergo deconjugation and subsequent dehydroxylation by the bacterial flora, resulting in the toxic secondary bile salts deoxycholate and lithocholate, and in the tertiary bile salt ursodeoxycholic acid (UDCA). UDCA is a hydrophilic, non-toxic bile salt compared with the hydrophobic, toxic CA. UDCA inhibits UCB-induced apoptosis in cultured rat neural cells.[310]

UDCA is used in the management of cholestatic liver diseases with conjugated hyperbilirubinemia. We propose that UDCA might be used for treatment of unconjugated hyperbilirubinemia as well. Solubilization of UCB by bile salts and interactions between UCB and bile salts occur.[311-313] Furthermore, dietary supplementation with UDCA has been suggested to impair fat absorption in some individuals.[314] Since we hypothesized that increasing fecal fat excretion reduces the enterohepatic circulation of UCB via intestinal capture of UCB by fat, we investigated whether UDCA treatment decreases plasma UCB concentrations in Gunn rats (see chapter 2). In contrast to our hypothesis, Méndez-Sánchez *et al.* have hypothesized that dietary UDCA supplementation induces enterohepatic cycling of UCB by causing bile salt malabsorption, which elevates colonic bile salt levels, promoting solubilization and reabsorption of UCB.[315] Their hypothesis has not been proven by ^{3}H-UCB kinetic studies.

This chapter mainly discussed bilirubin metabolism and treatment options for unconjugated hyperbilirubinemia in Crigler-Najjar disease to provide background information regarding the research described in this thesis. We propose two oral treatment options for unconjugated hyperbilirubinemia: orlistat, and bile salts. In chapter 2 the outline of this thesis is presented.

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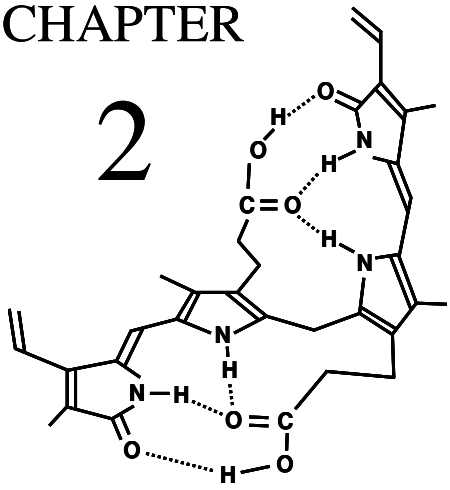
Outline of this thesis

CHAPTER 2



OUTLINE OF THIS THESIS

This thesis focuses on oral treatment of unconjugated hyperbilirubinemia. Permanent unconjugated hyperbilirubinemia occurs in patients with Crigler-Najjar disease due to a genetic deficiency of the hepatic enzyme bilirubin-UDP-glucuronosyltransferase (UGT1A1). Glucuronidation of unconjugated bilirubin (UCB) via UGT1A1 greatly enhances its biliary secretion and subsequent fecal excretion.[1] Accumulation of UCB in the body 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 daily phototherapy for up to 12 hours. Long-term daily phototherapy 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.[4-6]

We aimed to develop an alternative or additional treatment for unconjugated hyperbilirubinemia in Crigler-Najjar disease that is based on oral administration. The oral treatment approach is based on previously demonstrated strategies for intestinal capture of UCB (Figure 1). Particularly when plasma UCB levels are high as in Crigler-Najjar disease, UCB can diffuse from the blood into the intestinal lumen across the intestinal mucosa.[7;8] In addition, under conditions of absent or strongly diminished conjugation, small amounts of UCB enter the intestine via biliary secretion.[9;10] Intestinal capture of UCB followed by enhanced fecal excretion has been shown to reduce the enterohepatic circulation[11;12] of UCB and to decrease plasma UCB concentration. Binding of UCB in the intestine increases the gradient for unbound UCB from blood to intestinal lumen, enhancing transmucosal diffusion.

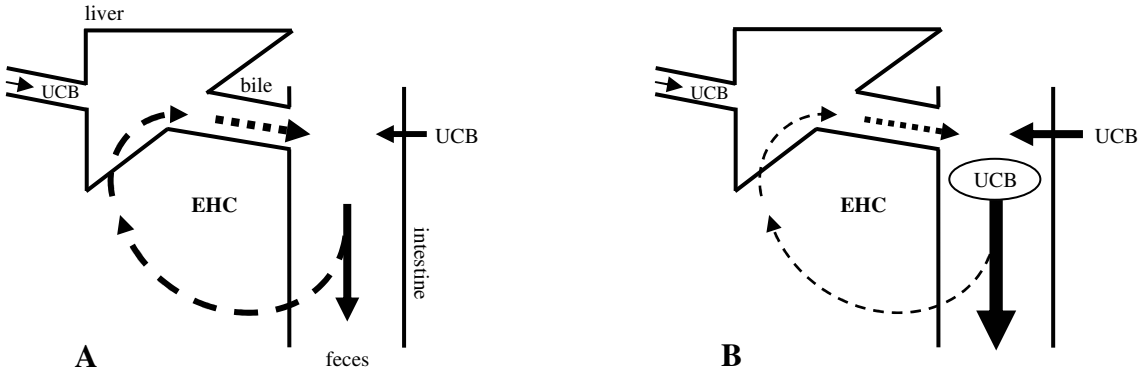


Figure 1. Intestinal capture of UCB. **A:** In Crigler-Najjar disease, UCB can enter the intestine via biliary secretion and via transepithelial diffusion from the blood into the intestinal lumen, across the intestinal mucosa. UCB and its metabolites are partly excreted with the feces, and partly reabsorbed into the enterohepatic circulation (EHC). **B:** Capture of UCB in the intestine followed by enhanced fecal excretion reduces the enterohepatic circulation of UCB. Binding of UCB in the intestine increases the gradient for unbound UCB from blood to intestinal lumen, enhancing transmucosal diffusion.

Considering the relatively lipophilic character of UCB, we hypothesized that UCB could associate with (unabsorbed) fat in the intestine,[13] although no direct evidence exists for

increased binding. In **chapter 3** of this thesis we investigated whether stimulation of fecal fat excretion affected plasma UCB concentrations in Gunn rats. The Gunn rat is a well-established animal model for permanent unconjugated hyperbilirubinemia (Crigler-Najjar disease type I). Fecal fat excretion was increased by dietary supplementation with the lipase inhibitor orlistat. Orlistat inhibits intestinal hydrolysis of dietary triglycerides and thereby reduces dietary fat absorption. Gunn rats received short-term (≤3 weeks) or long-term (24 weeks) orlistat treatment. Effects of orlistat treatment on plasma UCB concentrations and on fecal excretion of fat and UCB were determined.

In **chapter 4** we compared the effects of orlistat treatment with the effects of a previously demonstrated oral treatment for unconjugated hyperbilirubinemia, calcium phosphate. Calcium phosphate treatment had effectively reduced plasma UCB levels in Gunn rats,[14] but efficacy was less pronounced in patients with Crigler-Najjar disease.[15] We determined the separate and the combined effects of orlistat and calcium phosphate treatment on plasma UCB concentrations in Gunn rats. Also, we compared the efficacy of orlistat and/or calcium phosphate with the conventional treatment for unconjugated hyperbilirubinemia, phototherapy. To determine whether the effects of orlistat and calcium phosphate were influenced by dietary fat content, we conducted our experiments during a low-fat and high-fat diet.

In **chapter 5** we investigated in Gunn rats the mechanism(s) underlying the effects of orlistat, phototherapy and of combined treatment on UCB homeostasis. Using ^{3}H-UCB kinetics we determined the effects of the three treatments on fractional turnover of UCB, and on biliary secretion and net transmucosal excretion of UCB. We developed a new method of estimating the steady-state enterohepatic circulation and intestinal flux of UCB and its derivatives.

Chapter 6 describes the effects of orlistat treatment on plasma UCB concentrations in patients with Crigler-Najjar disease. A randomized placebo-controlled cross-over trial was conducted in 16 patients, simultaneous with their regular treatment with phototherapy and/or phenobarbital. Patients received orlistat or placebo, each for 4-6 weeks with 2 weeks interval. We determined the effects of orlistat treatment on plasma UCB concentrations and on fecal excretion of fat and UCB.

In **chapter 7** we performed initial experiments towards another strategy for oral treatment of unconjugated hyperbilirubinemia. The bile salt ursodeoxycholic acid (UDCA) had been suggested to impair fat absorption.[16] Since our previous studies indicated that stimulation of fecal fat excretion decreased plasma UCB concentrations, we investigated in Gunn rats whether dietary supplementation with UDCA or with a different bile salt, cholic acid (CA), affected plasma UCB concentrations and fecal excretion of fat and UCB.

Chapter 8 provides a summary and general discussion of the research described in this thesis, including conclusions and perspectives for future studies.

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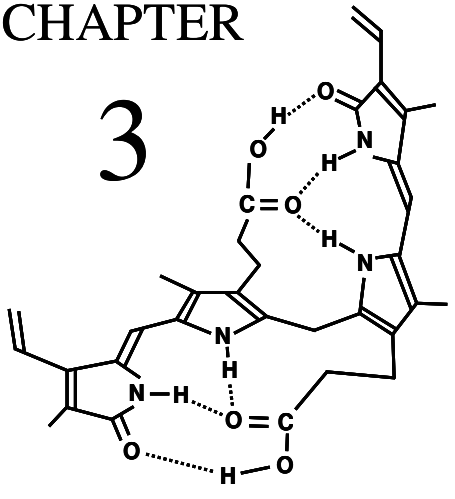
Orlistat treatment increases fecal bilirubin excretion and decreases plasma bilirubin concentrations in hyperbilirubinemic Gunn rats

CHAPTER

3



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Tomoji Nishioka, Anja M. Hafkamp, Rick Havinga,
Pieter P.E. van Lierop, Herman Velvis, Henkjan J. Verkade

Department of Pediatrics, Division of Pediatric Gastroenterology,
Center for Liver, Digestive and Metabolic Diseases, University
Medical Center Groningen, Groningen, The Netherlands

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,[1] which occurs for example during increased hemolysis, Gilbert syndrome,[2] Crigler-Najjar disease[3] or neonatal hyperbilirubinemia. UCB can accumulate in several organs, including the central nervous system, where it can lead to toxicity (kernicterus) and death.[3-5] 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,[6;7] and that UCB can be reabsorbed from the intestinal lumen.[8-11] Prevention of intestinal reabsorption of UCB, for example by agar, cholestyramine, or calcium phosphate, has been demonstrated to decrease its plasma concentrations.[12-17] 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.[12;14;15;18-21]

Recently, we hypothesized that an inverse relation exists between neonatal unconjugated hyperbilirubinemia and the degree of neonatal fat malabsorption.[22] 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.[22] 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).[23;24] Fecal fat excretion was stimulated by feeding Gunn rats high-fat diets supplemented with orlistat.[25] 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.[26;27]

METHODS

Animals

Homozygous male Gunn rats (RHA/jj,[7] 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.[17] Possibly this phenomenon occurs because lab chow, in contrast to a semisynthetic diet, may contain naturally occuring 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 (T_0) 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.[28;29] Total biliary bile salts were assayed by the 3α-hydroxysterol dehydrogenase method.[30] Biliary cholesterol and phospholipid were measured after lipid extraction,[31] according to the methods of Gamble *et al.*[32] and Bötcher *et al.*,[33] 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.[34] 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,[25;35;36] 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.

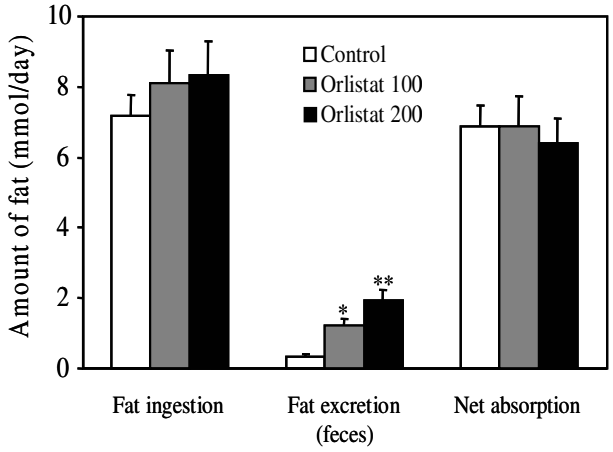

Figure 1. Dietary administration of orlistat to Gunn rats on a high-fat diet induces a dose-dependent increase in fecal fat excretion. Amounts of dietary fat ingestion, fecal fat excretion and net fat uptake in Gunn rats fed a high-fat diet without supplement (control) or supplemented with orlistat (100 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. *$p<0.05$, **$p<0.01$.

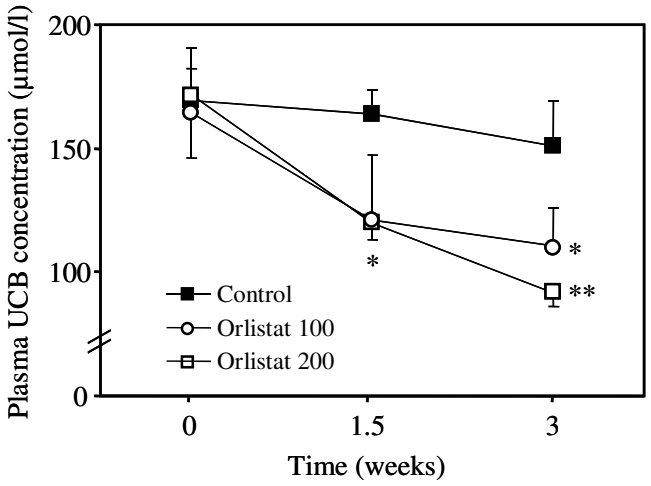

Figure 2. Dietary administration of orlistat to Gunn rats on a high-fat diet induces a decrease in plasma UCB concentration. Plasma UCB concentration determined by high-performance liquid chromatography in Gunn rats fed high-fat diet without supplement (control) or supplemented with orlistat (100 mg/kg chow or 200 mg/kg chow). Experimental diets were fed for 3 weeks, after a 4 week run-in period on high-fat diet. After 3 weeks on experimental diet, plasma UCB concentrations had decreased by 26% in orlistat 100- ($p<0.05$) and by 40% in orlistat 200-treated rats ($p<0.01$), compared with controls. Values reflect mean ± SD, n = 4-5 per group. *$p<0.05$, **$p<0.01$.

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 ($r = -0.87$, $p<0.001$).

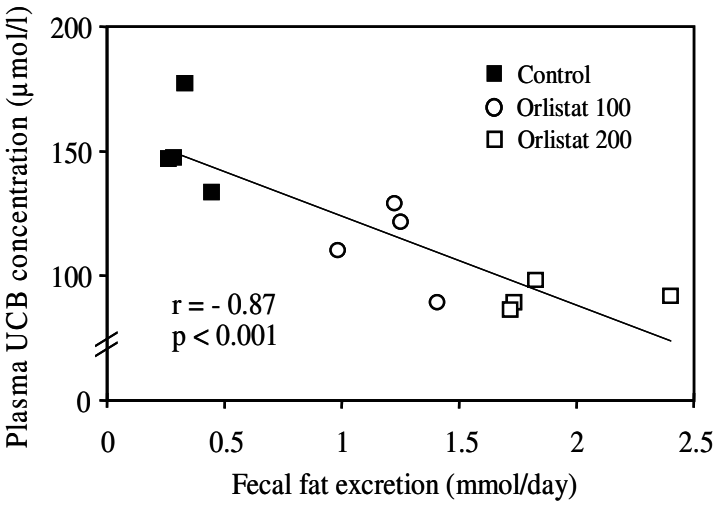

Figure 3. Plasma UCB levels appear strongly, negatively related to fecal fat excretion after 3 weeks of feeding Gunn rats a high-fat diet with orlistat. Relation between fecal fat excretion, determined by gas chromatography based on a 72-hour fat balance, and plasma UCB concentration, determined by high-performance liquid chromatography, in Gunn rats fed high-fat diet without supplement (control) or supplemented with orlistat (100 mg/kg chow or 200 mg/kg chow). Each symbol represents values obtained in an individual animal. Linear regression analysis results in the equation $[UCB]_{plasma} = 154 - 32.5\ [fat\ excretion]_{feces}$, $r = -0.87$, $p<0.001$.

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.

	Control	Orlistat 100	Orlistat 200	*P* value
Bile flow *(µl/h/100 g)*	183 ± 8	203 ± 40	184 ± 37	NS
Bilirubin *(nmol/h/100 g)*	8.1 ± 3.3	6.2 ± 2.8	5.4 ± 0.7	NS
Bile salts *(µmol/h/100 g)*	6.3 ± 1.0	8.2 ± 1.8	7.2 ± 1.0	NS
Cholesterol *(nmol/h/100 g)*	66 ± 19	77 ± 6	56 ± 16	NS
Phospholipids *(µmol/h/100 g)*	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	NS

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.

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).

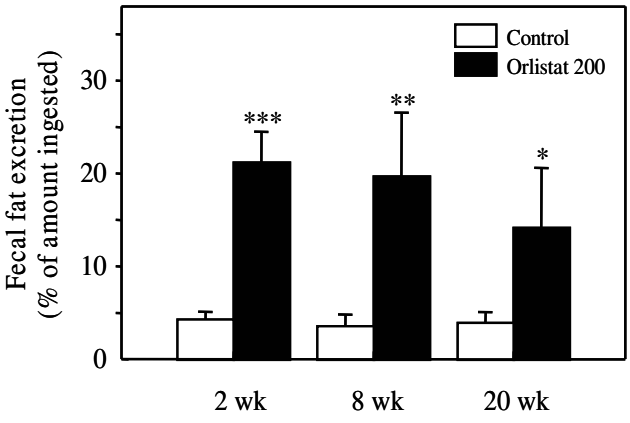

Figure 4. Prolonged dietary administration of orlistat to Gunn rats on a high-fat diet is associated with a sustained increase in fecal fat excretion. Amounts of fecal fat excretion in Gunn rats fed high-fat diet without supplement (control) or supplemented with orlistat (200 mg/kg chow). Experimental 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<0.05, **p<0.005, ***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.

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

Table 2. Orlistat treatment for 24 weeks of Gunn rats on a high-fat diet does not affect growth, body weight or plasma lipids. Gunn rats fed 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. Net amount of fat uptake was defined as the difference between the amount of fat ingested and the amount of fat excreted via the feces. Data represent mean ± SD of control and orlistat treated rats (n = 4 per group). NS, not significant.

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).

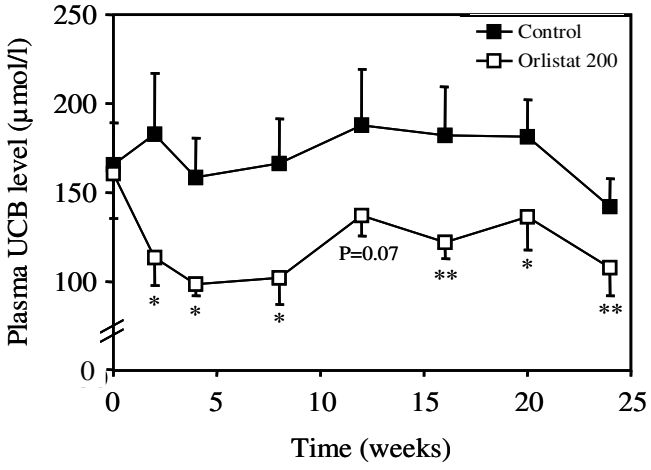

Figure 5. The effects of orlistat on plasma UCB concentrations in Gunn rats on a high-fat diet are sustained during treatment for 24 weeks. Plasma UCB concentration, determined by high-performance liquid chromatography, in Gunn rats fed a high-fat diet without supplement (control) or supplemented with 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.[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.

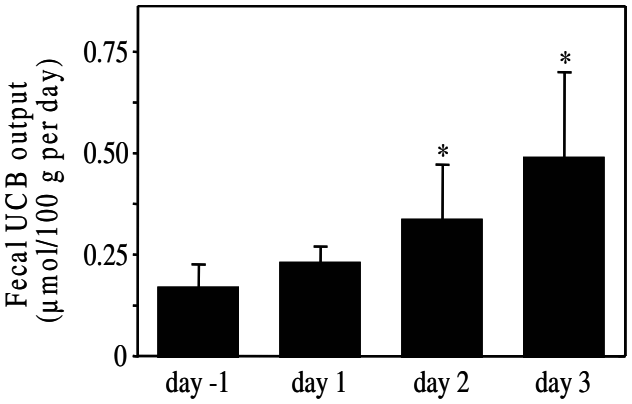

Figure 6. Effects of starting orlistat treatment on fecal excretion of UCB in Gunn rats on a high-fat diet. Fecal UCB excretion, determined by high-performance liquid chromatography, in Gunn rats that were switched from a high-fat control diet (day –1, run-in period 4 weeks), to the high-fat diet with 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.[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

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,[6;37-40] the elevated plasma and organ concentrations of UCB must be accompanied by a similar net disposal of UCB from the body by metabolism[41-43] 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.[6;7] The efficacy of UCB disposal into the intestinal lumen in terms of UCB excretion, however, is counteracted by reabsorption of UCB.[7;8;10;44-46] Previously, it had been demonstrated that the intraluminal presence of calcium phosphate, which binds UCB *in vitro*,[47] decreases plasma UCB concentrations in Gunn rats.[17] 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.[26;27] We recently demonstrated that orlistat supplementation to high-fat diets allowed for regulated manipulation of the coefficient of fat absorption in rats.[25] Orlistat is a chemically synthesized derivative of the natural product lipstatin, and specifically inhibits lipases at their catalytic triad $serine_{153}$-$histidine_{264}$-$aspartate_{177}$ by covalent binding to the serine residue.[48] 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.[49] Orlistat treatment has been applied for up to two years in weight reduction programs for persons with obesity, without serious side effects.[50] 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.[51;52] Another potential side effect of long-term orlistat treatment, namely deficiency of fat-soluble vitamins,[51] 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

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.[57;58] 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.[57] Yet, the similar biliary secretion rates of bile salts are not compatible with major differences in the intestinal concentrations of bile salts.[57] 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.[59] 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.[22] 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;[50] orlistat has been applied in studies in adults for two years without serious side effects;[50] 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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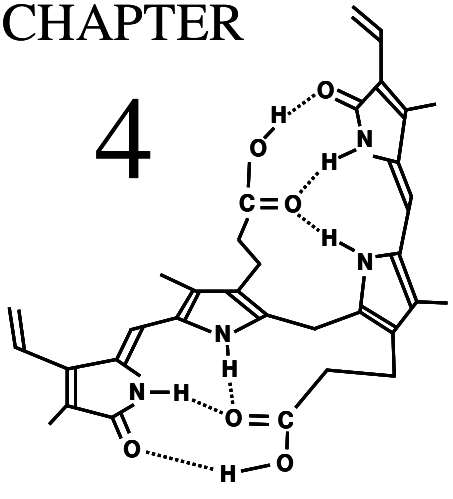
Effective oral treatment of unconjugated hyperbilirubinemia in Gunn rats

CHAPTER

4



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Anja M. Hafkamp,[1] Rick Havinga,[1]
Maarten Sinaasappel,[2] Henkjan J. Verkade[1]

[1]Department of Pediatrics, Division of Pediatric Gastroenterology, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, Groningen, The Netherlands

[2]Department of Pediatrics, Erasmus Medical Center, Sophia Children's Hospital, University Medical Center Rotterdam, The Netherlands

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.[1] Severe unconjugated hyperbilirubinemia can lead to bilirubin encephalopathy, kernicterus, and death.[2;3] Phenobarbital treatment can usually control unconjugated hyperbilirubinemia in Crigler-Najjar type II patients via residual enzyme induction.[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,[6;7] to a decrease in the surface area to body mass ratio,[8] and to a diminishing compliance to the intensive phototherapy regimen, which may take up to 12 hours per day.[6] To prevent irreversible brain damage due to kernicterus, many patients with Crigler-Najjar disease type I undergo liver transplantation in their second decade.[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[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.[13] In addition, UCB can diffuse from the blood, across the intestinal mucosa, into the intestinal lumen.[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.[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.[16] Amorphous calcium phosphate was shown to bind to UCB *in vitro*,[17] and intestinal capture of UCB by calcium phosphate decreased plasma UCB concentrations in Gunn rats,[18] a well-established animal model for Crigler-Najjar disease type I.[19;20] In Crigler-Najjar patients however, the effects of calcium phosphate treatment were less pronounced.[7] Other capturing agents like agar,[21] activated charcoal,[22] and cholestyramine[23] are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects.[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.[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.[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.[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.[31] 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 $\mu W/cm^2/nm$ 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 $\mu W/cm^2/nm$.

