Chapter 2

Pharmacological Therapies for Unconjugated Hyperbilirubinemia

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Adapted from: Current Pharmaceutical Design 2009;**15**:2927-38

2.1 Abstract

Severe unconjugated hyperbilirubinemia, seen mainly in neonates, may cause kernicterus and death. Conventional treatment for severe unconjugated hyperbilirubinemia consists of phototherapy and exchange transfusion. Phototherapy, however, has several known disadvantages while exchange transfusion is associated with a significant morbidity, and even mortality. These harmful effects indicate the need to develop alternative pharmacological treatment strategies for unconjugated hyperbilirubinemia. Generally, these strategies aim to decrease the plasma concentration of unconjugated bilirubin (UCB) by inhibiting production, stimulating hepatic clearance, or interrupting the enterohepatic circulation of the pigment.

To be considered for routine clinical use, an alternative treatment strategy should be less invasive and at least as effective and safe as phototherapy. Several pharmacological therapies such as metalloporhyrins, clofibrate, bile salts, laxatives and bilirubin oxidase may meet these criteria in the future, but none of them has yet been evaluated sufficiently to allow routine application. This chapter aims to discuss the state of the art and future perspectives in pharmacological treatment of neonatal jaundice.

2.2 Introduction

Unconjugated bilirubin (UCB) is produced by the degradation of heme, derived mainly from the catabolism of erythrocyte hemoglobin. After its production, the hydrophobic UCB is bound to albumin in the plasma and transported to the liver. In the liver, the enzyme bilirubin-UDP-glucuronosyltransferase (UGT1A1) conjugates UCB with glucuronic acid. The relatively hydrophilic bilirubin conjugates are readily excreted into the bile and transit the biliary tree to the intestine. In the intestinal lumen, the bilirubin conjugates are partly hydrolyzed to UCB, which can be reabsorbed from the intestine, reduced to urobilinoids, or excreted with the feces (Fig. 1). Reabsorbed UCB is transported to the liver via the vena porta, where it again can be conjugated and excreted *via* the bile, thus constituting an enterohepatic circulation.

Unconjugated hyperbilirubinemia, the accumulation of UCB in the body, can result from increased production, decreased hepatic clearance, or enhanced enterohepatic circulation of the pigment (Fig. 1). This is illustrated by the transient unconjugated hyperbilirubinemia during the first postnatal week. This so-called physiological jaundice is due to a combination of high erythrocyte turnover (increased UCB production),[1] immature hepatic conjugation of the pigment (decreased hepatic clearance),[2] and a delayed intestinal transit (enhanced enterohepatic circulation of UCB).[3,4] For the majority of neonates, unconjugated hyperbilirubinemia is a benign transitional phenomenon that may be beneficial, due to the potent antioxidant properties of the pigment.[5] Under specific conditions, however, plasma UCB concentrations can rise to hazardous levels. This occurs for example in patients with neonatal hemolytic disease, due to excessive UCB production, or in patients with inherited deficiencies of UCB conjugation,[6,7] due to a severely decreased biliary excretion of the pigment. Crigler-Najjar disease, the most serious of these inherited deficiencies, is characterized by a genetically absent (type I) or decreased (type II) activity of UGT1A1.[6] With severely deficient conjugation, the excretion of bilirubin into the bile is diminished, resulting in a lifelong unconjugated hyperbilirubinemia in Crigler-Najjar patients and in Gunn rats, the animal model for this disease.

The harm from severe unconjugated hyperbilirubinemia is due to the potential deposition of UCB in the central nervous system, causing bilirubin-induced neurological dysfunction (BIND), kernicterus, and death.[8] The conventional treatment for unconjugated hyperbilirubinemia is phototherapy. Phototherapy induces photo-isomerization of the hydrophobic UCB to polar isomers that can readily be excreted into the bile without conjugation.[9] Although generally effective, phototherapy has several disadvantages. Most notably, short-term phototherapy does not always decrease plasma UCB to non-toxic levels in

neonates, whereas long-term phototherapy, such as needed for patients with Crigler-Najjar disease type I, becomes less effective with age and has a profound impact on social life.[10,11] Under conditions of very severe unconjugated hyperbilirubinemia or of hyperbilirubinemia with an insufficient response to phototherapy a "rescue" treatment consists of exchange transfusion, in which the hyperbilirubinemic blood is removed and replaced with non-jaundiced blood. Exchange transfusion, however, has a considerable morbidity, especially in sick preterm newborns, and even mortality has been reported.[12]

The potential neurotoxicity of unconjugated hyperbilirubinemia and the disadvantages of present treatments have prompted investigation of alternative treatment strategies. These strategies have concentrated on pharmacological therapies that decreased the production, increased the biliary excretion, and/or interrupted the enterohepatic circulation of UCB. Recently, several excellent reviews have appeared that discuss the clinically available therapies for unconjugated hyperbilirubinemia. Interestingly, and in spite of a large body of experimental and clinical studies, only one systematic review of proven and experimental pharmaceutical treatment strategies has been published recently.[13] In the present review, we critically discuss the background, effectiveness, applicability, and future perspectives of pharmacological treatment options for severe unconjugated hyperbilirubinemia.