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.[30] 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,[32] 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.[14;30] After evaporation under nitrogen, the residue was re-dissolved in chloroform and analyzed by reversed-phase HPLC, as previously described,[33;34] 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.[35] Cholesterol and phospholipids were measured after lipid extraction,[36] according to methods of Gamble *et al.*,[37] and Bötcher *et al.*,[38] 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,[39] 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% HNO_3 was added and the mixture was heated at 95ºC for 5 minutes, after which 5 ml of 0.1% lanthanum chloride ($LaCl_3$) was added. After mixing, the samples were centrifuged for 10 minutes at 1500g. The supernatant was diluted 20 times with 0.1% $LaCl_3$ 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,[30] 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.[30]

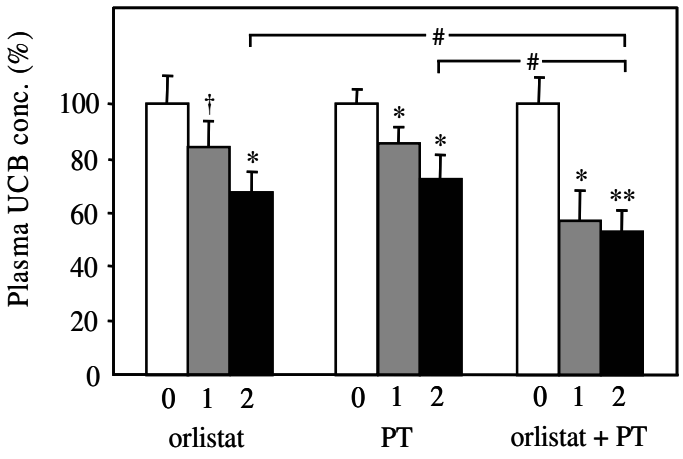

Figure 1. Effects of orlistat, continuous phototherapy (PT) and combined treatment (orlistat + PT) on plasma UCB concentrations in Gunn rats. Animals (n = 4-5 per group) were fed HF diet for 4 weeks, followed by treatment for 2 weeks with dietary orlistat supplementation, PT, or orlistat + PT. Blood samples were taken before treatment (□), and after 1 (▩) and 2 weeks of treatment (■). Plasma UCB values at T_0 (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.

Table 1. Plasma parameters after two weeks of treatment

		orlistat	phototherapy	orlistat + phototherapy
Hb	(mmol/l)	8.6 ± 0.3	8.4 ± 0.3	8.6 ± 0.4
Ht	(l/l)	0.41 ± 0.01	0.40 ± 0.01	0.41 ± 0.02
AST	(U/l)	21.0 ± 4.7	23.6 ± 2.8	24.4 ± 9.4
ALT	(U/l)	63.0 ± 12.2	57.6 ± 12.1	48.2 ± 11.7
Cholesterol	(mmol/l)	1.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.2
Triglycerides	(mmol/l)	2.2 ± 0.8	1.9 ± 0.6	1.7 ± 0.3

Table 1. 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. Blood samples were taken after 2 weeks of treatment. Data represent mean ± SD from n = 4-5 animals per group.

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

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

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,[30] 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 ($p<0.05$).

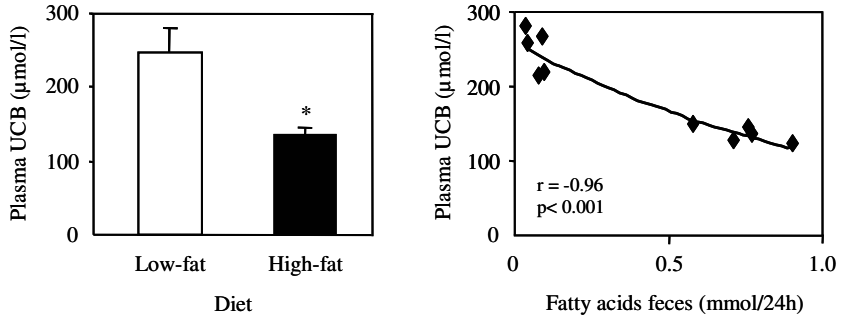

Figure 2. Effect of dietary fat content on plasma UCB concentrations in Gunn rats (left panel), and relationship between fecal fat excretion and plasma UCB concentrations (right panel). Gunn rats (n=5) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Data after 3 and 6 weeks of diet are 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.

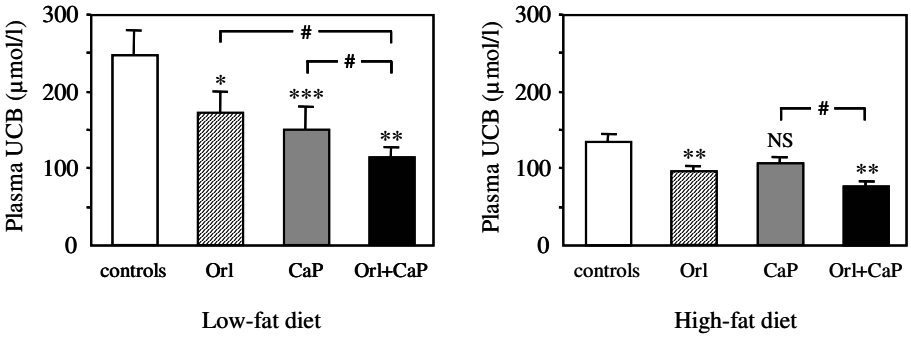

Figure 3. Effects of orlistat (Orl), calcium phosphate (CaP), and their combination (Orl + CaP) on plasma UCB concentrations in Gunn rats, during LF diet and HF diet. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), 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.

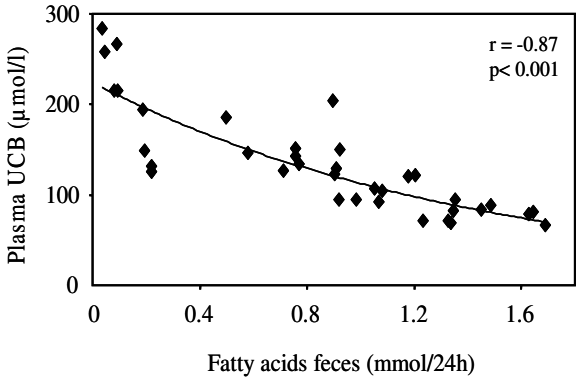

Figure 4. Relationship between fecal fat excretion and plasma UCB concentrations in Gunn rats. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), or supplemented with orlistat, calcium phosphate, or both. Feces were 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.

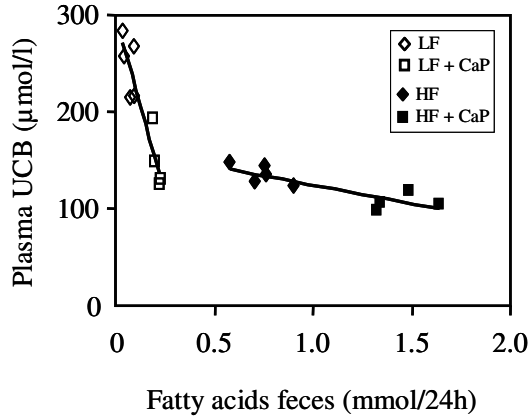

Figure 5. Relationship between fecal fat excretion and plasma UCB concentrations in Gunn rats, analyzed separately for controls and calcium phosphate treated animals during LF and HF diet (see Figure 4).

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

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.

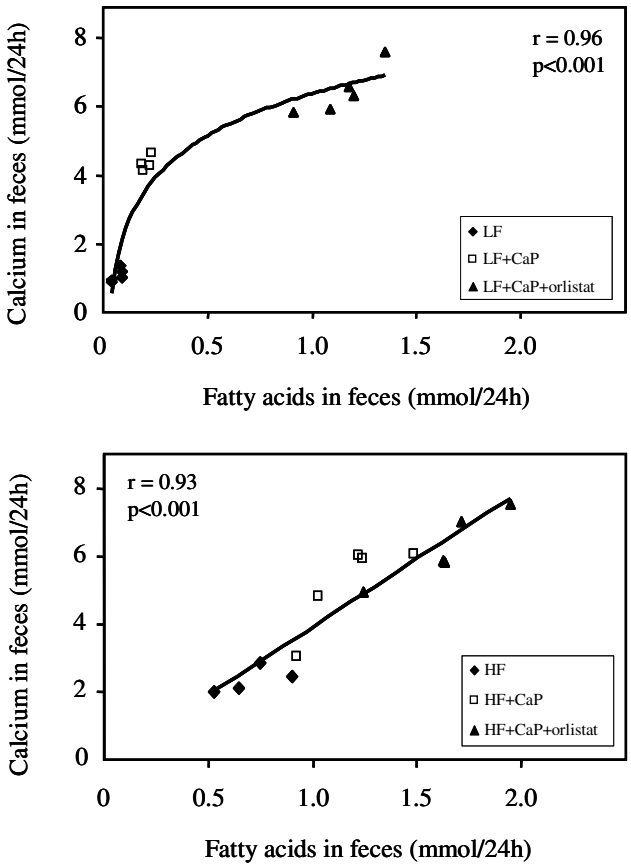

Figure 6. Relationship between fecal fat excretion and fecal calcium excretion in Gunn rats. Gunn rats (n = 4-5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Diets were either not supplemented (controls), or supplemented with calcium phosphate, or calcium phosphate + orlistat. 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.

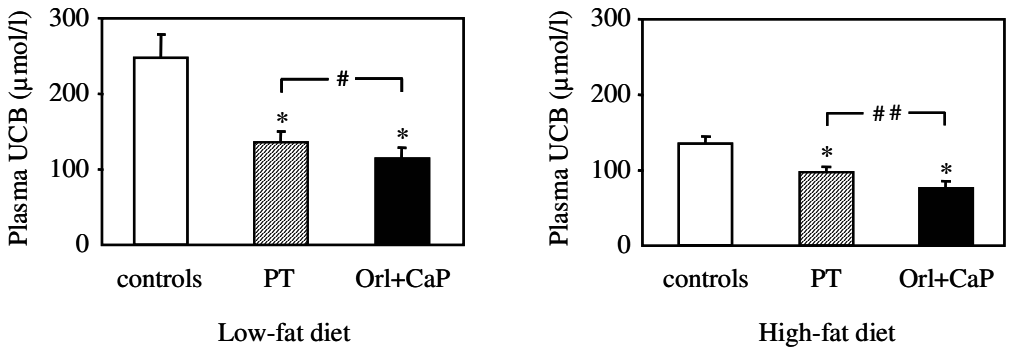

Figure 7. Efficacy of combined oral treatment with orlistat and calcium phosphate compared with continuous phototherapy in Gunn rats. Gunn rats (n = 5 per group) were fed LF diet for 3 weeks, followed by HF diet for 3 weeks. Animals were either not treated (controls), or treated with continuous phototherapy (PT), or with orlistat + calcium phospate (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 $\mu W/cm^2/nm$) 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 $\mu W/cm^2/nm$.[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 *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 *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%,[18] similar to our current LF diet results. Calcium phosphate treatment in Crigler-Najjar type I patients, however, decreased plasma UCB levels only by 18%.[7]. 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.[46;47] 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[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.[30] In humans, orlistat acts locally in the gastrointestinal tract and systemic absorption is minimal (~1%).[41] Orlistat is widely applied for treatment of obesity. Clinical trials in adults lasting up to two years have not reported serious side effects.[48] Recent studies in obese adolescents[49] and prepubertal children[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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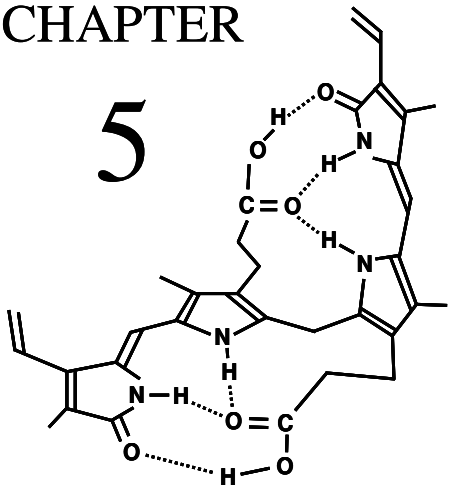
Novel kinetic insights into treatment of unconjugated hyperbilirubinemia: phototherapy and orlistat treatment in Gunn rats

CHAPTER

5



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Anja M. Hafkamp,[1] Rick Havinga,[1] J. Donald Ostrow,[2] Claudio Tiribelli,[3] Lorella Pascolo,[3] Maarten Sinaasappel,[4] Henkjan J. Verkade[1]

[1]Department of Pediatrics, Division of Pediatric Gastroenterology, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, Groningen, The Netherlands

[2]GI-Hepatology Division, University of Washington School of Medicine, Seattle, Washington, USA

[3]Department of Biochemistry, Biophysics and Chemistry of Macromolecules, University of Trieste, Trieste, Italy

[4]Department of Pediatrics, Erasmus University Medical Center, Sophia Children's Hospital, Rotterdam, The Netherlands

ABSTRACT

Treatment with phototherapy or with the lipase inhibitor orlistat decreases plasma unconjugated bilirubin (UCB) concentrations in hyperbilirubinemic Gunn rats. We investigated the mechanism(s) underlying the effects of orlistat, phototherapy, and combined treatment, using steady-state ^{3}H-UCB kinetics. After 3 weeks of treatment with orlistat (200 mg/kg chow), phototherapy (19 μW/cm^2/nm) or combined treatment, tracer ^{3}H-UCB was administered i.v. to treated and untreated (control) Gunn rats. Plasma samples and feces were collected every 12h for 60h, and bile for 30 min at 60h. The following results were obtained: a) each treatment decreased plasma bilirubin levels compared with controls: orlistat -19% ($p<0.05$), phototherapy -32% ($p<0.01$), combined treatment -53% ($p<0.001$); b) plasma bilirubin concentrations were strongly, negatively correlated with fractional bilirubin turnover ($r = -0.87$, $p<0.001$); c) orlistat treatment induced *net* transmucosal excretion of UCB into the intestinal lumen, whereas phototherapy increased biliary UCB excretion rate; d) all treatments profoundly increased the enterohepatic circulation of UCB derivatives, indicating enhanced metabolism by intestinal bacteria. In conclusion, orlistat and phototherapy lower plasma bilirubin concentrations in Gunn rats by increasing (net) intestinal influx of UCB, either by transmucosal excretion (orlistat), or increased biliary secretion (phototherapy). The mechanism of phototherapy and orlistat treatment involves increasing the availability of UCB in the intestinal lumen for fecal excretion and for metabolism by intestinal bacteria.

INTRODUCTION

Severe unconjugated hyperbilirubinemia can cause bilirubin-induced neurologic dysfunction (BIND), kernicterus, and death.[1] Conventional treatment for unconjugated hyperbilirubinemia involves phototherapy, which induces photoisomerization of unconjugated bilirubin (UCB) to more water-soluble derivatives that can be excreted into bile.[2] Short-term phototherapy has relatively minor side effects and is considered safe.[3]

Crigler-Najjar disease is characterized by permanent, unconjugated hyperbilirubinemia due to a genetic deficiency in bilirubin glucuronidation.[4] Conventional treatment for Crigler-Najjar disease (type I) involves life-long daily phototherapy. Disadvantages of long-term phototherapy are a declining efficacy with age and a profound impact on the quality of (social) life.[5;6] Since the introduction of phototherapy, serious long-term effects have not been reported. However, there have been reports of phototherapy-induced DNA damage to human cell lines *in vitro* and bilirubin was found to enhance this damage.[7;8]

An alternative treatment strategy for unconjugated hyperbilirubinemia is based on intestinal capture of UCB. UCB can diffuse across the intestinal mucosa from the blood into the intestinal lumen[9;10] and vice versa.[11] Capture of UCB in the intestine, by orally administered unabsorbable binders of UCB, can prevent its reabsorption into the enterohepatic circulation. Such agents, like agar, activated charcoal, and cholestyramine are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects. Intestinal capture of UCB by calcium phosphate was very effective in Gunn rats,[12] a well-established animal model for Crigler-Najjar disease.[13] In Crigler-Najjar patients however, the decrease in plasma UCB concentration was less pronounced, and occurred only in patients treated simultaneously with phototherapy.[5] Phototherapy profoundly enhances the very limited biliary secretion of UCB that can occur in the absence of conjugation,[14] thus increasing the amount of UCB available for capture in the intestine.

Recently, we demonstrated in Gunn rats that dietary supplementation with the lipase inhibitor orlistat decreased plasma UCB levels, parallel to an increase in fecal fat excretion.[15;16] Plasma UCB concentrations were strongly, negatively correlated with the amount of fat excreted via the feces. Orlistat treatment was equally effective as continuous phototherapy in Gunn rats, and combined treatment was more effective than either treatment alone,[16] which suggests that the two treatments operate by different mechanisms. The mechanism by which orlistat treatment reduces plasma bilirubin levels has not been elucidated so far. In the present study, we used steady-state ^{3}H-bilirubin kinetics to assess the mechanism(s) underlying the effects of orlistat, phototherapy, and combined treatment in Gunn rats.

METHODS

Animals. Homozygous male Gunn rats (RHA/jj, 220-320 g), were obtained from our breeding colony (University Medical Center Groningen, The Netherlands). Animals were housed in an environmentally controlled facility, were fed *ad libitum*, and were caged individually or, in case of phototherapy, per treatment group. The Ethics Committee for Animal Experiments (University of Groningen, The Netherlands) approved experimental protocols.

Phototherapy lamps. Two phototherapy devices were developed according to the prototype designed by Ostrow.[2] 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 $\mu W/cm^2/nm$; 380-480 nm; measured by an Elvos-LM-1010 Lux meter at 20 cm distance), was administered continuously to Gunn rats, shaven on their backs and flanks.

Chemicals. 2,3-^{3}H-labeled 5-aminolevulinic acid (specific activity 2.0 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Heptadecanoic acid (C17:0) and bilirubin were purchased from Sigma (St Louis, MO). Orlistat was from Roche Nederland (Woerden, The Netherlands).

Preparation of ^{3}H-labeled UCB. ^{3}H-labeled UCB (specific activity $48.8x10^3$ dpm/µg = 12.9 µCi/µmol) was prepared by biosynthetic labeling of bilirubin in dog bile from precursor labeled 2,3-^{3}H-5-aminolevulinic acid.[17] The ^{3}H-UCB was isolated and recrystalized twice.[18]

Preparation of ^{3}H-UCB solution for i.v. administration. Under dim light, immediately before injection into the Gunn rats, 1.5 mg of ^{3}H-UCB was dissolved in 1.0 ml dimethyl sulfoxide (DMSO). Then, 12.5 ml of Wistar rat plasma was slowly added with continuous, thorough mixing. 0.2 ml/100 g body weight (BW), containing 22 µg UCB/100 g BW, and 0.49 µCi/100 g BW, was administered to each Gunn rat via the penile vein.

Diets. Hope Farms (Woerden, The Netherlands) produced the semisynthetic, purified control diet (code 4063.02), that contained 13 energy% fat / 5.2 wt% long-chain fatty acids. As in previous studies, all Gunn rats were fed the control diet for a run-in period of 4 weeks.[15;16] The orlistat-supplemented diet was prepared by thorough mixing of orlistat (Xenical®, 200 mg/kg chow) into the control diet.

Study Design

Pilot experiment to determine non-toxic limit of injected DMSO

To study ^{3}H-UCB kinetics under steady-state conditions, it was essential to prevent DMSO-induced hemolysis, by minimizing the volume of administered DMSO, without compromising solubilization of bilirubin. Intravenous administration of 0.3 or 0.15 ml of DMSO (diluted 1:4 with Wistar rat plasma) to ~300 g Gunn rats caused transient hemolysis, characterized by increased plasma UCB concentrations, decreased hemoglobin (Hb) and hematocrit (Ht), and reticulocytosis (data not shown). By contrast, when an 8 mM solution of bilirubin in DMSO was diluted 1:16 with Wistar rat plasma, and 0.2 ml/100g BW was administered i.v. to three

adult Gunn rats, samples of tail vein blood obtained 0, 6, 12, 24, 30 and 48 hours after injection revealed no evidence of hemolysis. Thus, in an adult male Gunn rat, i.v. administration of ~0.04 ml of DMSO, diluted 1:16 with rat plasma, should not alter steady-state ^{3}H-UCB kinetics.

Effects of orlistat and/or phototherapy on steady-state bilirubin kinetics

After 4 weeks run-in on the control diet, Gunn rats were randomly assigned to one of 4 groups (n = 4-6 per group), to receive either the control diet, the orlistat-supplemented diet, continuous phototherapy, or the combination of orlistat-supplemented diet and continuous phototherapy. Previously, we showed that 2 weeks of orlistat treatment is sufficient to reach steady-state plasma UCB concentrations and fecal UCB excretion.[15] After 3 weeks of treatment (T_0h), heparinized samples of tail vein blood were obtained under isoflurane anesthesia. We then administered ^{3}H-UCB (~0.49 μCi/100 g BW) via the penile vein and collected blood samples every 12h for 60h to determine plasma ^{3}H and bilirubin concentration. After ~60h, under pentobarbital anesthesia, bile was collected for 30 minutes under light-protected conditions after cannulation of the common bile duct. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. After bile collection, blood was obtained by vena cava inferior puncture for determination of Hb, Ht, reticulocytes, aspartate-aminotransferase activity (AST), and alanine-aminotransferase activity (ALT). Three days before T_0h, feces were collected for 72h to determine steady-state fecal fat excretion. From T_0h onwards, feces were collected every 12h for 60h to determine fecal ^{3}H excretion.

Calculation of fluxes based on steady-state ^{3}H-UCB kinetics

Fractional turnover of ^{3}H-UCB (%/h) was calculated from semilogarithmic plots of ^{3}H-UCB specific activity. Bilirubin pool size was calculated by dividing the calculated specific activity at T_0h (Y-axis intercept) by the administered dose of ^{3}H-UCB radioactivity. Total bilirubin turnover was calculated as the product of fractional turnover and pool size,[19] and assumed to equal steady-state fecal excretion of UCB and derivatives. The data allowed estimation of steady-state fluxes of UCB and derivatives in the enterohepatic circulation during the various treatments. Individual fluxes were calculated as follows. Biliary excretion of UCB was measured by HPLC (see: Analytical Methods). With biliary excretion of UCB and specific activity in plasma at the time of bile collection, the percentage of ^{3}H in bile which originated from ^{3}H-UCB was calculated. Biliary excretion of derivatives was calculated by subtracting ^{3}H-UCB radioactivity from total biliary ^{3}H-radioactivity measured by liquid scintillation, and expressed as equivalent percent of the exchangeable bilirubin pool excreted per time period. *Net* transmucosal flux of UCB was estimated by subtracting biliary UCB excretion from fractional UCB turnover. Total fecal excretion of UCB and its derivatives was calculated by dividing the total amount of dpm in feces between T_{12} and T_{60} hours, by the mean specific activity of UCB in plasma between T_{12} and T_{60} hours.

Analytical Methods

All analytical procedures were performed in dim light.

Plasma. Bilirubin levels were determined by reflectance spectrophotometry on a Vitros-950 analyzer (Johnson & Johnson, Tilburg, The Netherlands), and were confirmed by HPLC analysis. Hb, Ht, and reticulocytes were determined on a Sysmex-XE-2100 hematology analyzer (Goffin Meyvis, Etten-Leur, The Netherlands). AST, and ALT were determined on a Mega analyzer (Merck, Darmstadt, Germany). ^{3}H content was determined by liquid scintillation (Tri-Carb 2500-TR Liquid Scintillation Analyzer, Packard Bioscience, Meriden, CT), using two ml of Ultima Gold-XR (Packard Bioscience) scintillation fluid added to 100 µl of plasma. Samples were counted for 10 min and quench corrected using ^{133}Barium as external standard.

Bile. For UCB measurement, bile was submitted to alkaline methanolysis and chloroform extraction. After evaporation under nitrogen, the residue was re-dissolved in chloroform and analyzed by reversed-phase HPLC, as described previously.[16] ^{3}H content in 100 µl of bile was determined by liquid scintillation as described above.

Feces. Feces were freeze-dried and mechanically homogenized. For determination of fatty acids, aliquots of feces were extracted, hydrolyzed and methylated,[20] using methanol/hexane. Resulting fatty acid methyl esters were determined by gas chromatography (HP-Ultra-1 column, Hewlett-Packard, Palo-Alto, CA). Fatty acid contents were calculated in molar amounts, using C17:0 as internal standard. For duplicate determination of ^{3}H content, two aliquots (~100 mg) of feces were decolorized by incubation with 0.6 ml of NaOCl for 30 minutes at 37ºC. Then 60 µl of NH_3 was added to neutralize free chloride. After vortexing, 10 ml of scintillation fluid was added and thoroughly mixed. After 24h, ^{3}H content was determined by liquid scintillation.

Statistical Analyses

Analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). Results are expressed as mean ± SD. Based on a normal distribution of plasma bilirubin levels in large groups of Gunn rats,[15] parametric tests were used for statistical analysis. ANOVA with post-hoc Bonferroni correction was performed for comparisons among groups. The relationship between fractional ^{3}H-UCB turnover or pool size, and plasma bilirubin concentration was analyzed by linear regression analysis. *P* values <0.05 (two-tailed) were considered significant.

RESULTS

Effects of orlistat and/or phototherapy on body weights and food intake

Body weights at the start and end of 3 weeks of treatment were not significantly different among groups. All animals increased their body weight in 3 weeks (controls, 7 ± 2%; orlistat,

14 ± 5%; phototherapy (PT), 12 ± 8%, orlistat + PT, 7 ± 4%; not significant). As observed previously,[15] orlistat-treated animals appeared to compensate fecal fat loss by increasing their food intake, compared with controls (25.6 ± 5.1 vs. 20.0 ± 2.4 g/24h, respectively; $p<0.05$).

Effects of orlistat and/or phototherapy on plasma bilirubin concentrations

Figure 1 shows that after 3 weeks of treatment (T_0h) with orlistat or phototherapy, plasma bilirubin concentrations were significantly lower than in controls (-19%, $p<0.05$; and -32%, $p<0.01$, respectively). Efficacies of orlistat and phototherapy were not significantly different. Combined treatment with orlistat and phototherapy induced a more profound decrease in plasma bilirubin concentrations than either treatment alone (-53%, $p<0.001$). After i.v. ^{3}H-UCB administration, plasma UCB levels at 12h intervals did not change significantly compared with T_0h values (except at T_{24}h in the combined treatment group), in accordance with steady-state conditions. Also, none of the treatments significantly affected relevant hematological and liver function parameters (Table 1), confirming absence of significant hemolysis.

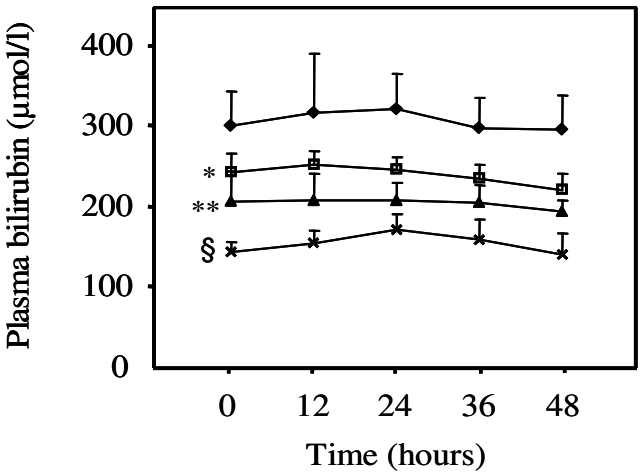

Figure 1. Steady-state plasma bilirubin concentrations after i.v. administration of ^{3}H-UCB. Four groups of Gunn rats (n = 4-6 per group) were either untreated (controls ♦), or treated for 3 weeks with orlistat (□), continuous phototherapy (PT ▲), or combined treatment (orlistat + PT ×). After 3 weeks (T_0h) ^{3}H-UCB was injected i.v.; blood samples were taken at 12h intervals. Data represent mean ± SD. *$p<0.05$, **$p<0.01$, $^{§}p<0.001$, T_0h data compared with controls.

Table 1. Hematological and liver function parameters after 3 weeks of treatment

	controls (n=6)	orlistat (n=5)	PT (n=4)	orlistat+PT (n=4)
Hemoglobin (mmol/l)	6.9 ± 0.4	7.1 ± 0.3	7.2 ± 0.2	7.4 ± 0.2
Hematocrit (v/v)	0.30 ± 0.02	0.32 ± 0.01	0.33 ± 0.02	0.33 ± 0.02
Reticulocytes ($^{0}/_{00}$)	52 ± 15	48 ± 18	56 ± 11	40 ± 24
AST (U/l)	94 ± 30	85 ± 12	94 ± 17	92 ± 12
ALT (U/l)	46 ± 7	55 ± 11	45 ± 10	55 ± 14

Table 1. Hematological and liver function parameters in Gunn rats after 3 weeks of either no treatment (controls), or treatment with orlistat, phototherapy (PT), or combined treatment (orlistat + PT). Data represent mean ± SD. No significant differences among groups.

Table 2. Steady-state ^{3}H-bilirubin kinetics and biliary UCB excretion after 3 weeks of treatment

	controls (n=6)	orlistat (n=5)	PT (n=4)	orlistat + PT (n=4)
Plasma bilirubin at T_0h (µmol/l)	301 ± 43	243 ± 23*††	206 ± 38**^{b}	143 ± 12§
Fractional turnover ^{3}H-UCB (%/h)	0.59 ± 0.23	0.91 ± 0.21^{a†}	1.00 ± 0.07*	1.29 ± 0.13§
Bilirubin pool size (µmol/100 g BW)	4.8 ± 1.1	3.8 ± 0.5	3.7 ± 0.5	2.8 ± 0.2**
Total turnover bilirubin (nmol/h per 100 g BW)	30.1 ± 6.5	34.6 ± 5.5	36.7 ± 4.8	36.6 ± 3.3
Biliary UCB excretion (nmol/h per 100 g BW)	29.3 ± 4.3	25.7 ± 6.9	36.7 ± 6.1†	19.7 ± 7.1
Total biliary excretion of UCB + derivatives (nmol UCB (equivalents)/h per 100 g BW)	109 ± 33	158 ± 16	155 ± 36	109 ± 42
Fecal ^{3}H excretion $T_{0\text{-}60}$h (10^3 dpm)	726 ± 63	951 ± 132*	972‡	1372‡
Total fecal excretion of UCB + derivatives (nmol UCB (equivalents)/h per 100 g BW)	42 ± 9	52 ± 12	59‡	63‡

Table 2. Steady-state ^{3}H-bilirubin kinetics in Gunn rats after 3 weeks of either no treatment (controls), or treatment with orlistat, continuous phototherapy (PT), or combined treatment (orlistat + PT). After 3 weeks (T_0h), a tracer dose of ^{3}H-UCB (~0.5 µCi/100 g BW) was administered i.v. Plasma ^{3}H and bilirubin levels were determined at 12 hour intervals for 60 hours. Feces was collected every 12 hours for 60 hours. After 60 hours bile was collected for 30 minutes. For calculation of fluxes see Methods section. Data represent mean ± SD. *p<0.05, ^{a}p=0.058 **p<0.01, §p<0.001, compared with controls. †p<0.05, ††p<0.01, ^{b}p=0.078, compared with orlistat + PT. ‡SD could not be calculated due to pooled feces per group.

Effects of orlistat and/or phototherapy on steady-state bilirubin kinetics

Table 2 shows biliary UCB secretion and results of the ^{3}H-UCB kinetics study. Similar to previous observations,[15] orlistat treatment did not significantly affect bile flow compared with control Gunn rats (4.2 ± 0.9 and 3.2 ± 0.7 µl/min per 100 g BW, respectively), and neither did phototherapy or combined treatment (3.9 ± 0.3 and 4.0 ± 0.5 µl/min per 100 g BW, respectively). Biliary excretion rate of UCB tended to be higher during phototherapy (+25%), compared with controls, and lower during orlistat treatment (-12%) and combined treatment (-33%), but none of these differences were statistically significant.

Figure 2 shows a semilogarithmic plot of plasma ^{3}H-UCB specific activities during 60h after ^{3}H-UCB administration. In each of the four groups, the logarithm of plasma ^{3}H-UCB specific activity declined in a linear fashion with time, in accordance with steady-state kinetics. Table 2 summarizes the effects of orlistat and/or phototherapy on steady-state ^{3}H-bilirubin kinetics. Bilirubin pool sizes, compared with controls, had decreased in each treatment group after 3 weeks (orlistat, -21%; PT, -23%; orlistat + PT, -42%), but statistical significance was only reached with combined treatment (p<0.01). Bilirubin pool sizes were strongly positively correlated with plasma bilirubin concentrations at T_0h (r = 0.87, p<0.001, not shown). Total bilirubin turnover was not significantly altered by any of the treatments, confirming steady-state conditions and absence of (DMSO-induced) hemolysis. The quantity of ^{3}H-UCB injected was <1.5% of the total UCB pool in all groups, thus representing a true tracer dose labeling of the UCB pool. Fractional turnover of ^{3}H-UCB, compared with controls, was increased by 54% during orlistat treatment (p=0.058), by 69% during phototherapy (p<0.05), and by 119% during combined treatment (p<0.01).

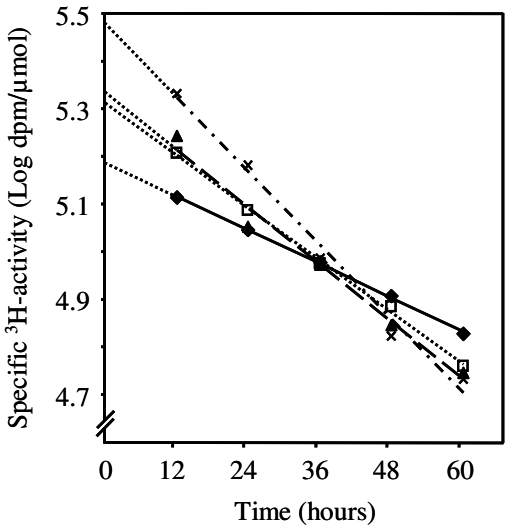

Figure 2. Semilogarithmic plots of ^{3}H-UCB specific activity after i.v. administration of ^{3}H-UCB to Gunn rats. Experimental groups, treatments and ^{3}H-UCB administration as in Figure 1. Blood samples were taken at 12h intervals. Data represent mean values for each group. ♦ controls, r = -1.00; □ orlistat, r = -1.00; ▲ PT, r = -0.99; × orlistat + PT, r = -0.99.

Figure 3 shows that fractional turnover of ^{3}H-UCB and plasma bilirubin concentrations at T_0h were negatively, linearly correlated (r = -0.87, p<0.001), indicating that the differences in plasma bilirubin concentration between controls and treated groups were related to stimulation of fractional turnover of bilirubin in the treated animals.

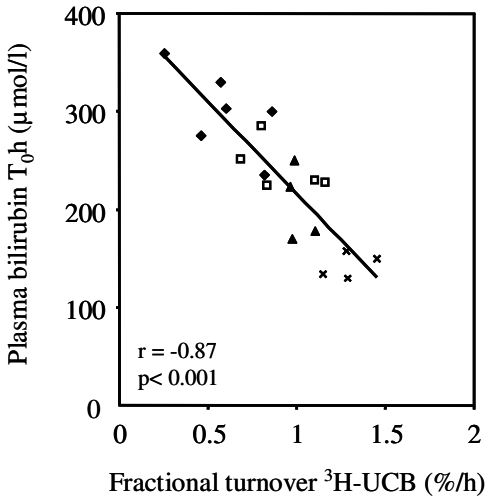

Figure 3. Negative linear relationship between fractional turnover of ^{3}H-UCB and steady-state plasma bilirubin levels at T_0h in Gunn rats. Experimental groups, treatments and ^{3}H-UCB administration as in Figure 1. Each symbol represents data obtained in an individual animal. r = -0.87, p<0.001. ♦ controls, □ orlistat, ▲ PT, × orlistat + PT.

Effects of orlistat on fecal fat and ^{3}H excretion

In agreement with our previous data,[15;16] orlistat treatment increased fecal fat excretion. Fatty acid concentration in feces collected for 72h was significantly higher in orlistat-treated animals (0.44 ± 0.08 mmol/g freeze-dried feces; p<0.001), compared with controls (0.04 ± 0.01). Orlistat treatment also increased dry fecal weight (3.7 ± 0.7 g/24h) compared with controls (2.5 ± 0.3; p<0.01), and cumulative fecal ^{3}H excretion between T_0h and T_{60}h (+31%, p<0.05, Table 2). Fecal fat excretion per individual animal could not be calculated for the phototherapy or combined treatment group because these animals were housed together per treatment group. In pooled fecal samples from these rats, fatty acid concentrations were respectively 0.32 mmol/g feces (combined treatment) and 0.05 mmol/g feces (phototherapy).

Cumulative ^{3}H excretion in the pooled fecal samples of the phototherapy group was comparable to the orlistat group. With combined treatment, cumulative ^{3}H excretion was almost twice control values (Table 2).

DISCUSSION

We investigated in Gunn rats the mechanism(s) underlying the hypobilirubinemic effects of orlistat, phototherapy and combined treatment, using steady-state ^{3}H-UCB kinetics. Our data indicate that orlistat treatment and phototherapy lower plasma bilirubin concentrations in Gunn rats by increasing intestinal influx of UCB, either by transmucosal excretion (orlistat), or increased biliary secretion (phototherapy). By increasing intestinal influx of UCB, it becomes available for fecal elimination and/or for metabolism by intestinal bacteria, both of which interrupt the enterohepatic circulation of UCB.

In the assessment of bilirubin turnover rates, it is crucial to work at steady-state conditions. We therefore performed a pilot experiment to define the threshold of i.v. DMSO administration to avoid hemolysis. Steady-state conditions in the ^{3}H-UCB kinetics study were confirmed by the following observations: 1) in each group, plasma bilirubin concentrations remained stable during the study (Fig. 1). Previously, we showed that plasma UCB concentrations in Gunn rats did not change significantly between 2 and 24 weeks of orlistat treatment;[15] 2) total bilirubin turnover was unaltered compared with controls (Table 2). Under steady-state conditions, total bilirubin turnover equals UCB production rate. Neither phototherapy nor orlistat treatment has been shown to affect UCB production rate;[21] 3) relevant hematological parameters were stable (Table 1); and, finally, 4) ^{3}H-UCB specific activities declined in a semilogarithmic fashion in each of the four groups (Fig. 2).

Not all individual parameters of bilirubin kinetics were significantly different between the various treatments and controls (Table 2). Yet, plasma bilirubin levels were strongly, negatively correlated with fractional turnover of ^{3}H-UCB (Fig. 3). This observation indicates that phototherapy and orlistat treatment both decrease plasma UCB concentrations via stimulation of bilirubin turnover. The present study also showed a strong, positive correlation between plasma UCB concentrations and bilirubin pool size, indicating that plasma UCB concentrations closely reflected UCB pool sizes during these (steady-state) treatments. The decrease in UCB pool size during phototherapy (-23%) is almost identical to results reported by Cohen et al. (-22%).[21] Pool sizes were essentially the same in orlistat versus phototherapy treated Gunn rats and efficacies of orlistat and phototherapy were not significantly different, as shown previously.[16] However, in the current study, orlistat seemed to be slightly less effective than phototherapy in decreasing plasma UCB concentrations. This suggests that there was a different distribution of the UCB pool between plasma and tissues in orlistat versus phototherapy-treated Gunn rats.

The present study allowed estimation of steady-state fluxes of UCB and its derivatives in the enterohepatic circulation under the four experimental conditions (Figure 4). Calculations are based on the assumption that quantitative disposal of bilirubin and its derivatives from the body occurs exclusively via the feces; *i.e.*, fecal excretion of UCB plus derivatives equals fractional bilirubin turnover from the UCB pool.

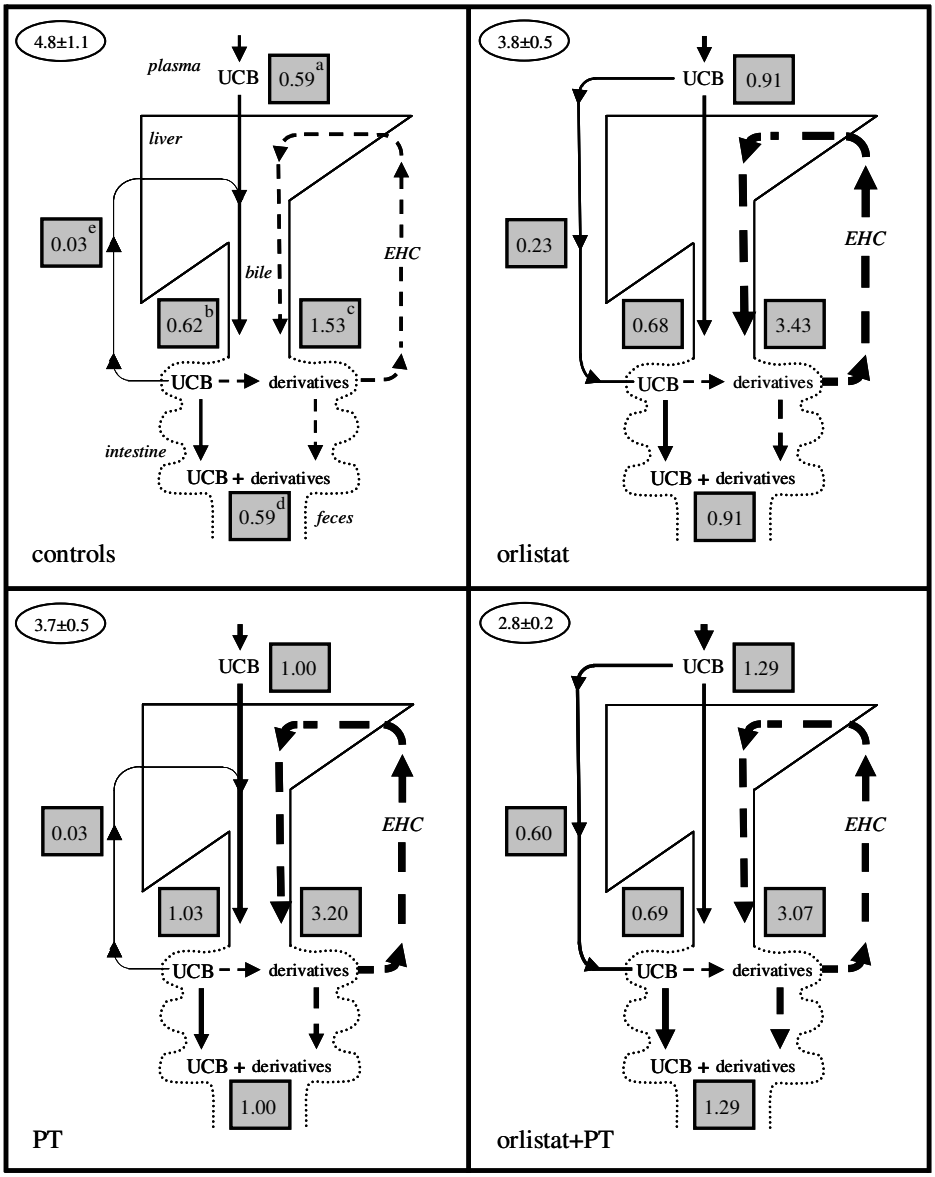

Figure 4. Fractional steady-state biliary and fecal fluxes of UCB and derivatives, and calculated *net* transmucosal flux of UCB in Gunn rats after 3 weeks of either no treatment (controls), or treatment with orlistat, continuous phototherapy (PT), or combined treatment (orlistat + PT). Fluxes of UCB and derivatives were determined from tracer ^{3}H-UCB kinetic data, as described in Methods. UCB fluxes are expressed as % of the exchangeable (plasma + tissue) bilirubin pool excreted per hour. Derivatives fluxes are expressed as equivalent% of the exchangeable bilirubin pool excreted per hour. [a] = fractional turnover of UCB; [b] = fractional biliary excretion of UCB; [c] = fractional biliary excretion of derivatives; [d] = fractional fecal excretion of UCB + derivatives (equals [a] in a steady-state, under the assumption that turnover of plasma UCB is completely determined by fecal excretion of UCB and derivatives); [e] = estimated *net* transmucosal flux of UCB, from intestinal lumen to blood or vice versa, calculated as [a]-[b]. EHC = enterohepatic circulation. Ovals in upper left of each panel show bilirubin pool size (µmol/100 g BW; mean ± SD).

This assumption seems reasonable, since, in Gunn rats, urinary secretion of labeled UCB and its derivatives (consisting of polar photoderivatives, urobilinoids and other bacterial metabolites) is limited (~5%).[10;22] Our calculations are also based on the assumption that metabolism of UCB to UCB-derivatives quantitatively occurs only in the intestinal lumen. It does, therefore, not take into account oxidative catabolism of UCB that could occur, *e.g.*, in the liver. This catabolism is, however, relatively small in the untreated Gunn rat.[21;23] *Net* transmucosal flux of UCB from the intestinal lumen into the blood or vice versa was calculated by subtracting biliary UCB excretion from fractional UCB turnover. It needs to be emphasized that the estimate reflects the *net difference* between the unidirectional fluxes of UCB from and into the intestinal lumen, whereas individual fluxes may be substantially different between treatments.

The main results obtained from the calculations are: a) phototherapy increases biliary excretion of UCB and its derivatives; b) orlistat treatment induces net transmucosal excretion of UCB into the intestinal lumen; c) treatment with phototherapy and/or orlistat enhances catabolism of UCB to derivatives that undergo efficient enterohepatic circulation; d) combined treatment with orlistat and phototherapy more effectively increases the amount of UCB in the intestinal lumen and its subsequent disposal than either treatment alone. The main results will now be discussed consecutively in more detail.

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. Phototherapy enhanced biliary excretion of UCB, as shown previously.[2] A novel finding of the present study is that phototherapy also enhances biliary excretion of UCB derivatives. The increased biliary excretion of derivatives in the phototherapy group is most likely the consequence of enterohepatic recirculation of urobilinogens and other metabolites due to enhanced supply of substrate in the intestinal lumen. Previously, Schmid *et al.*[10] and Kotal *et al.*[9;24] showed that labeled UCB derivatives in Gunn rat bile are mostly urobilinogen and other diazo-negative polar products. Interestingly, the fractional flux of derivatives undergoing enterohepatic circulation is increased during phototherapy, compared with controls. This suggests that enhanced conversion (metabolism) of UCB to derivatives in the intestine quantitatively contributes to the hypobilirubinemic effect of phototherapy in Gunn rats. Another novel finding of the present study is the highly efficient reabsorption and enterohepatic circulation of UCB-derivatives in all four groups. Our data do not permit calculation of derivative pool sizes, nor the exact percentages of derivatives that were reabsorbed into the enterohepatic circulation.

Detailed analysis of (radiolabeled) photoisomers was not performed. It has been shown previously that the contribution of photoisomers to plasma radioactivity, originating from tracer radiolabeled UCB administered i.v. to Gunn rats, is minor.[2] This is probably due to much more rapid hepatic clearance and biliary excretion of photoisomers by the Gunn rat.[25] That study also documented that the major photoisomer in bile was cyclized, stable

photobilirubin II, whereas unstable photobilirubins IA&B rapidly reverted to UCB. There is no reason to believe that results in our Gunn rats would be different.

Our estimation of fluxes assumes that almost all ^{3}H in blood is UCB. Others have shown previously with ^{14}C labeled UCB that only a very small amount of label is present in plasma urobilinogen. Our steady-state turnover rates are comparable with those found with ^{14}C labeled UCB.[2;10] The exchange rate of ^{14}C with other C atoms is much lower than that of ^{3}H with H-atoms, *e.g.* H_2O. Furthermore, the fact that we measure more radioactivity in bile relative to plasma turnover indicates that UCB derivatives apparently undergo rapid enterohepatic circulation, but do not enter the systemic circulation, presumably due to a rapid first-pass effect in the liver. Finally, ^{3}H-label exchange with water is not likely considering the following. We utilized ^{3}H-UCB prepared biosynthetically in dogs by administration of 2,3-^{3}H-5-aminolevulinic acid[17] based on the method of Howe et al.[26]. As stated by them, all tritium atoms in the labeled UCB are in stable positions on the side chains. They documented this directly by showing that, after simultaneous i.v. administration of ^{3}H-UCB and ^{14}C-UCB to humans, plasma disappearance curves of ^{3}H and ^{14}C radioactivity were superimposable for periods ranging from 12 hours to 16 days. Comparable results were obtained by Lester and Klein[27] with the supposedly less stable ^{3}H-UCB prepared from 3,5-^{3}H-5-aminolevulinic in rats; the ^{3}H/^{14}C-ratio of the two labeled UCB excreted in rat bile was constant over a period of 24 hours.

Orlistat reduces absorption of dietary fats through inhibition of gastrointestinal lipases.[28] Based on a strong negative correlation between the amount of fat excreted via the feces and plasma UCB concentrations in Gunn rats, we hypothesized that orlistat treatment diminishes the enterohepatic circulation of UCB via intestinal capture of UCB by unabsorbed fat.[15;16] In the two groups treated with orlistat (± phototherapy), there was indeed net transmucosal passage of UCB from the blood into the intestinal lumen, and enhanced fecal excretion of UCB and its derivatives, compatible with the proposed concept. In addition, however, the hypobilirubinemic effect of orlistat appears to result from increased metabolism of UCB to derivatives. As with phototherapy, the increased intraluminal pool of UCB during orlistat treatment appears to provide increased substrate for intestinal metabolism. Vitek *et al.*[29] recently showed that plasma UCB concentration increases significantly in Gunn rats in which conversion of UCB to urobilinoids by anaerobic intestinal flora was prevented by treatment with antibiotics. We cannot exclude the possibility that phototherapy or orlistat alters the composition of the intestinal microflora and/or their capacity to metabolize UCB, in addition to increasing the amount of UCB available in the intestinal lumen for microbial metabolism.

In the combined treatment group, the increased influx of UCB into the intestine appeared to be due mainly to enhanced net transmucosal UCB excretion, since biliary UCB secretion was lower in the combined treatment group than in the phototherapy group. This may be related to the greater decrease in steady-state plasma UCB concentration in the combined treatment group, since phototherapy is less effective at lower plasma UCB concentrations due

to depletion of the bilirubin pool in the skin, which is the main target of phototherapy.[21;30] In addition, one could speculate that orlistat treatment induces net transmucosal transport of photoisomers of UCB, although these compounds are considered relatively water-soluble.

In conclusion, orlistat treatment and phototherapy lower plasma bilirubin concentrations in Gunn rats by increasing intestinal influx of UCB, either by transmucosal excretion (orlistat), or increased biliary secretion (phototherapy). Combined treatment has an additive effect. The mechanism of phototherapy and of orlistat treatment involves increasing the availability of UCB in the intestinal lumen for subsequent fecal excretion and for metabolism by intestinal bacteria. We speculate that manipulation of the metabolizing capacity of the intestinal flora (antibiotics, probiotics) will influence the hypobilirubinemic effects of phototherapy, orlistat treatment, or combined treatment.

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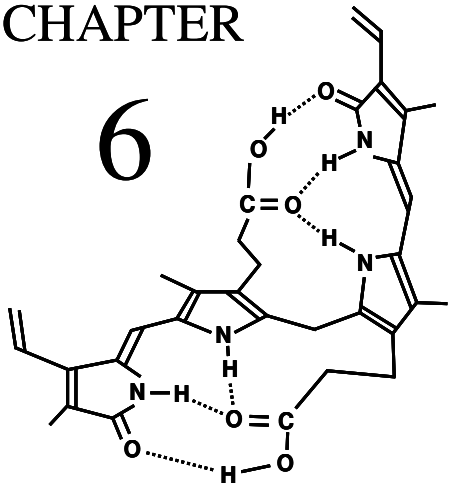
Orlistat treatment of unconjugated hyperbilirubinemia in Crigler-Najjar disease; A randomized controlled trial

CHAPTER

6



Submitted

Anja M. Hafkamp,[1] Rineke Nelisse-Haak,[2] Maarten Sinaasappel,[2]
Ronald P.J. Oude Elferink,[3] Henkjan J. Verkade[1]

[1]Department of Pediatrics, Division of Pediatric Gastroenterology, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, Groningen, The Netherlands

[2]Department of Pediatrics, Erasmus University Medical Center, Sophia Children's Hospital, Rotterdam, The Netherlands

[3]Amsterdam Liver Center, Academic Medical Center, Amsterdam, The Netherlands

ABSTRACT

Unconjugated hyperbilirubinemia in Crigler-Najjar (CN) disease is conventionally treated with phototherapy (PT) and/or phenobarbital (PB). Orlistat treatment decreases plasma unconjugated bilirubin (UCB) concentrations in Gunn rats, the animal model for CN disease. We determined in CN patients the effects of orlistat treatment on plasma UCB concentrations, and on fecal excretion of fat and UCB. A randomized, placebo-controlled, double-blind, cross-over trial was conducted in 16 patients, simultaneous with their regular treatment (PT, n = 11, and/or PB, n = 6). Patients received orlistat or placebo, each for 4-6 weeks (2 weeks interval). Plasma UCB concentrations and fecal excretion of fat and UCB were determined. We defined a clinically relevant response to orlistat treatment as a decrease in plasma UCB concentration of at least 10%. Compared with placebo, orlistat increased fecal fat excretion (+333%, $p<0.001$), and fecal UCB excretion (+43%, $p<0.05$). Orlistat treatment decreased plasma UCB concentration by 9% ($p<0.01$). In 7 of 16 patients, the decrease in plasma UCB levels was clinically relevant (*i.e.* more than 10%; mean 21%, $p<0.001$). Only in patients with a clinically relevant response, plasma UCB concentrations during orlistat were strongly, negatively correlated with fecal fatty acid excretion ($r = -0.93$, $p<0.01$). Clinically relevant response to orlistat treatment was not correlated with age, sex, CN-type, BMI, or co-treatment with PT or PB, but appeared correlated with a relatively low dietary fat intake of less than 35 energy% ($p=0.05$). In conclusion, orlistat treatment decreases plasma UCB concentrations, particularly in a subgroup of CN patients. Dietary fat intake may determine the responsiveness to orlistat treatment.

INTRODUCTION

Crigler-Najjar (CN) disease is a genetic disorder of bilirubin metabolism caused by deficiency of the hepatic enzyme bilirubin-UDP-glucuronosyltransferase (UGT1A1).[1] UGT1A1 catalyzes the conjugation of unconjugated bilirubin (UCB), the hydrophobic end product of heme degradation. UCB 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. CN patients suffer from permanent unconjugated hyperbilirubinemia which, if left untreated, can cause bilirubin-induced neurologic damage (BIND) and kernicterus, resulting in physical and mental handicaps or even death.[2;3] The prevalence of CN disease is estimated at 1:1,000,000.[4] In the Netherlands there are approximately 20 patients. Two types of CN disease exist. In type I there is no detectable UGT1A1 activity. Plasma UCB concentrations in untreated type I patients are above 350 µmol/l and can be as high as 800 µmol/l, especially during intercurrent febrile illness.[5] Plasma UCB levels in untreated type II patients are generally below 350 µmol/l because of some (~5%) residual enzyme activity, which can be enhanced by treatment with phenobarbital.[6;7]

All type I and some type II patients need daily phototherapy to reduce the risk of kernicterus. Phototherapy increases the hydrophilicity and biliary secretion of UCB by photoisomerization.[8] Phototherapy aims to keep plasma UCB levels below 350-400 µmol/l. Life-long daily phototherapy 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.[5;9;10] During exacerbations of jaundice, several measures in addition to continuous high-intensity phototherapy are taken to manage the disease safely, including albumin infusion if the bilirubin-albumin molar ratio is above 0.7, and the avoidance of drugs that displace bilirubin from albumin.[9] Several type I patients have undergone liver transplantation,[11-13] which restores UGT1A1 activity but has major risks and complications, and requires life-long immunosuppressive treatment. Future treatment for CN disease may be gene therapy. In the animal model for CN disease, the Gunn rat, gene therapy effectively restores UGT1A1 activity.[14-17] However, vector toxicity and concerns about long-term safety have so far prevented the use of gene therapy in humans.

An alternative treatment option for unconjugated hyperbilirubinemia is based on intestinal capture of UCB by oral treatment. Particularly when plasma UCB concentrations are high as in CN disease, UCB can diffuse from the blood into the intestinal lumen across the mucosa.[18;19] Additionally, very small amounts of UCB can be excreted into bile.[20;21] In type II patients the small amounts of conjugates that are formed can be deconjugated to UCB after biliary secretion. Intestinal capture of UCB followed by fecal excretion reduces the enterohepatic circulation of UCB and subsequently decreases plasma UCB concentration. Several orally administered non-absorbable binders of UCB have been applied for intestinal

capture. Agar,[22] activated charcoal[23] and cholestyramine[24] are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects.[25-27] Zinc sulphate was shown to inhibit enterohepatic cycling of UCB, but increased serum zinc levels.[28] Intestinal capture of UCB by calcium phosphate was very effective in Gunn rats,[29] but efficacy was less pronounced in patients with CN disease.[4]

Recently, we demonstrated in Gunn rats that orlistat treatment decreases plasma UCB concentrations parallel with increased fecal fat excretion, and induces net transmucosal excretion of UCB from the blood into the intestinal lumen.[30-32] In human adults, orlistat has been widely applied for treatment of obesity, without serious side effects.[33] Recent studies in obese adolescents and prepubertal children indicate that short-term orlistat treatment is well-tolerated by children and generally has only mild side effects.[34-38] In the present study, we determined the effects of orlistat treatment in patients with CN disease.

METHODS

Study Design. A randomized, placebo-controlled, double-blind, cross-over clinical trial was conducted in patients with CN disease. The study was performed in the winter season (Sept.-Dec.). All patients started at the same time with the trial to minimize environmental confounding factors, such as variations in sunlight and CYP1A1&2 inducers that catalyze oxidation of UCB. In all patients, current treatment with phototherapy (n = 11) and/or phenobarbital (n = 6) was continued during the trial, *i.e.* orlistat was tested as an adjunct treatment. Patients received orlistat or placebo in a cross-over design with 2 weeks interval, and each patient served as his/her own control. The initial duration of the trial was 12 weeks. The first two weeks was a control period in which the intra-individual variation in plasma bilirubin concentration was determined in three, weekly, samples. Orlistat or placebo would then be taken during 4 weeks, followed by 2 weeks (wash-out) interval and then the alternate treatment during 4 weeks. At the end of the study period, there was a post-treatment control period of 2 weeks. Upon conduction of the trial, however, the dosage of the trial medication had to be adapted after two weeks (see Medication section) and therefore 2 weeks were added to the trial. Total duration of the trial was thus 14 weeks, with treatment periods of 6 and 4 weeks and two control weeks before, in between, and after the treatment periods.

Blood samples were taken weekly to determine plasma UCB concentration. A clinically relevant response to orlistat 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% (mean variation 6 ± 2%). Control UCB values were a mean of 3 weekly samples taken before start of orlistat. Response UCB values were a mean of 2 weekly samples taken around the end of the orlistat treatment period. Samples were taken at approximately the same hour each week to exclude diurnal variation of plasma UCB levels as possible confounding factor,

and to be sure that each week there was an equal time period between nightly phototherapy and blood sampling. Six times during the trial hematological and biochemical parameters were assessed (see Analytical Methods section). In patients treated with phenobarbital, plasma levels were checked every two weeks. Feces were collected 10 times at regular intervals during the trial. Three times 72-hour samples were collected to determine fecal fat excretion; once during each period (control, orlistat, placebo). The other 7 times a small sample of feces was collected. Fecal fat- and UCB concentration was determined in all feces samples.

Patients. Sixteen patients participated in the trial; 13 from The Netherlands, 3 from Belgium. All patients, and/or their parents if the patient was younger than 18 years, gave written informed consent. The study protocol was approved by the Ethics Committees of the Erasmus University Medical Center in Rotterdam, The Netherlands, and of the Hôpital Universitaire des Enfants Reine Fabiola in Brussels, Belgium. To be included in the trial, patients had to be older than 7 years. Exclusion criteria were cholestasis, chronic malabsorption syndrome and pregnancy. Stop criteria were defined as a patient's wish to stop or serious adverse events, defined as death, life-threatening events, hospitalization or severe side effects (anaphylaxis, an increase of liver transaminases > 30%, an increase of plasma UCB concentration > 50% with risk of kernicterus, a plasma UCB level > 500 µmol/l, severe coagulation problems or diarrhea with > 10% weight loss). During the trial, body weight, blood pressure and pulse rate were recorded weekly and, if indicated, physical examination was performed.

Medication. Orlistat (Xenical®) is a selective inhibitor of gastrointestinal lipases that dose-dependently inhibits hydrolysis of dietary triglycerides.[39] At a recommended dose of 3 times daily (t.i.d.) 120 mg, dietary fat absorption is reduced by approximately 30%. Orlistat and placebo capsules were custom-made by the pharmacist of the Erasmus University Medical Center, Rotterdam. Initially, adults received 120 mg t.i.d. during a meal, and children ~66 mg/m^2 body surface area t.i.d. (= roughly equal amount as an adult per m^2 body surface area). However, after two weeks of treatment, some patients suffered from side effects (diarrhea, n = 3) and one patient (patient C, Table 1) from severely increased plasma UCB levels (maximum 451 µmol/l), after which the code was broken in close collaboration with the Ethics Committee. The symptoms were confined to orlistat treated patients, after which the orlistat dosage was reduced by one third. This resulted in the following dosages. Adults: during breakfast and lunch 60 mg, during dinner 120 mg; children: during breakfast and lunch ~33 mg/m^2 body surface, during dinner ~66 mg/m^2 body surface area. The study protocol was prolonged by two weeks, to allow patients to be compared for identical durations of treatment with the adapted orlistat dosage (*i.e.* 4 weeks). The Ethics Committee approved the new study protocol. Cellulose was used as placebo. Previously, cellulose was shown to have no effect on plasma bilirubin concentrations.[4]

Diet. Patients were instructed to eat their normal diet, but were requested not to eat heme-rich food, such as blood sausages or bloody red meat because heme would be converted to bilirubin. Food intake was assessed three times during the trial, once during each period (control, orlistat, placebo). Patients and/or their parents were instructed by a dietician to keep a detailed record of the diet for 3 successive days, at the same days the 72-hour feces samples were collected. Nutrient intake was calculated from a computerized Netherlands food composition database.

Compliance. The importance of medication compliance was stressed regularly during the trial. Compliance was checked by counting remaining capsules afterwards, by determination of fecal fat concentration, and by reviewing the diary patients had been instructed to keep. In this diary, patients had to record daily the times at which the medication was taken, as well as the number and consistency of bowel movements, and side effects, if any. Plasma levels of phenobarbital were determined every other week. With regard to phototherapy compliance, patients and/or their parents were instructed to use the lights for approximately the same amount of hours every day and to keep a detailed record of phototherapy times. Furthermore, light levels were checked weekly with a Lux meter. Since in 14 weeks light levels would deteriorate too much if the same light bulbs were used, half of the light bulbs were replaced by new bulbs two weeks before start of the trial; the other half of the bulbs were replaced halfway the trial in the wash-out period.

Analytical Methods

Blood. Directly after vein puncture, tubes were protected from light and kept cool until analysis. All analytical procedures were performed in dim light with routine clinical chemical procedures. For bilirubin measurements, blood samples were processed immediately. Plasma bilirubin levels were determined twice per sample. First, plasma total bilirubin level was determined on a Hitachi 912 analyzer (Roche, Mannheim, Germany). The remaining plasma was kept in the dark at -80ºC under nitric oxide gas, until analysis of plasma UCB concentration by reversed-phase HPLC, as described previously.[30;32] Hemoglobin (Hb), hematocrit (Ht) and reticulocytes were determined on a Advia 120 hematology analyzer (Bayer, Leverkusen, Germany). Aspartate-aminotransferase activity (AST) and albumin were determined on a Hitachi 912 analyzer, activated partial thromboplastin time (APTT) and protrombin time (PT) on a Sysmex CA-1500 analyzer (Sysmex UK, Milton Keynes, UK), and phenobarbital levels on a DDX-FLX analyzer (Abbott Diagnostics, Hoofddorp, The Netherlands). Vitamin E levels were determined by HPLC.

Feces. Feces were stored in the dark at -20ºC until analysis. For determination of fatty acids in feces, aliquots of homogenized feces were extracted, hydrolyzed and methylated according to the method of Lepage and Roy,[40] with the modification that methanol/hexane (4:1, v/v) 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. Fecal fat excretion was also determined via Near Infra Red Assay (NIRA) of homogenized 72-hour feces samples using a modification of the Van de Kamer method.[41] Total fat analysis was performed by using the Soxtec®Avanti 2050 system (FOSS Tecator AB, Hoganas, Sweden), which utilizes a new, patented four-step solvent (hexane) extraction technique. The entire process is fully automated, including hydrolysis, filtration, washing and drying. Before performing the automated extraction method, 4 gram of feces was dried overnight at 103°C. Fecal fat concentration was expressed as gram per 24-hour collection period. For determination of UCB in feces, aliquots of homogenized feces were submitted to alkaline methanolysis and chloroform extraction. After evaporation under nitrogen, the residue was re-dissolved in chloroform and analyzed by reversed-phase HPLC, as described previously.[32]

Statistical Analyses

Power analysis was performed before start of the trial.[42;43] Statistical analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). Results are expressed as mean ± SD. Based on a normal distribution of plasma UCB levels in CN patients, parametric tests were used for statistical analysis. Intra-individual differences were tested with the paired Student *t* test. The relationship between plasma UCB and fecal fat was analyzed by linear regression analysis. Correlation analysis was done with Pearson's cross-tables. *P* values less than 0.05 (two-tailed) were considered significant.

RESULTS

Patient characteristics

Table 1. Patient characteristics

Patient ID	CN Type	Age (years)	Sex (M/F)	Body Weight (kg)	BMI (kg/m ^{2})	Phototherapy (hours/day)	Phototherapy No. bulbs / Watt	Phenobarbital (mg/day)
A	I	10	F	41	20	10 -11	10 / 100	-
B	I	11	F	37	18	8 -10	12 / 100	-
C	I	14	M	56	19	11	10 / 100	-
D	I	14	F	61	25	9 -10	12 / 100	-
E	I	15	M	71	22	10 -11	14 / 150	-
F	I	18	F	82	30	6 -9	10 / 140	-
G	I	19	F	64	21	8	14 / 150	-
H	II	8	F	29	16	-	-	2 x 25
I	II	15	M	71	22	8	12 / 160	1 x 50, 1 x 75
J	II	17	M	56	18	1	20 / 100	3 x 50
K	II	27	F	63	23	-	-	Phenytoïne 1 x 200*
L$^+$	II	28	F	85	25	-	-	1 x 75, 2 x 50
M	II	30	F	80	30	1	10 / 150	-
N	II	32	F	79	26	2	24 / 100	2 x 35
O	II	48	F	64	23	-	-	-
P	II	51	M	74	23	-	-	-

BMI, Body Mass Index, calculated as: body weight (kg)/height (m^2). CN, Crigler-Najjar
*Phenytoine instead of phenobarbital. $^+$Patient quit during the trial after 10 weeks

Sixteen patients with Crigler-Najjar disease participated in the trial (Table 1). Seven patients had type I CN disease and 9 patients type II. Half of the patients were below the age of 18 years. Median age was 17.5 years (range 8-51 years). Male to female ratio was 5:11 (31%:69%). Median body weight was 64 kg (range 29-85 kg). Median BMI was 22.5 (range 16-30). Phototherapy treatment was necessary for 11 patients. The amount of hours of daily phototherapy ranged from an average of 9.4 hours in type I patients to 1 or 2 hours in some type II patients. Plasma UCB concentrations of one type II patient were in the high range of type I patients, but on the basis of a response to phenobarbital treatment this patient was diagnosed with type II CN disease. Five type II patients were treated with phenorbarbital and one with phenytoïne.

Table 2. Hematological and biochemical parameters

	control	orlistat
Hemoglobin (mmol/l)	8.8 ± 0.6	8.8 ± 0.7
Hematocrit (v/v)	0.41 ± 0.03	0.40 ± 0.03
Reticulocytes (%)	1.3 ± 0.3	1.3 ± 0.3
ALT (U/l)	67 ± 69	63 ± 47
Albumin (g/l)	45 ± 2	44 ± 3
APTT (sec)	33 ± 4	34 ± 4
PT (sec)	12 ± 1	12 ± 1
Vitamin E (mmol/l)	22 ± 5	18 ± 4*

*p<0.001

General effects of orlistat

Orlistat treatment did not significantly affect body weight (average body weight without orlistat: 64 ± 16 kg vs orlistat: 64 ± 17 kg, NS). Table 2 shows the effects of orlistat on hematological and biochemical parameters. Hemoglobin, hematocrit and reticulocyte count were not affected by orlistat treatment, in accordance with absence of relevant hemolysis and with stable bilirubin production during the trial. Plasma albumin concentrations, ALT, APTT, and PT levels were not affected by treatment. Orlistat treatment significantly decreased vitamin E levels (-18%, $p<0.001$). In patients treated with phenobarbital, plasma levels were stable during the experimental period, based on 2-weekly sampling (average levels without orlistat: 13.0 ± 3.5 mg/l, vs orlistat: 12.5 ± 4.0 mg/l, NS).

Effects of orlistat on plasma UCB concentrations

Figure 1 shows the effects of orlistat treatment on plasma UCB concentrations in Crigler-Najjar patients. Before orlistat treatment, plasma UCB levels ranged from 123 to 354 µmol/l among the patients (Figure 1A). Average plasma UCB concentrations per group were 317 ± 36 µmol/l for type I patients and 228 ± 71 µmol/l for type II patients. Within each patient, plasma UCB levels were fairly constant (mean variation 6 ± 2%). Plasma UCB levels measured by HPLC were used for calculations. Levels measured on the Hitachi analyzer were similar (mean difference <5%). Orlistat treatment decreased plasma UCB concentrations by 9

± 12% (p<0.01; range -32 to +8%; Figure 1B). Based on the definition of a clinically relevant response to treatment, 7 of the 16 patients (44%) responded, with a mean decrease of 21% in plasma UCB levels (range 11 to 32%).

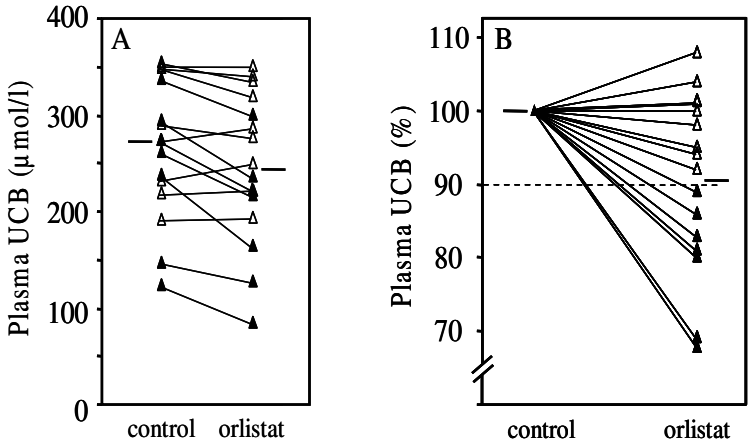


Figure 1. Effects of orlistat treatment on plasma UCB concentrations in Crigler-Najjar patients. Patients were treated for 4 weeks with orlistat. Plasma UCB concentration was measured weekly. Control UCB values were a mean of 3 weekly samples taken before start of orlistat. Orlistat UCB values were a mean of 2 weekly samples taken around the end of the orlistat treatment period. Each set of triangles represents data from one patient. Response to orlistat treatment was defined as a decrease in plasma UCB concentration of at least 10%.▲responders, △ non-responders.

Effects of orlistat on fecal fat- and UCB excretion

Orlistat treatment profoundly increased fecal fat concentration in all patients (+333 ± 249% according to gas chromatographic analysis of fecal fatty acids; p<0.001; data not shown), and total fecal fat excretion (+652 ± 516% according to the NIRA method of fecal fat analysis; p<0.001; Figure 2). Fecal excretion expressed per kilogram body weight increased from 0.05 ± 0.02 to 0.28 ± 0.12 g/kg/24h (+652 ± 516%, p<0.001). Fecal fat concentration and excretion were not different between orlistat responsive and non-responsive patients. Orlistat treatment also increased fecal concentration of UCB (+43%, p<0.05; range -46 to +223%; Figure 3). Under control conditions, fecal UCB concentrations varied between patients from 0.05 to 0.31 µmol/gram feces (mean 0.14 ± 0.08). Fecal UCB concentrations did not differ significantly between orlistat-responsive and non-responsive patients, either under control conditions or during orlistat treatment.

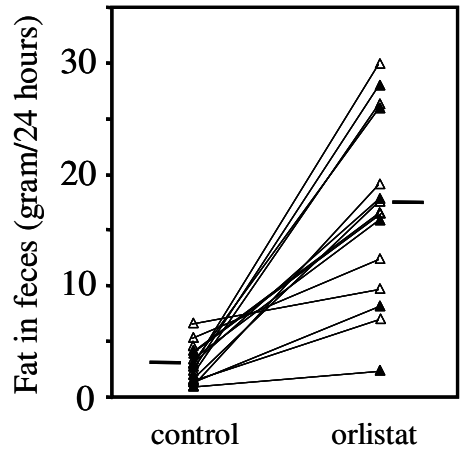


Figure 2. Effects of orlistat treatment on fecal fat excretion in Crigler-Najjar patients. Patients were treated for 4 weeks with orlistat. 72-hour fecal fat excretion was measured 3 times. Control values were a mean of two 72-hour samples taken during control and placebo periods. Orlistat values were single 72-hour samples taken during orlistat treatment. Each set of triangles represents data from one patient. Response to orlistat treatment was defined as a decrease in plasma UCB concentration of at least 10%. ▲ responders, △ non-responders.

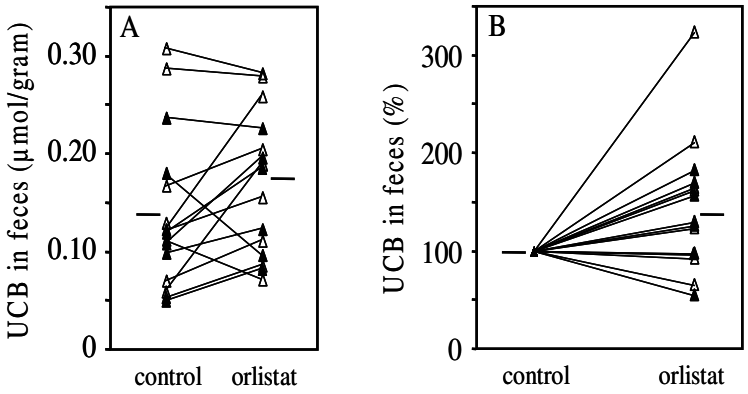


Figure 3. Effects of orlistat treatment on fecal UCB excretion in Crigler-Najjar patients. Patients were treated for 4 weeks with orlistat. Fecal UCB excretion was measured regularly. Control values were a mean of 5-6 samples taken during control and placebo periods. Orlistat values were a mean of 4-5 samples taken during orlistat treatment. Each set of triangles represents data from one patient. Response to orlistat treatment was defined as a decrease in plasma UCB concentration of at least 10%. ▲ responders, △ non-responders.

In previous studies in Gunn rats, the change in plasma UCB concentration was strongly correlated with the amount of fat excreted via the feces.[30;32] Figure 4 shows the relationship between parameters of fecal fat excretion and the orlistat-induced alteration in plasma UCB concentration. For all patients combined, the UCB response to orlistat was only minimally related to the relative stimulation of fecal fat excretion or to the absolute amount of fecal fat excretion (Figure 4; trend lines not shown). Upon separate analysis of responding and non-responding CN patients, however, marked differences were observed. Plasma UCB concentrations in responding CN patients were strongly, negatively related to parameters of fecal fat excretion ($r = -0.93$, $p<0.01$, Figure 4). In contrast, plasma UCB concentrations in non-responding patients were independent of fecal fat excretion. Fat absorption (calculated as the difference between total fat intake (g/24h) and fecal fat excretion (g/24h) appeared to be less in the responding patients compared with the non-responding patients (58 ± 31 and 86 ± 31 g/24h, respectively, $p=0.11$).

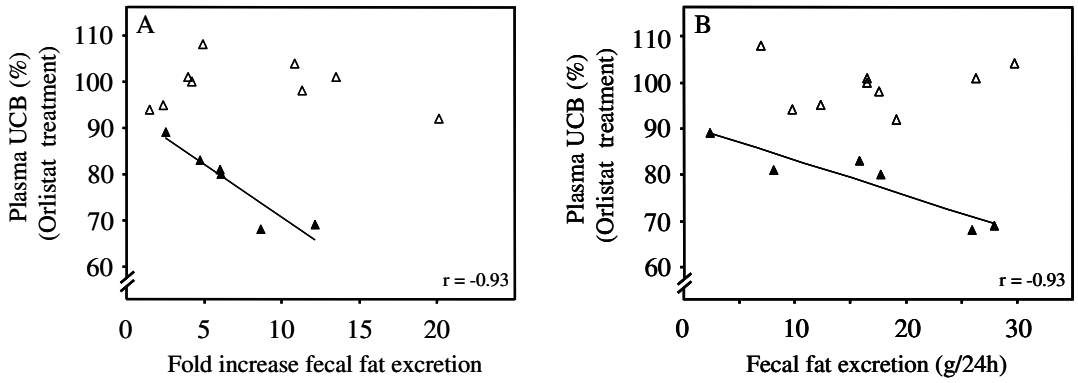


Figure 4. Negative linear relationship between plasma UCB concentration during orlistat treatment (compared with pre-treatment values) and fecal fat excretion during orlistat in responding Crigler-Najjar patients. Patients were treated for 4 weeks with orlistat. 72-hour fecal fat excretion was measured. Each triangle represents data from one patient. Response to orlistat treatment was defined as a decrease in plasma UCB concentration of at least 10%. ▲ responders, △ non-responders. One responder did not collect 72-hour feces.

Characteristics of responsive vs. non-responsive patients

Body weights of the patients varied between 29 and 85 kg (Table I). The observed differences between responding and non-responding patients were similar upon expression of fecal fat excretion relative to body weight. Also, the responders and non-responders did not significantly differ in the amount of saturated or unsaturated fatty acids excreted via the feces, nor in their relative (molar) ratio in feces (Figure 5). Accordingly, neither saturated nor unsaturated fecal fat appeared specifically related to plasma bilirubin concentrations.

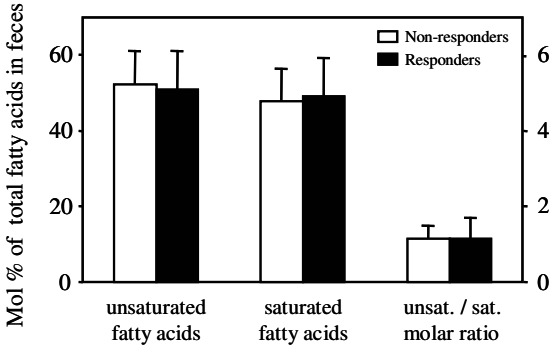


Figure 5. Distribution of unsaturated / saturated fatty acids in feces of Crigler-Najjar patients during orlistat treatment. Patients were treated for 4 weeks with orlistat. Fecal fatty acids were measured in 4-5 samples taken during orlistat treatment.

In a study in which Crigler-Najjar patients were treated with calcium phosphate, differences in response to treatment correlated with the type of CN disease (type I or II) and with co-treatment with phototherapy.[4] Since our data also indicated differences in responsiveness to orlistat treatment, we analyzed whether these or other patient characteristics correlated with (non-)responsiveness (Table 3). The (non-)responsiveness of plasma UCB concentrations to orlistat treatment did not significantly correlate with either CN type, age, sex, body weight, or co-treatment with phototherapy or phenobarbital. Interestingly, however, the majority of the responsive patients had a total dietary fat intake below 35 energy% (p=0.05), and all responsive patients had a BMI equal to or below 25 (p=0.10).

Table 3. Correlations (non)-respons vs patient characteristics / dietary intake

Patient characteristics		Non-responders (n = 9)	Responders (n = 7)	*P* value
CN type	I	44%	43%	0.95
Age	< 18 years	44%	57%	0.64
Sex	Female	56%	86%	0.22
BMI	≤ 25	67%	100%	0.10
Phototherapy	Yes	78%	57%	0.41
Phenobarbital*	Yes	33%	43%	0.72
Dietary intake < average per day		Non-responders (n = 9)	Responders (n = 7)	*P* value
Energy	< 2200 Cal	56%	57%	0.95
Total fat	< 35 energy%	22%	71%	0.05
Saturated fat	< 13 energy%	33%	43%	0.72
Cholesterol	< 220 mg	33%	71%	0.15
Calcium	< 800 mg	67%	71%	0.85
Fiber	< 17 g	67%	86%	0.42

*In one patient Phenytoine in stead of Phenobarbital

Dietary intake of saturated fat, cholesterol, calcium or fiber was not significantly different between orlistat responsive and non-responsive patients.

Side effects & compliance

As mentioned in the Methods section, after two weeks of treatment the dosage of orlistat had to be adapted because of side effects. Side effects were almost exclusively related to the gastrointestinal tract, such as diarrhea, oily leakage, stomach cramps, flatulence and fecal urgency. After dosage reduction the side effects decreased in severity and for the rest of the study they were generally mild, temporary, and tolerable for all patients. Oily leakage and diarrhea were remarkably yellow/orange colored. Mild side effects such as greasy appearance of the feces occurred in almost all patients. Some side effects (such as flatulence) were also reported during placebo treatment. Systemic side effects were not observed. Headache was incidentally reported, equally during placebo and orlistat treatment. Five patients reported increased energy levels and well-being during orlistat treatment. Three patients reported increased appetite during orlistat. One patient (patient C, table 1) stopped during one week with orlistat treatment because of an increase in plasma bilirubin levels after one week of treatment. After dosage reduction the treatment was restarted and one week was added to the treatment period of this patient. Another patient (patient L, table 1) dropped out from the trial at ten weeks, because of psychological issues (a.o. lack of concentration, mood swings). Compliance to medication appeared to be good in all patients, based on capsule counting, corresponding diary entries, and, by inference, from increased fecal fat excretion during orlistat treatment. With regard to phototherapy, patients spent the same number of hours under the phototherapy lights during control periods and placebo or orlistat treatment. Light levels checked weekly with a Lux meter, showed that lamps deteriorated during the study period by approximately 10%.

DISCUSSION

We determined the effects of orlistat treatment on plasma unconjugated bilirubin concentrations in patients with Crigler-Najjar disease. In general, orlistat treatment slightly decreased plasma UCB concentrations. Although this was a statistically significant decrease, it was not considered clinically relevant for the whole group, based on our definition of treatment response. Interestingly, however, the effect was more pronounced in a subgroup of patients. In this orlistat responsive subgroup, the decrease in plasma UCB concentration was strongly related to orlistat-induced fecal fat excretion. The responsiveness of CN patients could not be predicted by pre-treatment patient characteristics, except that the responsive patients tended to have a lower dietary fat intake and lower BMI. Our definition of treatment response (a decrease in plasma UCB concentration of at least 10%) was based on the variation in intra-individual plasma UCB concentrations before start of treatment, which was on

average below 10%. Our cutoff level of 35% energy% for separating a "high" versus "low" dietary fat intake was based on our dietician's guideline for a "healthy" diet that recommends a dietary fat intake between 30-35 energy%.

Previously, we demonstrated in Gunn rats that orlistat treatment decreased plasma UCB concentrations by 20-40%,[30;32] *i.e.* more pronounced than presently observed in CN patients. A species difference or the length of the intestine could possibly explain the different efficacies of orlistat treatment. Rats have a relatively much larger surface area of intestinal mucosa than humans and therefore transmucosal excretion of UCB during orlistat treatment might be more pronounced in rats than humans. Other potential reasons include type of diet and differences in intestinal microflora. Our observations are similar to those obtained with calcium phosphate treatment by Van der Veere *et al.* Calcium phosphate strongly decreased plasma bilirubin levels in Gunn rats, but the efficacy was limited in CN patients.[4;29] Also, calcium phosphate was only effective in a subgroup of patients, namely in patients receiving phototherapy (mostly type I patients).

The responsiveness to orlistat treatment was independent of co-treatment with phototherapy or phenobarbital, CN-type, body weight, sex, or age. However, the subgroup of patients that responded to orlistat treatment did appear to have a lower dietary fat intake and lower BMI than non-responsive patients. Previously, we showed in Gunn rats that orlistat treatment was more effective on a low-fat than a high-fat diet.[30] Dietary fat intake thus appears to be a relevant factor in determining efficacy of orlistat treatment. 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. Present data clearly identify the subgroup in one specific phenomenon: only in the CN patients with a clinically relevant response to orlistat treatment, plasma UCB levels were strongly, negatively correlated with fecal fat excretion. Previously, we showed a similar relationship upon orlistat treatment in Gunn rats.[32] The mechanism underlying the presence or absence of this relationship in specific patients remains partly unclear. We speculate that, in addition to dietary fat intake, intestinal factors such as composition or metabolic activity of the intestinal microflora play an important role. Previously, we showed in Gunn rats that the hypobilirubinemic effect of orlistat is partially the result of increased metabolism of UCB to derivatives.[31] Others have shown the importance of the intestinal microflora for UCB metabolism.[44] Gastrointestinal lipase activity levels of the patient may also play a role. The dose of orlistat should perhaps be individualized to generate a certain degree of fat malabsorption with a certain distribution of fatty acids or partially hydrolyzed triglycerides in the intestinal lumen to create an optimal environment for capture of UCB.

The number of CN patients that could participate in the trial was inevitably limited due to the low prevalence of the disease. To overcome this, a cross-over study design was chosen. We obtained several indications that both the duration of the treatment and of the wash-out

period may have been too short, and may have resulted in underestimation of the orlistat effect. Firstly, the majority of patients appeared not to have reached a steady-state in bilirubin homeostasis during the treatment, based on the increased fecal bilirubin content at the end of orlistat treatment. Secondly, in the first week of the wash-out period, plasma UCB concentrations did not readily return to pretreatment values. We cannot exclude the possibility that the hypobilirubinemic effects of orlistat are more pronounced upon prolonged treatment.

Orlistat treatment, at the (eventual) dose used, did not cause major side effects in the CN patients. 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.[33;34;36;38] Side effects were almost exclusively related to the gastrointestinal tract. Orlistat treatment decreased plasma vitamin E concentrations. The fat-soluble vitamin E was shown by others to decrease when orlistat was used to treat obesity.[33] Low plasma vitamin E levels (or other fat-soluble vitamins) due to prolonged orlistat treatment could easily be prevented by dietary supplementation.[45]

In conclusion, present data indicate that orlistat can be useful for treatment of unconjugated hyperbilirubinemia, particularly so in a subgroup of CN patients. Dietary fat intake may determine the responsiveness to orlistat treatment. It will be a challenge for future research to identify other relevant parameters or patient characteristics which could predict the responsiveness of CN patients to orlistat treatment, or indeed other patients with unconjugated hyperbilirubinemia. In addition, it needs to be addressed whether prolonged orlistat treatment could safely and reliably decrease the need for phototherapy.

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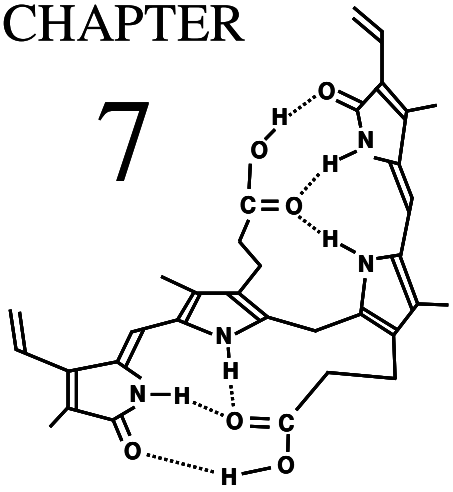
Effects of bile salts on unconjugated hyperbilirubinemia in Gunn rats

CHAPTER

7



Submitted

Anja M. Hafkamp, Thierry Claudel,
Frans J.C. Cuperus, Rick Havinga, Henkjan J. Verkade

Department of Pediatrics, Division of Pediatric Gastroenterology,
Center for Liver, Digestive and Metabolic Diseases, University
Medical Center Groningen, Groningen, The Netherlands

ABSTRACT

Previously, we demonstrated that stimulation of fecal fat excretion in hyperbilirubinemic Gunn rats decreased plasma unconjugated bilirubin (UCB) concentrations. Ursodeoxycholic acid (UDCA) has been suggested to decrease fat absorption. We studied the effects of dietary supplementation with UDCA or with cholic acid (CA) on plasma UCB concentration in Gunn rats, and on fecal excretion of fat and UCB. Dietary UDCA supplementation (0.5% w/w) for 3 weeks decreased plasma UCB concentration by 32% compared with control Gunn rats ($p<0.01$). The UDCA effect was not specific, since dietary CA supplementation (0.5% w/w) reduced plasma UCB concentration to a similar level as UDCA (8 days treatment, -27% and -34%, compared with controls, each $p<0.001$, respectively). UDCA did not affect fecal fat excretion, whereas CA decreased fecal fat excretion (-67%, $p<0.001$). 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. In conclusion, 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.

INTRODUCTION

Gunn rats have a permanent unconjugated hyperbilirubinemia due to a genetic deficiency of bilirubin glucuronidation, similar to patients with Crigler-Najjar disease.[1;2] Previously, we demonstrated in Gunn rats that stimulation of fecal fat excretion by a high-fat diet or by orlistat treatment decreases plasma unconjugated bilirubin (UCB) concentrations.[3;4] Orlistat treatment decreases UCB pool size and enhances fecal UCB excretion and fractional turnover of UCB.[4;5] Ursodeoxycholic acid (UDCA) treatment has been suggested to impair fat absorption in some individuals.[6] Previously, we showed that even a relatively minor increase in fecal fat excretion profoundly decreased plasma UCB concentrations.[3]

UDCA is widely used in the management of cholestatic liver diseases with conjugated hyperbilirubinemia. In patients with primary biliary cirrhosis, UDCA treatment decreases plasma conjugated bilirubin levels and jaundice.[7] The mechanism by which UDCA ameliorates conjugated hyperbilirubinemia may involve induction of the expression of the conjugate export pump Mrp2 (multidrug resistant protein 2, Abcc2) and the basolateral transporter Mrp3 (multidrug resistant protein 3, Abcc3). Both were shown to be upregulated by UDCA treatment in, respectively, liver, kidney and intestine (Mrp2) and liver (Mrp3) of mice and rats.[8;9] It has not been determined whether UDCA affects unconjugated hyperbilirubinemia. Mrp3 might be involved in transport of (oxidized) UCB, based on the fact that Mrp 3 expression is increased in liver and kidney of Gunn rats.[10] UDCA might interfere with solubilization of UCB.[11-13] In the present study, we determined in Gunn rats the effects of dietary bile acid supplementation on plasma UCB concentrations, biliary UCB secretion and fecal excretion of fat, UCB, and urobilinoids, and expression of relevant transport proteins in liver and intestine. Initially, we studied the effects of UDCA treatment, considering the hydrophilic, non-toxic nature and lack of appreciable side effects.[7;14] To address the kinetics and (non) specificity of the UDCA effects, we then compared UDCA treatment with treatment with cholic acid (CA), a relatively hydrophobic bile salt.

METHODS

Animals. Homozygous male Gunn rats (RHA/jj), weighing 240-340 g, were obtained from our breeding colony at the Central Animal Facility (University Medical Center Groningen, The Netherlands). Animals were housed individually in an environmentally controlled facility with a 12-12 hour light-dark cycle, were fed *ad libitum* and had free access to water. The Ethics Committee for Animal Experiments (University of Groningen, The Netherlands) approved experimental protocols.

Chemicals. Ursodeoxycholic acid (UDCA), cholic acid (CA) and heptadecanoic acid (C17:0) were purchased from Sigma Chemical Co. (St. Louis, MO). Urobilin was obtained from Frontier Scientific Inc. (Logan, UT).

Diets. Hope Farms BV (Woerden, The Netherlands) produced the semisynthetic, purified control diet (code 4063.02), that contained 13 energy% fat / 5.2 wt% long-chain fatty acids. As in previous studies, all Gunn rats were fed the control diet for a run-in period of at least 4 weeks.[3-5] Bile salt-supplemented diets were prepared by mixing of UDCA (0.5% w/w; code 4063.08) or CA (0.5%, w/w) into the control diet.

Study Design

Long-term UDCA treatment (3 weeks)

After 4 weeks run-in on the control diet, 2 groups of Gunn rats (n = 5 per group) were randomly assigned to receive either the control diet or the UDCA-supplemented diet for 3 weeks. Blood samples were obtained weekly via tail bleeding under isoflurane anesthesia. Feces were collected twice for 72h (before start and at the end of the experiment) to determine fecal fat excretion. Food intake was assessed at the same time. After 3 weeks, under pentobarbital anesthesia, bile was collected for 30 minutes under light-protected conditions after cannulation of the common bile duct. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. After bile collection the animal was sacrificed and the liver was removed.

Short-term UDCA or CA treatment (8 days)

After 5 weeks run-in on the control diet, 3 groups of Gunn rats (n = 5-7 per group) were randomly assigned to either continue with the control diet, or to start with the UDCA-supplemented diet or the CA-supplemented diet for 8 days. Blood samples were obtained at days 0, 1, 2, 4 and at the end of the experiment (day 8) via tail bleeding under isoflurane anesthesia. Feces were collected twice for 72h (before start and at the end of the experiment) to determine fecal fat excretion, and three times for 24-48 hours. Food intake was assessed several times. After 8 days, bile was collected for 30 minutes as described above. After bile collection the animal was sacrificed and the liver and intestine were removed.

Analytical Methods

All analytical procedures were performed in dim light.

Plasma. Bilirubin levels were determined by reflectance spectrophotometry on a Vitros-950 analyzer (Johnson & Johnson Ortho-Clinical Diagnostics, Tilburg, The Netherlands), and were confirmed by HPLC analysis.

Bile. For UCB measurement, bile was submitted to alkaline methanolysis and chloroform extraction. After evaporation under nitrogen, the residue was re-dissolved in chloroform and analyzed by reversed-phase HPLC, as described previously.[3] Urobilinoids were measured by spectrophotometry (Shimadzu UV-2401PC, Duisburg, Germany; software HyperUV), according to the method of Kotal *et al.*[15] The second derivative was calculated to correct for background pollution.[16;17]

Feces. Feces were freeze-dried and mechanically homogenized. For determination of fatty acids, aliquots of feces were extracted, hydrolyzed and methylated according to the method of Lepage and Roy,[18] with the modification that methanol/hexane (4:1, v/v) 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 urobilinoids was performed as described above for bile.

Hepatic and intestinal gene expression. Total RNA was isolated from livers or intestinal cells using the TriReagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions and quantified using Ribogreen (Molecular Probes, Inc., Eugene, Oregon, USA). Real-time quantitative PCR[19] was performed using an Applied Biosystems 7700 sequence detector according to the manufacturer's instructions. Primers were obtained from Invitrogen (Breda, The Netherlands). Fluorogenic probes, labeled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine, were made by Eurogentec (Seraing, Belgium), all sequences are available upon request. All expression data were subsequently standardized for 18S rRNA.

Statistical Analyses

Analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). Results are expressed as mean ± SD. Based on a normal distribution of plasma bilirubin levels in large groups of Gunn rats in previous studies,[4] parametric tests were used for statistical analysis. Analysis of variance (ANOVA) with post-hoc Bonferroni correction was performed for comparisons among groups. *P* values <0.05 (two-tailed) were considered significant.

RESULTS

Long-term UDCA treatment (3 weeks)

Figure 1 shows that after 1, 2 and 3 weeks of treatment with UDCA, plasma UCB concentrations were significantly lower than in control Gunn rats (-18%, $p<0.05$; -23%, $p<0.01$; and -32%, $p<0.01$; respectively).

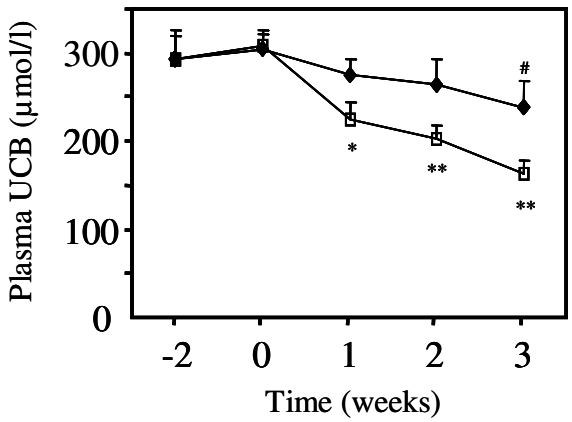


Figure 1. Effects of long-term dietary UDCA supplementation on plasma UCB concentrations in Gunn rats. After 4 weeks run-in on the control diet, Gunn rats (n = 5 per group) received the same diet without (controls ♦) or with UDCA (0.5% w/w, □) for 3 weeks. Data represent mean ± SD. *p<0.05, **p<0.01 compared with controls. #p<0.05, compared with T_0.

Compared with T_0, 3 weeks of UDCA treatment decreased plasma UCB levels by 47% ($p<0.001$). Plasma UCB concentrations in control animals also decreased (compared with T_0, -22%, $p<0.05$). Body weights were similar in controls and UDCA treated Gunn rats before treatment (301 ± 25 and 311 ± 20 g, respectively), and increased in both groups by 2% in 3 weeks. Food intake and feces production before and during treatment were similar in controls and UDCA treated animals. Taken together, these data showed that UDCA significantly decreased plasma UCB concentrations in Gunn rats and that the treatment was not toxic.

UDCA treatment was suggested to decrease fat absorption in humans.[6] Since we have shown that fat malabsorption can increase the fecal excretion of UCB and decrease plasma UCB levels in Gunn rats, we determined fecal fat excretion during UDCA treatment. Interestingly, UDCA treatment did not affect fecal fat excretion compared with controls (Figure 5).

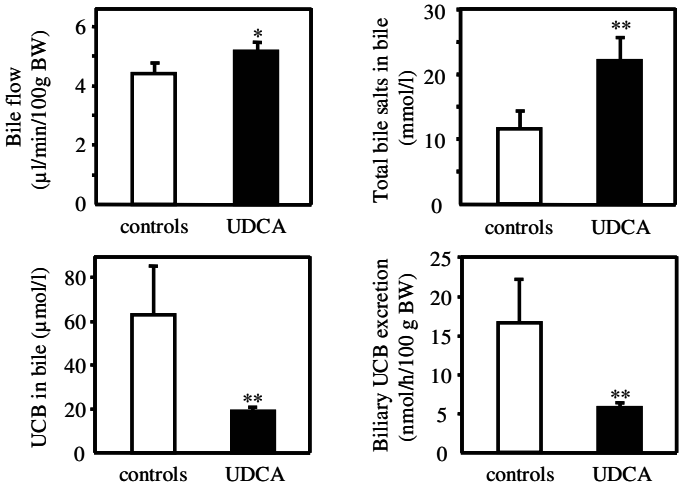


Figure 2. Effects of long-term dietary UDCA supplementation on bile flow, biliary bile salt and UCB concentration, and biliary secretion of UCB in Gunn rats treated for 3 weeks with UDCA (0.5% w/w, ■), compared with controls (□). Bile was collected for 30 minutes via cannulation of the common bile duct. Data represent mean ± SD of n = 5 animals per group. *p<0.05, **p<0.01, compared with controls.

The conventional clinical treatment for unconjugated hyperbilirubinemia is phototherapy. We recently showed that the mechanism by which phototherapy decreases plasma UCB concentrations in Gunn rats involves stimulation of biliary UCB secretion, followed by enhanced catabolism of UCB to derivatives.[5] In the present study, UDCA treatment did not increase, but rather decreased biliary UCB secretion (-65% compared with controls, $p<0.01$; Figure 2). Compared with controls, UDCA increased bile flow (+17%, $p<0.05$), and biliary secretion of bile salts, cholesterol and phospholipids (+123%, $p<0.001$; +83%, $p<0.01$; and, +90%, $p<0.01$; respectively; Figure 3). Biliary excretion of urobilinoids was not significantly changed. Liver weights were similar in controls and UDCA treated animals (11.4 ± 0.6 and 11.5 ± 0.3 g, respectively).

Short-term UDCA or CA treatment (8 days)

Body weights were similar in all groups before treatment (controls 285 ± 30, UDCA 287 ± 24, CA 281 ± 29 g). Body weights did not change significantly during treatment (controls, ~0%, UDCA -1%, CA -2%). Bile salt supplementation did not persistently affect feces

production (controls 2.0 ± 0.2, UDCA 2.2 ± 0.3, CA 2.3 ± 0.4 g dry weight/24h; not significant). At one day after starting treatment, feces production was transiently higher in UDCA- (+ 57%, p<0.001) and CA- (+56%, p<0.05) treated animals, compared with controls (data not shown). Food intake was similar in the three groups (controls 14 ± 2, UDCA 16 ± 3, CA 17 ± 6 g/24h), except for a higher intake at day two after starting treatment in the UDCA (+69%, p<0.001) and CA (+121%, p<0.001) groups, compared with controls (data not shown). Liver weights were similar in controls (3.5 ± 0.3) and treated animals (UDCA 3.4 ± 0.1, CA 3.5 ± 0.2 g; not significant).

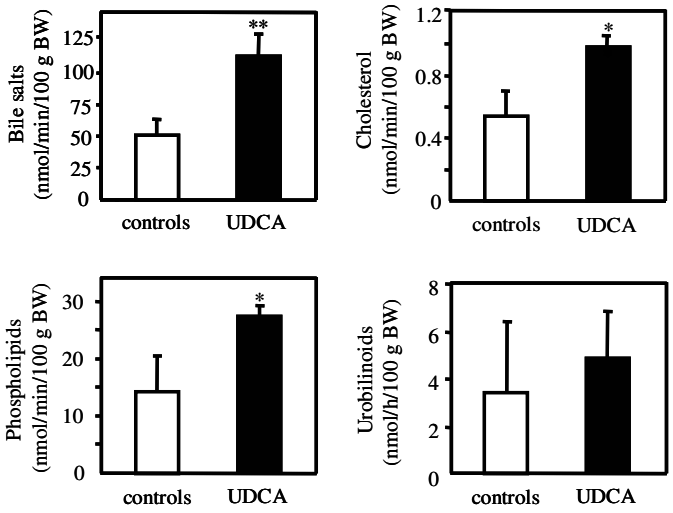


Figure 3. Effects of long-term dietary UDCA supplementation on biliary secretion of bile salts, cholesterol, phospholipids and urobilinoids in Gunn rats treated for 3 weeks with UDCA (0.5% w/w, UDCA ■), compared with controls (□). Bile was collected for 30 minutes via cannulation of the common bile duct. Data represent mean ± SD of n = 5 animals per group. *p<0.01, **p<0.001, compared with controls.

Figure 4 shows the effects of short-term UDCA and CA treatment on plasma UCB concentrations. Both bile salts decreased plasma UCB levels, although with slightly different kinetics. UDCA treatment decreased plasma UCB concentration within 2 days of treatment (-24%, p<0.05, compared with T_0), whereas CA treatment was not effective until day 4. At the end of the experiment, UDCA and CA had similarly decreased plasma UCB concentrations (-27% (p<0.001) and -34% (p<0.001) respectively, compared with controls). Plasma UCB levels in control animals decreased slightly during the course of the experiment (compared with T_0, -15%, p<0.05).

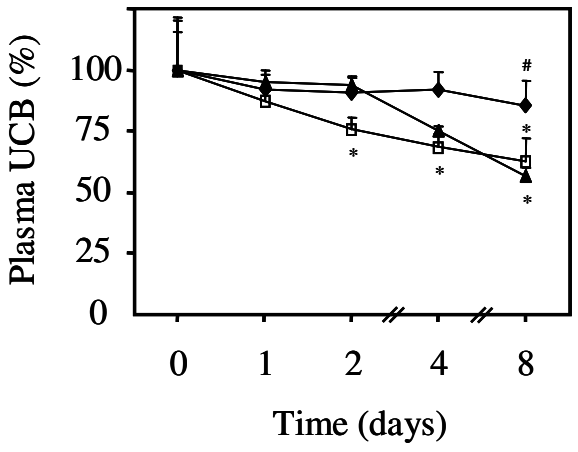


Figure 4. Effects of short-term dietary supplementation with UDCA or CA on plasma UCB concentrations in Gunn rats. After 4 weeks run-in on the control diet, Gunn rats received the same diet without (controls ♦, n = 7) or with UDCA (0.5% w/w, □, n = 7) or CA (0.5% w/w, ▲, n = 5) for 8 days. Data represent mean ± SD. *p<0.001, compared with controls. #p<0.05, compared with T_0.

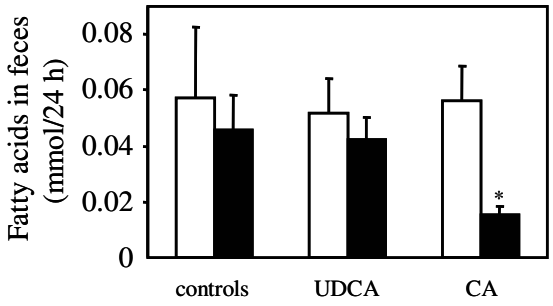


Figure 5. Effects of short-term dietary supplementation with UDCA or CA on fecal excretion of fatty acids in Gunn rats. Feces were collected for 72 hours before (□) and at the end (■) of 8 days of treatment with UDCA (0.5% w/w, n = 7) or CA (0.5% w/w, n = 5). Data represent mean ± SD. *p<0.001, compared with controls (n = 7).

Figure 5 shows that CA treatment decreased fecal fat excretion (-67%, p<0.001) compared with controls. UDCA supplementation did not affect fecal fat excretion. Fecal fat excretion in control animals was similar throughout the experiment. Fecal UCB excretion was similar in all groups before treatment. During treatment with UDCA, fecal UCB excretion was considerably higher compared with CA or no treatment (Figure 6). On average, fecal UCB excretion was 152% higher in UDCA treated rats compared with controls. Fecal fat and UCB excretion were not correlated in the three groups (linear regression lines, R^2<0.5, not shown). There were no significant differences in urobilinoid excretion during treatment (Figure 6).

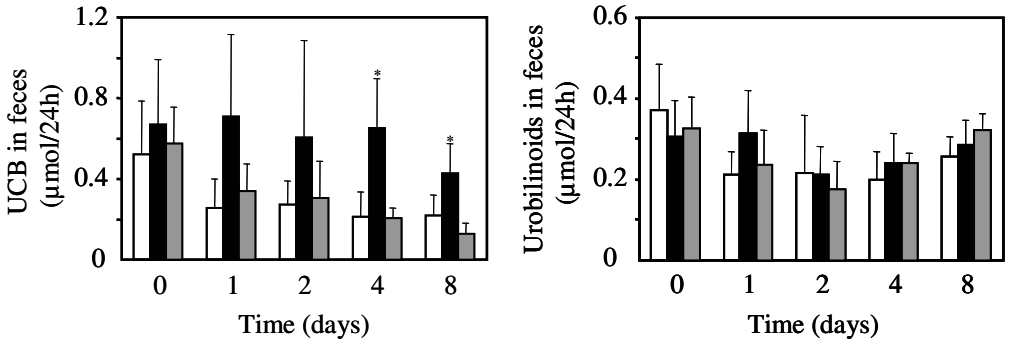


Figure 6. Effects of short-term dietary supplementation with UDCA or CA on fecal excretion of UCB and urobilinoids in Gunn rats. Feces were collected at the end of 8 days of treatment with UDCA (0.5% w/w, ■, n = 7) or CA (0.5% w/w, ▩, n = 5). Data represent mean ± SD. *p<0.01, compared with controls (□, n = 7).

We determined the effects of bile salt supplementation on the composition of the bile salt pool in Gunn rats. UDCA and CA profoundly changed the composition of the bile salt pool (Figure 7).

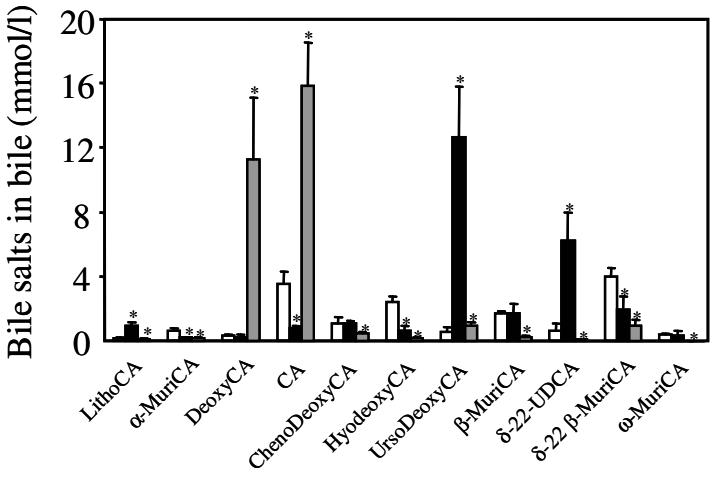


Figure 7. Effects of short-term dietary supplementation with UDCA or CA on the composition of the bile salt pool in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7) or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). Bile was collected for 30 minutes via cannulation of the common bile duct. Data represent mean ± SD. *p<0.05-p<0.001, compared with controls.

During UDCA treatment, biliary bile salts were mainly composed of UDCA, δ-22-UDCA, and δ-22-β-muricholic acid. During CA treatment, the contribution of CA and deoxycholic acid to biliary bile salts increased, indicating an increased hydrophobicity of the bile salt pool.

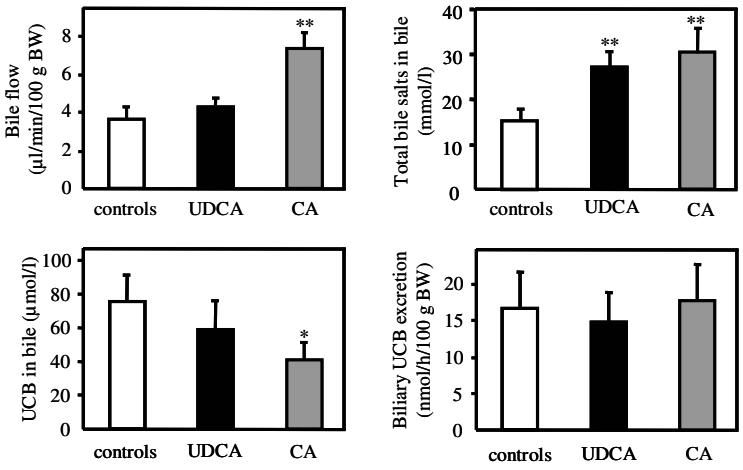


Figure 8. Effects of short-term dietary supplementation with UDCA or CA on bile flow, biliary bile salt and UCB concentration, and biliary secretion of UCB in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7) or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). Bile was collected for 30 minutes via cannulation of the common bile duct. Data represent mean ± SD. *p<0.01, **p<0.001, compared with controls.

Figure 8 shows that CA treatment increased bile flow (+101%, p<0.001), whereas UDCA did not, in contrast to long-term UDCA treatment (see above). Both bile salts increased biliary bile salt concentration (UDCA +75%, p<0.01; CA +100%, p<0.01). Biliary secretion of UCB was not different among the 3 groups after 8 days of treatment. Short-term CA treatment, in contrast to UDCA, greatly enhanced biliary secretion of bile salts (+306%, p<0.001), cholesterol (+134%, p<0.001) and phospholipids (+166%, p<0.001), compared with controls (Figure 9). Biliary secretion of urobilinoids tended to decrease during UDCA treatment (-43%, NS) and decreased significantly during CA treatment (-59%, p<0.05, Figure 9).

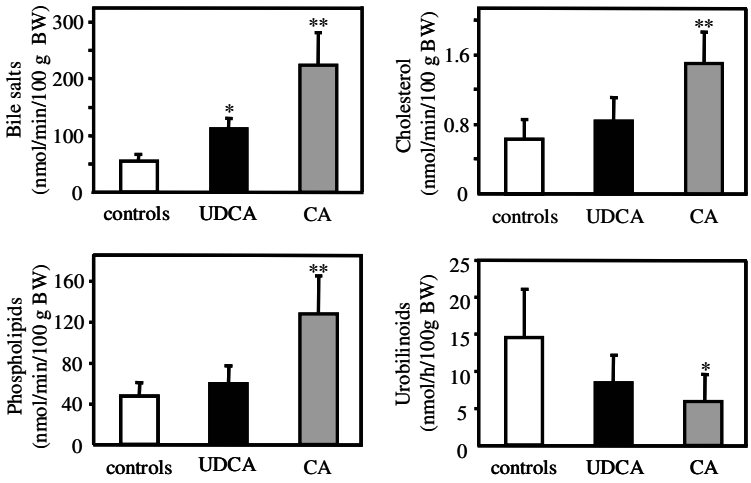


Figure 9. Effects of short-term dietary supplementation with UDCA or CA on biliary secretion of bile salts, cholesterol, phospholipids and urobilinoids in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7) or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). Bile was collected for 30 minutes via cannulation of the common bile duct. Data represent mean ± SD. *p<0.05, **p<0.001, compared with controls.

Effects of bile salts on various transporters and enzymes

Figure 10 shows the effects of long-term UDCA treatment on hepatic transporters. UDCA treatment enhanced the expression of Mrp2 and Bsep (Bile salt efflux pump, Abcb11) in Gunn rats, as demonstrated previously by others in different strains of rats and mice.[8;9] The

mRNA expression of Cyp7a1, encoding for cholesterol 7α-hydroxylase that exerts a rate-controlling role in bile salt synthesis, was also increased. The nuclear receptor FXR (Farnesoid X receptor) negatively regulates Cyp7a1 via activation of SHP (Small heterodimer partner). UDCA treatment did not induce SHP.

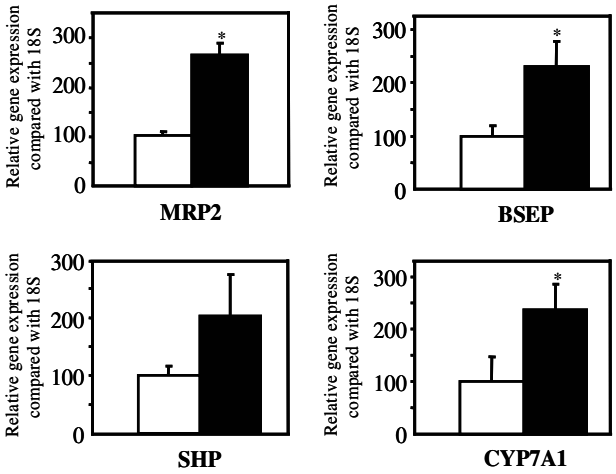


Figure 10. Effects of long-term UDCA supplementation on hepatic gene expression levels of Mrp2, Bsep, Shp and Cyp7a1 in Gunn rats treated for 3 weeks with UDCA (0.5% w/w, ■), compared with controls (□). RNA levels were determined by Q-RT-PCR. Values were normalized to 18S levels and presented as data relative to controls. Data represent mean ± SD of n = 5 animals per group. *p<0.05, compared with controls.

Figure 11 shows that short-term treatment with UDCA or CA did not enhance Mrp2 or Bsep. Short-term treatment with UDCA or CA decreased, as expected, mRNA expression of Cyp7a1 (UDCA, -54%, CA, -60%, each p<0.01) in the liver and induced Ibabp (Ileal bile acid-binding protein)[20] gene expression at the intestinal level, thus demonstrating that bile acid treatments induced the expected gene regulations. Sterol 12α-hydroxylase (Cyp8b1), the enzyme that controls the ratio in which cholate and chenodeoxycholate are formed, was greatly reduced by CA supplementation (-99%, p<0.001).

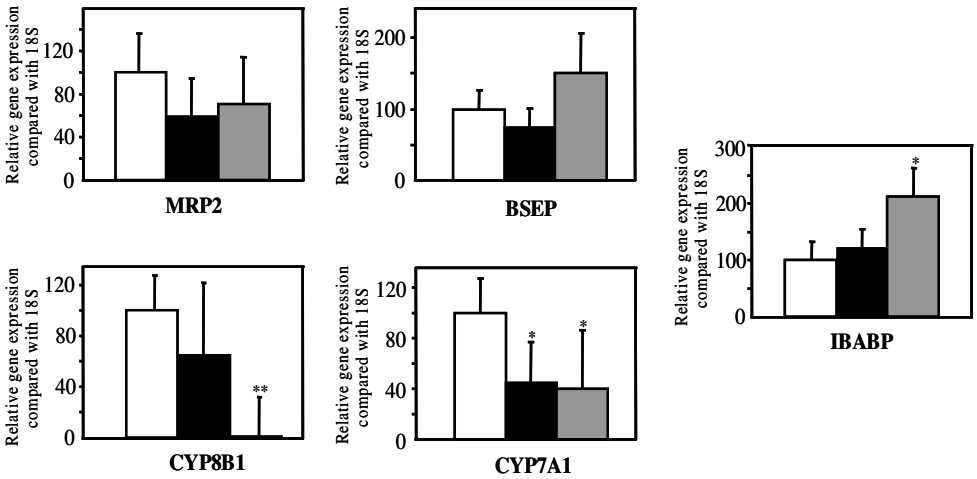


Figure 11. Effects of short-term UDCA or CA supplementation on hepatic gene expression levels of Mrp2, Bsep, Cyp8b1 and Cyp7a1, and on intestinal gene expression levels of Ibabp in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7), or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). RNA levels were determined by Q-RT-PCR. Values were normalized to 18S levels and presented as data relative to controls. Data represent mean ± SD. *p<0.01, **p<0.001, compared with controls.

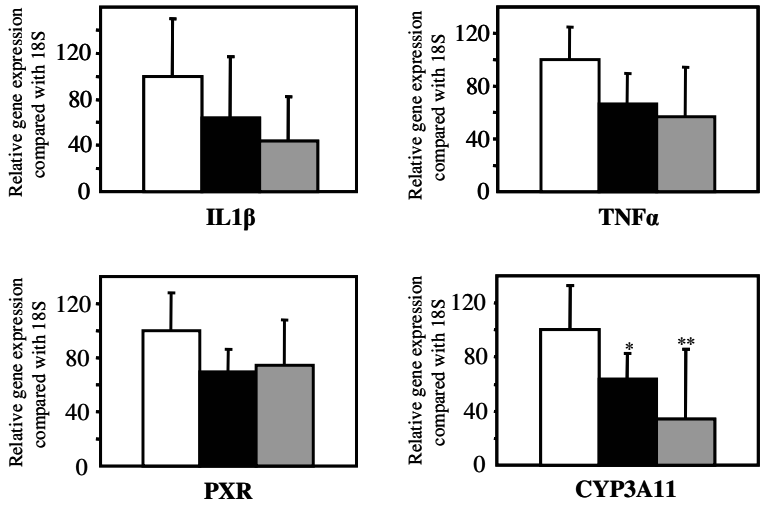


Figure 12. Effects of short-term UDCA or CA supplementation on hepatic gene expression levels of IL1β, TNFα, PXR and Cyp3a11 in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7), or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). RNA levels were determined by Q-RT-PCR. Values were normalized to 18S levels and presented as data relative to controls. Data represent mean ± SD. *p<0.05, **p<0.01, compared with controls.

Interestingly, inflammation markers IL1β and TNFα tended to decrease by UDCA and CA supplementation (Figure 12). The Pregnane X receptor (PXR) is known to be involved in bilirubin detoxification and elimination pathways in animals that can conjugate bilirubin.[21;22] Bile salt precursors were shown to activate PXR.[23] In our Gunn rats, PXR expression was not significantly changed by bile salt supplementation (Figure 12), but Cyp3a11 expression, a well known PXR target gene was decreased. Moreover, hepatic gene expressions of genes involved in bilirubin detoxification like Cyp1a2, a cytochrome P450 enzyme involved in oxidative catabolism of UCB, and Mrp4 were decreased, whereas Mrp3 expression in the liver was unchanged by UDCA or CA (Figure 13), demonstrating that bilirubin elimination induced by bile acid treatments lead to a de-activation of both detoxification and inflammatory pathways.

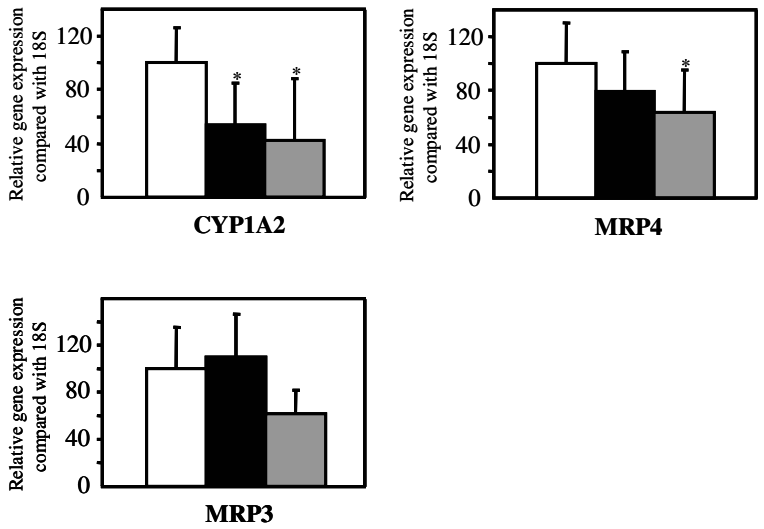


Figure 13. Effects of short-term UDCA or CA supplementation on hepatic gene expression levels of Cyp1a2, Mrp4, and Mrp3 in Gunn rats treated for 8 days with UDCA (0.5% w/w, ■, n = 7), or CA (0.5% w/w, ▩, n = 5), compared with controls (□, n = 7). RNA levels were determined by Q-RT-PCR. Values were normalized to 18S levels and presented as data relative to controls. Data represent mean ± SD. *p<0.05, compared with controls.

DISCUSSION

We demonstrate in Gunn rats that dietary supplementation with UDCA or CA decreases plasma UCB concentrations by approximately 30%. The mechanism(s) behind the effect is (are) yet unclear. Previously, we demonstrated that stimulation of fecal fat excretion decreases plasma UCB levels in Gunn rats.[3;4] In the present study, however, UDCA had no effect on fecal fat excretion and CA even decreased fecal fat excretion, indicating that these bile acids decrease plasma UCB concentration via a different mechanism. We previously also demonstrated that phototherapy, the conventional treatment for unconjugated hyperbilirubinemia, decreases plasma UCB levels by increasing biliary secretion of UCB.[5] Treatment with UDCA or CA for 8 days did not change biliary secretion of UCB. Long-term UDCA treatment even decreased biliary UCB secretion, possibly related to decreased plasma UCB concentrations and pool size.[5]

The mechanism(s) underlying the hypobilirubinemic effects of UDCA and CA in Gunn rats is (are) thus independent from enhancing biliary UCB excretion or stimulation of fecal fat excretion. Fecal UCB excretion was essentially higher in UDCA treated animals compared with controls or CA treated animals. An increased fecal UCB excretion with a decreased biliary UCB secretion in UDCA-treated animals is suggestive for a decreased enterohepatic circulation of UCB. Possibly UDCA interferes with the solubilization of UCB.[11-13] UDCA is known to decrease intestinal cholesterol absorption, although the exact mechanism is unknown.[24;25] However, our present results also indicate that UDCA did not increase fecal UCB excretion, indicating that the decrease in plasma UCB concentrations could not be attributed to (transiently) increased fecal loss of unmetabolized UCB. Another explanation might be a change in bacterial flora due to UDCA, leading to a quantitatively or qualitatively altered metabolism to derivatives. Fecal UCB and urobilinoid excretion appeared decreased in control animals. Although the decrease was not significant, it does suggest that the control animals were not in steady-state. Several results from long-term versus short-term UDCA treatment were different, such as hepatic expression levels of various transporters. These differences are unexplained, but may be related to differences in the bile salt pool or UCB pool. We feel that for better understanding of the (level of the) mechanism(s) underlying the hypobilirubinemic effect of UDCA, kinetic studies under steady-state conditions are needed.

Regarding the CA results, it appears that CA may decrease plasma UCB levels via a different mechanism than UDCA. CA was not effective until after 4 days of supplementation, whereas UDCA decreased plasma UCB concentrations already after 2 days of treatment. CA did not affect fecal UCB excretion, whereas UDCA-treated rats excreted more UCB than controls or CA-treated rats. As expected, the composition of biliary bile salts was very different between UDCA- and CA-treated animals. CA supplementation caused a much greater hydrophobicity of bile, which likely had an effect on the solubilization of UCB in the intestine. Another difference between CA and UDCA treatment is the decreased fecal fat

excretion during CA treatment. Based on our previous studies where an increased fecal fat excretion decreases plasma UCB levels in Gunn rats,[3-5] we would expect increased plasma UCB concentrations during CA treatment. Apparently the hypobilirubinemic effect of CA overrules the decrease in fecal fat excretion. CA greatly enhanced bile flow, in contrast to UDCA. Normally, a very small amount of UCB is excreted into bile, possibly via interaction with mixed micelles.[26;27] Ostrow *et al.* showed that taurocholate infusion enhances bile-salt mediated secretion of UCB into bile of Gunn rats.[28] Enhancement of bile-salt mediated secretion of UCB could be useful in the management of unconjugated hyperbilirubinemia if it involved a non-toxic bile salt. CA decreased the concentration of UCB in bile, so it is not likely that this mechanism is involved. Another possibility would be decreased UCB production. This is not likely considering the fact that retained hydrophobic bile salts are known to induce hemolysis. Another possible mechanism could be a shift in the UCB pool from plasma to tissues. Clearly, kinetic studies are needed to elucidate how CA decreases plasma UCB levels in Gunn rats.

Mendez-Sanchez *et al.*[29] showed in non-hyperbilirubinemic mice and rats that dietary UDCA supplementation increased biliary UCB secretion and plasma UCB levels, and postulated that UDCA induces enterohepatic cycling of UCB by causing bile salt malabsorption. UDCA supplementation causes bile salt malabsorption by competition for ileal bile acid transporters.[29;30] Free UDCA is able to diffuse passively but it will be largely conjugated after the first pass by the hepatocytes and will then start to compete and displace the endogenous bile salts. Comparing Mendez-Sanchez's and our study is rather difficult. We used the Gunn rat, the animal model for unconjugated hyperbilirubinemia, to study the effects of UDCA on plasma UCB concentrations. They used C57L/J mice and Sprague-Dawley rats that have normal bilirubin conjugation. Interpretation of their results is difficult since steady-state bilirubin kinetic studies investigating transepithelial transport of UCB were not performed.

Oxidation is an alternative pathway for removal of UCB under hyperbilirubinemic conditions. In young Gunn rats, cytochrome P450 enzymes Cyp1a1 and Cyp1a2 induce microsomal UCB degradation.[31] Inducers of Cyp1a1 and Cyp1a2 decrease plasma UCB levels by production of polar UCB derivatives that can be excreted into bile.[32-34] In the present study, however, we do not have positive indications that oxidation is a likely mechanism by which UDCA and CA decreased plasma UCB concentrations. First of all, liver weight was not increased by treatment with UDCA or CA, what would have been expected when liver microsomal hydroxylase activity is enhanced. Furthermore, Cyp1a2 was not increased, but rather repressed by UDCA or CA treatment. However, the induction of UCB oxidation cannot not be excluded since only mRNA (not protein) was measured and only Cyp1a2. The repression of Cyp1a2 by UDCA or CA treatment may be explained by the fact that bile salt supplementation decreased plasma (and thus likely tissue) UCB levels, that in turn deactivated the inflammation, since IL1β and TNFα expressions were reduced. The decreased

tissue UCB levels may also have deactivated the detoxification pathways controlled by PXR, since gene expression of Cyp3a11, a marker of PXR activation, was decreased and concomitantly Cyp1a2 and various Mrp's expressions were reduced. Taken together our data show not only that UDCA and CA treatments efficiently decreased plasma UCB levels, but also that the improvement in bilirubin metabolism translated into interesting potential therapeutic consequences, namely a lower inflammation and a lower stress/detoxification induction. Interestingly, despite the fact that bile acids are known to activate various nuclear receptors, it is unlikely that the treatments effects were due to an enhanced PXR activity or enzyme metabolism.

In conclusion, 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. Kinetic studies with ^{3}H-UCB which provide detailed information about fractional turnover, production and pool size of UCB and about the enterohepatic circulation of UCB and its derivatives may elucidate the mechanism(s) underlying the hypobilirubinemic effect of bile salts.

ACKNOWLEDGEMENTS

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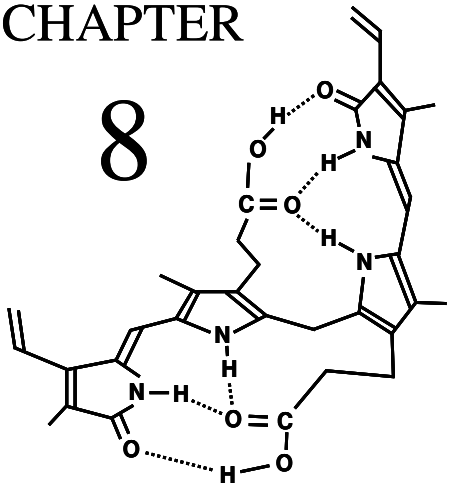
Summary and general discussion

CHAPTER

8



SUMMARY AND GENERAL DISCUSSION

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 ~85 µmol/l. Untreated, plasma UCB levels in Crigler-Najjar patients range between 350-800 µmol/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.[4-6]

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).

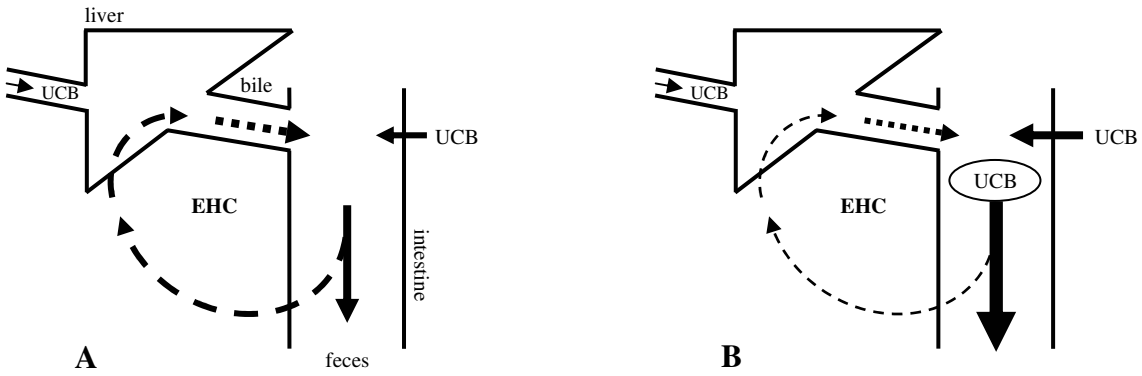


Figure 1. Intestinal capture of UCB. **A:** In Crigler-Najjar disease, UCB can enter the intestine via biliary secretion and via transepithelial diffusion from the blood into the intestinal lumen, across the intestinal mucosa. UCB and its metabolites are partly excreted with the feces, and partly reabsorbed 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.[12] 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,[13] but efficacy was less pronounced in patients with Crigler-Najjar disease.[14] 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.*[13;14] 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,[15] 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 (^{3}H) labeled (*i.e.* radioactive) UCB. Gunn rats were either not treated (controls), or treated for 3 weeks with orlistat, phototherapy, or combined treatment. ^{3}H-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,[16] 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 ^{3}H-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.[20;21;23;24] 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.[25] 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.[16;18;19] Ursodeoxycholic acid (UDCA) had been suggested to impair fat absorption.[26] UDCA is a hydrophilic, non-toxic bile salt that is used in the

management of *conjugated* hyperbilirubinemia.[27;28] 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.[27;28] 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.

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Nederlandse samenvatting
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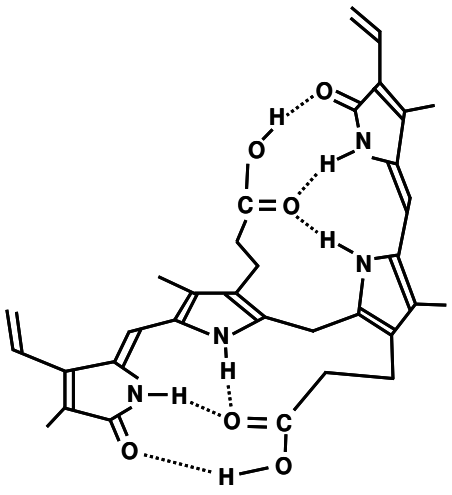
Publicaties

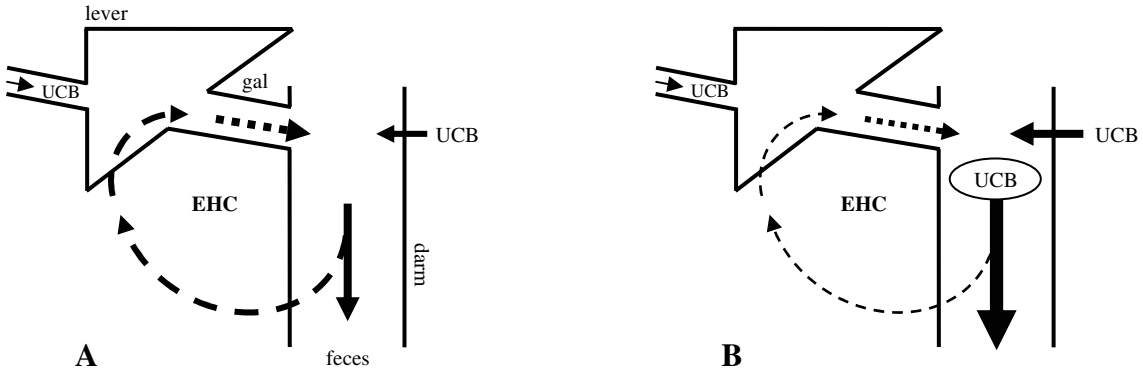


NEDERLANDSE SAMENVATTING

Het onderzoek in dit proefschrift bestudeert orale behandeling van ongeconjugeerde hyperbilirubinemie. Ongeconjugeerd bilirubine (**UCB**) is een afbraakproduct van rode bloedcellen. Ophoping van UCB in het lichaam (hyperbilirubinemie) veroorzaakt geelzucht (icterus) en kan onbehandeld leiden tot neurologische schade en kernicterus, resulterend in lichamelijke en geestelijke handicaps en zelfs overlijden.[1;2] UCB wordt via het enzym bilirubine-UDP-glucuronosyltransferase (UGT1A1) in de lever beter wateroplosbaar gemaakt (geconjugeerd) waardoor het kan worden uitgescheiden in de gal. Geconjugeerd bilirubine komt via de gal in de darm terecht en kan vervolgens met de ontlasting het lichaam verlaten.

Bij de ziekte van Crigler-Najjar ontbreekt UGT1A1 waardoor er een permanente ongeconjugeerde hyperbilirubinemie bestaat sinds de geboorte.[3] Onbehandeld variëren plasma UCB concentraties bij Crigler-Najjar patiënten van 350-800 µmol/l (normaalwaarden voor plasma UCB concentraties bij volwassenen: 7-15 µmol/l). De conventionele behandeling voor de ziekte van Crigler-Najjar is fototherapie, lichttherapie middels een soort zonnebank met blauwe lampen waaraan de huid tot zo'n 12 uur per dag moet worden blootgesteld. Langdurige, dagelijkse fototherapie heeft aanzienlijke nadelen. De voornaamste problemen zijn een verminderende effectiviteit van fototherapie bij stijgen van de leeftijd en een grote impact van het intensieve fototherapieregime op de kwaliteit van (sociaal) leven.[4-6]

Het doel van ons onderzoek was het ontwikkelen van een alternatieve of additionele behandeling voor ongeconjugeerde hyperbilirubinemie die is gebaseerd op orale toediening en "intestinal capture" van UCB. UCB ondergaat een enterohepatische (darm-lever) kringloop (zie figuur 1).[7;8] Nadat UCB door de lever wordt geconjugeerd, komt het via de gal in de darm terecht waar het wordt gedeconjugeerd. Het UCB kan vervolgens: 1) het lichaam verlaten via de ontlasting (feces), 2) worden omgezet in UCB-metabolieten die worden uitgescheiden via de feces, en/of 3) worden heropgenomen vanuit de darm in de enterohepatische circulatie.



Figuur 1. "Intestinal capture" van UCB. **A.** Bij de ziekte van Crigler-Najjar komt UCB in de darm terecht via de gal en via diffusie vanuit het bloed naar het darmlumen, over de darmwand heen. UCB en UCB-metabolieten worden gedeeltelijk uitgescheiden met de feces en gedeeltelijk heropgenomen in de enterohepatische kringloop (EHC). **B.** "Intestinal capture" van UCB gevolgd door versterkte fecale uitscheiding vermindert de enterohepatische kringloop van UCB en verlaagt daardoor de plasma UCB concentratie.

UCB kan niet alleen vanuit de darm in het bloed worden heropgenomen. Als de UCB concentratie in het bloed hoog is, zoals bij de ziekte van Crigler-Najjar, dan kan UCB vanuit het bloed naar het darmlumen diffunderen.[9;10] "Intestinal capture", het vangen van UCB in de darm en vervolgens bevorderen van de fecale UCB uitscheiding vermindert de enterohepatische kringloop en verlaagt de plasma UCB concentratie. Onze hypothese was dat UCB in de darm zou kunnen associëren met vet, gezien het relatief lipofiele karakter van UCB.[11] Volgens onze hypothese zou verhoging van de fecale vetuitscheiding de fecale UCB uitscheiding verhogen en de enterohepatische kringloop van UCB verminderen.

Orlistat behandeling verhoogt de fecale UCB concentratie en verlaagt de plasma UCB concentratie in hyperbilirubinemische Gunn ratten

In **hoofdstuk 3** hebben we in het diermodel voor ongeconjugeerde hyperbilirubinemie, de Gunn rat, onderzocht of stimulatie van de fecale vetuitscheiding de fecale UCB uitscheiding verhoogt en de plasma UCB concentratie verlaagt. We hebben de fecale vetuitscheiding verhoogd door de lipase remmer orlistat toe te voegen aan het voer. Orlistat remt de vertering van vetten (triglyceriden) en daardoor wordt de opname van voedingsvet verminderd.[12] Korte- en lange termijn effecten van orlistat behandeling werden bestudeerd. Binnen enkele dagen verhoogde orlistat behandeling de fecale uitscheiding van zowel vet als UCB. Na 3 weken behandeling met de hoogste dosis orlistat (200 mg/kg voer) was de plasma UCB concentratie met 46% verlaagd. Plasma UCB concentraties waren sterk, negatief gecorreleerd met de fecale vetuitscheiding, dus hoe hoger de fecale vetuitscheiding, hoe lager de plasma UCB concentratie. De verhoging van de fecale UCB uitscheiding was tijdelijk, in tegenstelling tot permanent verlaagde plasma UCB concentraties, wat suggereert dat er een nieuw evenwicht in de UCB homeostase was bereikt. Korte termijn behandeling met orlistat was dus succesvol. Op lange termijn hielden de effecten aan. Bij behandeling van Gunn ratten gedurende 24 weken met orlistat bleven plasma UCB concentraties significant lager (~35%) in behandelde ratten vergeleken met controle ratten. Er werden geen bijwerkingen gevonden. Orlistat behandeling had geen invloed op lichaamsgewicht, groei of plasma concentraties van vetten of vetoplosbare vitamines. Netto vetopname was niet beïnvloed omdat de ratten een verhoogde fecale vet uitscheiding compenseerden door meer te gaan eten.

Effectieve orale behandeling van ongeconjugeerde hyperbilirubinemie in Gunn ratten

Vervolgens hebben we de relatieve effectiviteit van orlistat behandeling onderzocht ten opzichte van de effectiviteit van de conventionele behandeling voor ongeconjugeerde hyperbilirubinemie, fototherapie. De meerderheid van de Crigler-Najjar patiënten is afhankelijk van fototherapie. Mogelijk zouden orlistat en fototherapie kunnen worden gebruikt als elkaar aanvullende behandelingen. In **hoofdstuk 4** hebben we getracht een orale behandeling voor ongeconjugeerde hyperbilirubinemie te ontwikkelen die even effectief of effectiever is dan fototherapie. We hebben gefocust op "intestinal capture" van UCB door een

combinatie van vet en calciumfosfaat. Anderen hebben laten zien dat calciumfosfaat effectief was bij Gunn ratten,[13] maar de effectiviteit was minder uitgesproken bij patiënten met de ziekte van Crigler-Najjar.[14] Wij hebben de individuele en gezamenlijke effecten van orlistat en calciumfosfaat onderzocht in Gunn ratten en hebben de effectiviteit vergeleken met continue fototherapie. Eén of twee weken orlistat behandeling was even effectief in het verlagen van de plasma UCB concentratie als continue fototherapie, en de combinatie van orlistat met fototherapie was effectiever dan de behandelingen afzonderlijk. Orale behandeling met een combinatie van orlistat en calciumfosfaat gedurende 3 weken verlaagde de plasma UCB concentratie met ~50% en was effectiever dan continue fototherapie in Gunn ratten. Om te bepalen of de effecten van orlistat en calciumfosfaat werden beïnvloed door de hoeveelheid vet in het voer, hebben we de effecten onderzocht tijdens een laag-vet (13 energie%) en hoog-vet (35 energie%) dieet. De hoeveelheid voedingsvet had een groot effect op plasma UCB concentraties in Gunn ratten. Vervanging van een laag-vet door een hoog-vet dieet verlaagde de plasma UCB concentratie met 46% na 3 weken. Bij nadere analyse bleek dat de hoeveelheid voedingsvet sterk de fecale vetuitscheiding beïnvloedde en dientengevolge de plasma UCB concentratie. Orlistat behandeling verlaagde de plasma UCB concentratie onafhankelijk van de hoeveelheid voedingsvet. Calciumfosfaat was echter alleen significant effectief tijdens een laag-vet dieet. Wij hypothetiseerden dat de hoeveelheid voedingsvet voor een deel de lage effectiviteit van calciumfosfaat bij patiënten met de ziekte van Crigler-Najjar kan verklaren, zoals onderzocht door Van der Veere *e.a.*[14] Het humane (westerse) hoog-vet dieet bevat 35-40 energie% vet, terwijl de Gunn ratten in Van der Veere's studie een laag-vet (13 energie%) dieet kregen,[13] identiek aan het laag-vet dieet in onze experimenten.

Zoals eerder aangetoond verhoogde fototherapie de uitscheiding van UCB in de gal.[15] De observatie dat fototherapie de effectiviteit van orlistat verhoogde, ondersteunde ons voorgestelde concept dat orlistat behandeling de enterohepatische kringloop van UCB vermindert. De resultaten van dit onderzoek ondersteunden de mogelijkheid van een effectieve orale behandeling van patiënten met ongeconjugeerde hyperbilirubinemie.

Nieuwe kinetische inzichten in de behandeling van ongeconjugeerde hyperbilirubinemie: fototherapie en orlistat behandeling in Gunn ratten

De observatie dat gecombineerde behandeling met orlistat en fototherapie de plasma UCB concentratie effectiever verlaagt dan een van beide behandelingen afzonderlijk, suggereert dat de twee behandelingen werken via verschillende mechanismen. In **hoofdstuk 5** hebben we de mechanismen achter de effecten van orlistat, fototherapie en combinatietherapie onderzocht met behulp van tritium- (^{3}H) gelabeld (radioactief) UCB. Gunn ratten werden ofwel niet behandeld (controles), of behandeld met orlistat, fototherapie of gecombineerde behandeling gedurende 3 weken waarna de plasma UCB concentraties stabiel bleven ("steady-state"). Met behulp van ^{3}H-kinetiek gegevens tijdens steady-state condities konden we de verschillende metabole fluxen van UCB in het lichaam berekenen, zoals de UCB productie, turnover en

pool grootte (de totale hoeveelheid UCB in het lichaam). Daarnaast konden we de dynamiek van de enterohepatische kringloop karakteriseren. Zoals we eerder lieten zien verlaagde gecombineerde behandeling met orlistat en fototherapie de plasma UCB concentraties sterker dan een van beide behandelingen afzonderlijk.[16] Steady-state plasma UCB concentraties waren sterk, negatief gecorreleerd met de fractionele turnover van ^{3}H-UCB, wat aangaf dat fototherapie en orlistat behandeling beiden de plasma UCB concentratie verlagen door de bilirubine turnover te stimuleren. De totale bilirubine turnover, een maat voor de UCB productie, was niet significant veranderd door de behandelingen, wat steady-state condities bevestigde. Het feit dat gecombineerde behandeling het meest effectief de plasma UCB concentraties en bilirubine pool grootte verminderde, ondersteunde de opzet van een patiëntenstudie waarbij orlistat zou worden gebruikt als additieve behandeling naast fototherapie.

Door de experimentele opzet en het mathematische model van de huidige studie konden we een schatting maken van de turnover en het metabolisme van zowel gelabeld UCB als UCB-metabolieten in de enterohepatische kringloop. Fototherapie verhoogde de uitscheiding van UCB in de gal, zoals eerder aangetoond.[15;16] Een nieuwe bevinding van deze studie was dat fototherapie ook de uitscheiding van UCB-metabolieten verhoogde. Wij postuleerden dat de verhoogde uitscheiding van UCB-metabolieten in de gal tijdens fototherapie het gevolg was van enterohepatische circulatie van urobilinogeen en urobilinoïden door ofwel een verhoogd aanbod van substraat (=UCB) aan het darmlumen, en/of door verandering van de metabole activiteit van de bacteriële flora door fototherapie. Orlistat behandeling induceerde netto uitscheiding van UCB vanuit het bloed, over de darmwand heen, naar het darmlumen. Deze observatie kwam overeen met ons voorgestelde concept dat orlistat behandeling de enterohepatische kringloop van UCB vermindert door “intestinal capture” van UCB. Daarnaast leek het hypobilirubinemische effect van orlistat tevens te berusten op een verhoogd metabolisme van UCB naar UCB-metabolieten. Zoals hierboven besproken voor fototherapie zou orlistat de hoeveelheid substraat kunnen hebben verhoogd, of de samenstelling van de darmflora veranderd. De darmflora is van groot belang voor het metabolisme van bilirubine.[17] Wij speculeerden dat manipulatie van de metabole capaciteit van de darmflora door antibiotica of specifieke bacteriën (“design probiotica”) de hypobilirubinemische effecten van orlistat, fototherapie of gecombineerde behandeling kan beïnvloeden. Momenteel zijn er studies gaande naar de interactie tussen bacteriële flora en bilirubine homeostase in Gunn ratten.

Orlistat behandeling van ongeconjugeerde hyperbilirubinemie bij de ziekte van Crigler-Najjar; een gerandomiseerd gecontroleerd onderzoek

De goede resultaten van onze studies met orlistat behandeling van Gunn ratten[16;18;19] en de afwezigheid van serieuze bijwerkingen van orlistat bij Gunn ratten[16;18;19] en bij patiënten met obesitas,[20-24] bracht ons tot het opzetten van een klinisch onderzoek naar de effecten van

orlistat behandeling bij patiënten met de ziekte van Crigler-Najjar. **Hoofdstuk 6** beschrijft de resultaten van ons gerandomiseerde placebo-gecontroleerde dubbelblinde cross-over onderzoek naar de effecten van orlistat behandeling op plasma UCB concentraties en op de fecale uitscheiding van vet en UCB. Zestien Crigler-Najjar patiënten participeerden in het onderzoek. Hun reguliere behandeling met fototherapie en/of fenobarbital werd gecontinueerd. Orlistat werd dus getest als aanvullende behandeling. Patiënten kregen oraal orlistat of een placebo, elk gedurende 4-6 weken met 2 weken interval. Een klinisch relevante respons op behandeling werd gedefinieerd als een verlaging van de plasma UCB concentratie met ten minste 10%. Deze definitie was gebaseerd op de variatie in intra-individuele plasma UCB concentraties in de controle periode voor start van de behandeling, welke gemiddeld lager dan 10% was. Orlistat behandeling verlaagde plasma UCB concentraties in de groep als geheel met 9%. Hoewel dit een statistisch significante daling was, hebben we dit niet beschouwd als een klinisch relevante daling voor de gehele groep, gebaseerd op onze definitie van behandelrespons. Echter, in een subgroep (7 van 16 patiënten, 44%) van patiënten verlaagde orlistat behandeling plasma UCB concentraties met meer dan 10% (gemiddeld 21%, spreiding 11-32%). Een klinische relevante respons op orlistat behandeling was niet gecorreleerd met leeftijd, geslacht, Crigler-Najjar type, body-mass index of gelijktijdige behandeling met fototherapie of fenobarbital. Echter, een relatief (t.o.v. de groep) lagere voedingsvet inname (<35 energie%) leek vaker voor te komen in de patiënten die reageerden op orlistat behandeling ten opzichte van de patiënten die geen respons vertoonden. Daarnaast waren alleen in de patiënten met een klinisch relevante respons op behandeling de plasma UCB concentraties tijdens orlistat sterk, negatief gecorreleerd met fecale vet uitscheiding. Mogelijk hebben patiënten met een hoge vet inname (en dientengevolge hogere fecale vetuitscheiding) al een maximum niveau van UCB capture door vet bereikt en heeft het (verder) verhogen van de vet inname en/of uitscheiding geen verlaging van de plasma UCB concentratie tot gevolg.

Naast dieetvet intake zijn er waarschijnlijk andere parameters die de repons van Crigler-Najjar patiënten op orlistat behandeling bepalen. Het is verleidelijk om te speculeren dat de mate van lipase activiteit in het maagdarmkanaal een rol speelt. De dosis orlistat zou misschien geïndividualiseerd moeten worden om een bepaalde mate van vetuitscheiding te bewerkstelligen, of een bepaalde optimale samenstelling van vetzuren of gedeeltelijk gesplitste triglyceriden in de darm. De optimale omgeving voor “intestinal capture” van UCB is mogelijkerwijs ook afhankelijk van de bacteriële flora van de patiënt.

Orlistat behandeling veroorzaakte geen serieuze bijwerkingen in ons onderzoek. De milde, tijdelijke en draaglijke bijwerkingen die optraden waren vergelijkbaar met de bijwerkingen die zijn gerapporteerd in onderzoeken waarbij orlistat werd gebruikt voor de behandeling van obesitas.[20;21;23;24] Bijwerkingen waren vrijwel geheel gerelateerd aan het maagdarmkanaal (bijv. winderigheid, olie-incontinentie, diarree, buikkrampen). Orlistat behandeling verlaagde de plasma concentratie van vitamine E (-18%). Echter, lage plasma

vitamine concentraties door langdurige orlistat behandeling zouden gemakkelijk kunnen worden voorkomen door de voeding te supplementeren met vitamines.[25] Het effect van orlistat behandeling was beperkt en de individuele respons op behandeling onvoorspelbaar. Voordat orlistat overwogen zou kunnen worden als additionele behandeling naast de conventionele behandeling van de ziekte van Crigler-Najjar, zijn wij van mening dat het noodzakelijk is om de mogelijke relatie tussen behandelrespons en voedingsvet inname te verhelderen, en om relevante parameters of patiënt karakteristieken te identificeren die de repons op orlistat behandeling kunnen bepalen of voorspellen. Nader onderzoek zou ons kunnen vertellen of orlistat behandeling mogelijk ook nuttig kan zijn voor de behandeling van andere patiënten met ongeconjugeerde hyperbilirubinemie, bijvoorbeeld voor de behandeling van geelzucht bij de pasgeborene.

Effecten van galzouten op ongeconjugeerde hyperbilirubinemie in Gunn ratten

In de vorige hoofdstukken hebben we bepaald dat een verhoogde fecale vetuitscheiding de plasma UCB concentraties in Gunn ratten verlaagt.[16;18;19] Ursodeoxycholaat (UDCA) zou mogelijk de vetopname kunnen verminderen.[26] UDCA is een hydrofiel, niet-toxisch galzout dat wordt gebruikt bij de behandeling van *geconjugeerde* hyperbilirubinemie.[27-28] In **hoofdstuk 7** hebben we in Gunn ratten onderzocht wat het effect van is van orale suppletie met UDCA of met het meer hydrofobe cholaat (CA). We hebben de effecten van deze galzouten op de plasma UCB concentraties en op de fecale uitscheiding van vet en UCB bepaald. UDCA en CA verlaagden beiden de plasma UCB concentratie met ongeveer 30% na 1-3 weken. Echter, UDCA behandeling had geen invloed op de fecale vetuitscheiding en CA behandeling verlaagde zelfs de fecale vetuitscheiding (-67%). Desalniettemin was de fecale UCB concentratie hoger in de ratten die met UDCA behandeld waren vergeleken met CA behandelde of controle ratten. Geen van beide behandelingen verhoogde de UCB uitscheiding in de gal of de fecale urobilinoïden uitscheiding. De onderliggende mechanismen van het hypobilirubinemische effect van deze galzouten zijn momenteel onbekend. Het is onwaarschijnlijk dat behandeling met galzouten de UCB productie verandert. Het is eveneens onwaarschijnlijk dat galzouten microsomale oxidatie (afbraak) in de lever bevorderen aangezien de levergewichten niet waren toegenomen en de Cyp1a2 expressie niet was verhoogd. Echter, verminderde UCB productie of toegenomen UCB oxidatie kunnen momenteel niet worden uitgesloten. Een mogelijk mechanisme van UDCA zou kunnen zijn dat het interfereert met de oplosbaarheid van UCB in de darm en het daardoor de enterohepatische kringloop van UCB vermindert. Een andere verklaring zou een verandering in de bacteriële flora door galzouten kunnen zijn die leidt tot een verhoogd metabolisme tot UCB-metabolieten. Wij hebben geconcludeerd dat orale suppletie met UDCA of CA de plasma UCB concentratie bij Gunn ratten verlaagt door een vooralsnog onopgehelderd mechanisme dat onafhankelijk is van stimulatie van UCB uitscheiding in de gal of stimulatie van de fecale vetuitscheiding. Het is waarschijnlijk dat UDCA en CA de plasma UCB

concentraties via een verschillend mechanisme verlagen gezien het verschil in hydrofiliciteit en de snellere repons op UDCA behandeling. Aanvullende experimenten met ^{3}H-UCB zijn gepland om te bepalen of de fractionele turnover in UDCA en CA behandelde ratten inderdaad verschilt. Gedetailleerde gegevens over UCB productie, pool grootte en de enterohepatische kringloop van UCB en UCB-metabolieten zouden mogelijk de onderliggende mechanismen van het hypobilirubinemische effect van galzouten kunnen onthullen.

Conclusies en toekomstperspectieven

De conventionele behandeling van ongeconjugeerde hyperbilirubinemie is fototherapie. Ons doel was een alternatieve of additionele behandeling te ontwikkelen die is gebaseerd op orale toediening. Onze behandelstrategie was gebaseerd op "intestinal capture" van UCB, wat de enterohepatische kringloop van UCB vermindert en vervolgens de plasma UCB concentraties. Wij hypothetiseerden dat vet gebruikt zou kunnen worden voor "intestinal capture" van UCB. In dit proefschrift hebben wij aangetoond dat stimulatie van de fecale vetuitscheiding de plasma UCB concentraties verlaagt in Gunn ratten en in een subgroep van patiënten met de ziekte van Crigler-Najjar. In Gunn ratten verlaagde orlistat behandeling plasma UCB concentraties en bilirubine pool grootte, vooral in combinatie met fototherapie. Met ^{3}H-UCB kinetiek gegevens werd bepaald dat orlistat behandeling de fractionele turnover and fecale uitscheiding van UCB verhoogt, netto uitscheiding van UCB vanuit het bloed richting darmlumen induceert en het metabolisme van UCB tot UCB-metabolieten verhoogt. Onze huidige gegevens tonen niet rechtstreeks aan dat UCB associeert met (delen van) niet opgenomen vet in de darm. Theoretisch zou UCB kunnen associëren met gedeeltelijk gesplitste triglyceriden, vrije vetzuren of fosfolipiden. Naast het effect op de vetopname zou orlistat een (in)direct effect kunnen hebben op een tot dusver onbekende parameter die vervolgens de UCB uitscheiding beïnvloedt. De oplosbaarheid van UCB door galzouten zou kunnen veranderen in een veranderde lipofiele omgeving. *In vitro* experimenten zijn nodig om het exacte mechanisme hiervan te verhelderen.

Zoals hierboven besproken, verlaagde orlistat behandeling de plasma UCB concentraties bij patiënten met de ziekte van Crigler-Najjar, maar waren de effecten relatief beperkt in de groep van 16 patiënten als geheel. Echter, een klinisch relevante repons was aanwezig in een subgroep van patiënten. Een relatief lage voedingsvet inname correleerde met de respons op behandeling. Op dit moment kan orlistat niet worden aanbevolen als algemene behandeling voor de ziekte van Crigler-Najjar. Eerst zouden wij patiënt karakteristieken of andere relevante parameters willen identificeren die kunnen voorspellen welke patiënten baat zouden kunnen hebben bij orlistat behandeling.

Orale UDCA of CA suppletie verlaagde plasma UCB concentraties in Gunn ratten via een vooralsnog onopgehelderd mechanisme dat onafhankelijk was van verhoging van de uitscheiding van UCB in de gal of stimulatie van de fecale vetuitscheiding. UDCA wordt

wereldwijd gebruikt bij de behandeling van cholestatische leveraandoeningen, zonder serieuze bijwerkingen.[27;28] Opheldering van het onderliggende mechanisme van het UDCA effect zou een belangrijke stap kunnen zijn in de richting van een nieuwe behandeloptie voor patiënten met ongeconjugeerde hyperbilirubinemie.

In dit proefschrift hebben we diverse strategieën voor orale behandeling van ongeconjugeerde hyperbilirubinemie onderzocht. Ontwikkelen van een alternatieve of additieve behandeling zou de behoefte aan fototherapie of wisseltransfusies kunnen verminderen. Dit is niet alleen van belang voor patiënten met de ziekte van Crigler-Najjar voor wie dagelijkse fototherapie noodzakelijk is. Onder strikte voorwaarden zouden ook pasgeborenen met geelzucht theoretisch gezien in aanmerking kunnen komen voor orale (thuis) behandeling in plaats van fototherapie. Een orale behandeling zou mogelijk beter dan fototherapie geïmplementeerd kunnen worden in delen van de wereld waar fototherapie nog niet gemakkelijk beschikbaar is. De huidige studies geven aan dat orale behandeling van ongeconjugeerde hyperbilirubinemie haalbaar lijkt en dat andere strategieën dan “intestinal capture” verwachtingen scheppen voor de toekomst. Wij zijn van mening dat er voor het beoordelen van de klinische toepasbaarheid van orale behandeling de volgende stappen zouden moeten worden gezet: *in vitro* studies naar de associatie van UCB met vet en galzouten, kinetiek studies om de *in vivo* mechanismen te demonstreren, studies die het effect bestuderen van orlistat of UDCA op de samenstelling van de darmflora, en een klinisch onderzoek met orlistat of UDCA bij pasgeborenen met geelzucht.

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In maart 2001 begon ik als grasgroene arts-onderzoeker aan het avontuur op het researchlab Kindergeneeskunde. Inmiddels ben ik wat minder groen op researchgebied; het groen is geel geworden. Door mijn onderzoek zal ik de kleur geel voortaan associëren met bilirubine en vanzelfsprekend is mijn proefschrift dan ook geel geworden. Onderzoek doen was (en blijft) erg leerzaam, leuk en uitdagend, maar soms ook frustrerend. Voor elke onderzoeksvraag die beantwoord werd kreeg ik er drie nieuwe terug. Dit proefschrift markeert het eind van een geweldige periode, maar is eigenlijk slechts een begin, onderzoek stopt nooit.

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Don Ostrow and Claudio Tiribelli, dear “Fellows of the Yellow”, with your help chapter five of this thesis became the chapter I am most proud of. Studying ^{3}H-UCB kinetics was difficult and the experiments were time consuming, but your expertise and encouragement made it much easier. I thoroughly enjoyed working with you and spending time together in Trieste. Hopefully we will meet again soon!

Dr. Sinaasappel, beste Maarten, met plezier kijk ik terug op de Crigler-Najjar patiëntenstudie, ons frequente overleg, de wetenschappelijke avonden en de informele bijeenkomsten. De patiënten missen straks een zeer betrokken kinderarts. Beste patiënten en ouders van patiënten, zonder jullie geweldige inzet en doorzettingsvermogen was het orlistat onderzoek niet gelukt. Hoofdstuk 6 van dit proefschrift is te danken aan jullie! Helaas zijn de

resultaten van de studie misschien niet wat we op basis van het voorafgaande proefdieronderzoek hadden verwacht, maar dit onderzoek heeft desondanks belangrijke resultaten opgeleverd. Heel hartelijk dank voor jullie vertrouwen en geduld. Ik hoop jullie in de toekomst nog eens te zien op een Crigler-Najjar Buitendag of op een wetenschappelijke avond in Rotterdam. Ik ben eveneens veel dank verschuldigd aan het bestuur van de Najjar Stichting voor de financiering van mijn aanstelling, de betrokkenheid bij mijn onderzoek en de uitnodigingen voor de Buitendagen. Rineke, ik weet niet hoe ik de patiëntenstudie zonder jou had moeten doen. Ik denk nog vaak aan ons eindeloze regelwerk, aan de vele kilometers en lange dagen die we samen gemaakt hebben en aan de gezellige ritjes naar Brabant of België op de vroege ochtend. Dank je wel! Hartelijk dank ook aan alle anderen die hebben bijgedragen aan de patiëntenstudie, in het bijzonder Prof. dr. J.H.P. Wilson, Dr. P. Goyens, M. Van Eygen en de mensen van het lab in Rotterdam.

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vragen hopelijk door jou beantwoord gaan worden. Er komen vast een paar hele interessante artikelen uit voort. Veel succes, ik zal een paar knallers bewaren voor jouw promotie.

Naast de nodige uurtjes op het lab Kindergeneeskunde heb ik heel veel tijd doorgebracht "in" de zuurkasten op het lab van Henk B. Hartelijk dank dat ik van de faciliteiten daar gebruik mocht maken. Eén collega in het bijzonder verdient veel lof. Herman ("carpe diem") jij hebt mij (met Pieter van L. toen ik net begon) veel geleerd over bilirubine, over de bepalingen, de HPLC techniek, de bilikolom en het "gevoel" krijgen voor "mijn" HPLC, een oud maar zeer betrouwbaar apparaat. Ik kon altijd met vragen bij je terecht, of bij Marchien, Enge, Jasper, Ingrid of een van de andere collega's, allemaal bedankt! Ingrid, naast je drukke werkzaamheden was je altijd bereid om mij te helpen bij het ontcijferen van mijn vetzuurpiekjes en om het computerprogrammaleed te delen; dank voor de leuke gesprekken! Mensen van het lab op de 1^{e} verdieping, dank dat jullie op de meest onmogelijke tijden bereid waren om mijn rattenmonsters te analyseren.

Na 4 jaar onderzoek te hebben gedaan was het erg fijn om weer in de kliniek te werken. Collega arts-assistenten en kinderartsen, met name supervisor Gerbrich en de oncologen en cardiologen op afdeling M2, jullie hebben de overgang van onderzoek naar kliniek een stuk makkelijker gemaakt. Dank voor de morele steun en de belangstelling voor mijn onderzoek. Onderzoek doen is erg leuk (sommige arts-assistenten zijn daarvan moeilijk te overtuigen), maar op een afdeling onderdeel uitmaken van teamzorg voor een ziek kind heeft mijn voorkeur. Ik hoop kliniek en onderzoek ooit nog eens te kunnen combineren. Eerst maar eens genieten van wat meer vrije tijd en beginnen aan de stapel ongelezen boeken.

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Anja

CURRICULUM VITAE

Anja M. Hafkamp werd op 3 mei 1973 geboren te Raalte. Na het behalen van haar VWO diploma aan het Florens Radewijns College te Raalte werd zij in 1991 in eerste instantie uitgeloot voor de studie Geneeskunde. Na enkele weken werd zij gelukkig nageplaatst aan de Vrije Universiteit in Amsterdam, waar zij in 1996 cum laude haar doctoraalexamen behaalde. Tijdens haar studie werd de basis voor wetenschappelijk onderzoek gelegd tijdens haar wetenschappelijke stage bij de Neonatal Unit van het John Radcliffe Hospital te Oxford, Engeland. Daar onderzocht zij gedurende 6 maanden onder begeleiding van Prof. dr. A.R. Wilkinson en Prof. dr. H.N. Lafeber de prognose van aangeboren hartafwijkingen bij prematuur versus a term geboren kinderen. In 1998 behaalde zij cum laude het artsdiploma. Na ruim 2 jaar werkzaam te zijn geweest als arts-assistent Kindergeneeskunde in Haarlem en Enschede werd zij in 2001 aangesteld als arts-onderzoeker bij de vakgroep Kindergeneeskunde van de Beatrix Kinderkliniek van het Universitair Medisch Centrum Groningen. Onder begeleiding van haar promotores Prof. dr. H.J. Verkade en Prof. dr. P.J.J. Sauer (opleider) werkte zij aan het in dit proefschrift beschreven promotieonderzoek naar de orale behandeling van ongeconjugeerde hyperbilirubinemie. Sinds april 2005 is zij in opleiding tot kinderarts in de Beatrix Kinderkliniek van het UMCG.

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