2.3 Treatment strategies

2.3.1 Treatments that decrease the production of UCB

At the end of their life span, red blood cells are degraded in the reticuloendothelial system (RES), mainly located in the liver, spleen, and bone marrow. In the RES, the released heme is phagocytized by macrophages. These macrophages contain two essential enzymes for heme degradation: heme oxygenase (HO) and biliverdin reductase. The microsomal HO catalyzes the conversion of heme into the blue-green biliverdin, the first step in heme degradation. The cytosolic biliverdin reductase then converts biliverdin to the yellow-orange UCB (Fig. 2A). Heme and biliverdin are water-soluble, non-toxic compounds. It is believed they are converted into the water-insoluble, potentially toxic UCB because of its anti-oxidative properties and ability to cross the placenta.[5,14]

Decreasing UCB production is a rational approach to treat unconjugated hyperbilirubinemia. This strategy has the benefit of actually *preventing* UCB

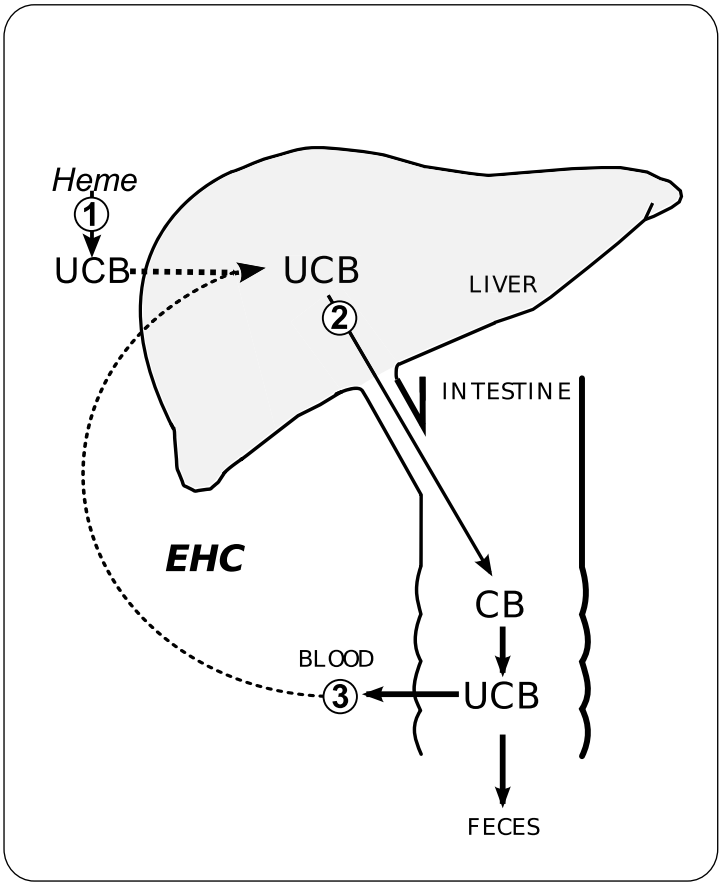

Figure 1. Unconjugated hyperbilirubinemia can result from increased production ①,(decreased hepatic clearance ②, or enhanced enterohepatic circulation of UCB ③. After its production from heme, the hydrophobic UCB is transported to the liver. In the liver, the enzyme bilirubin-UDP-glucuronosyltransferase conjugates UCB with glucuronic acid. The relatively hydrophilic conjugated bilirubin (CB) is readily excreted *via* the bile into the intestine. In the intestinal lumen, CB is partly hydrolyzed to UCB, which can be reabsorbed from the intestine, reduced to urobilinoids (not shown), or excreted with the feces. Reabsorbed UCB is transported back to the liver *via* the vena porta (the enterohepatic circulation; EHC).

accumulation, rather than removing already accumulated UCB from the body. UCB production can be decreased *via* inhibition of HO activity with metalloporphyrins or D-penicillamine (Fig. 2B). Although HO inhibition effectively blocks heme catabolism, the efficient biliary excretion of heme prevents its accumulation in the body.[15] In theory, inhibition of biliverdin reductase could also decrease UCB production. However, inhibitors of biliverdin have not been explored, most likely because their use in neonates would produce green babies.

Metalloporphyrins

Metalloporhyrins are synthetic heme analogues, in which other metals such as zinc (Zn), tin (Sn), and chromium (Cr) replace the central iron atom of heme.[16] Metalloporphyrins competitively inhibit HO, and thus decrease heme degradation and UCB production (Fig. 2B).[17] Of the metalloporphyrins selected from the early *in vitro* studies, only tin, zinc, manganese, and chromium competitively inhibited HO *in vivo*,[18] but zinc and chromium were excluded from human use due to detrimental tissue effects.[19-21] Also, zinc-, chromium-, and manganese- protoporphyrins inhibited heme oxygenase, but induced biliverdin reductase during chronic administration in newborn rats.[22]

Clinical research eventually focused on Sn-mesoporphyrin (SnMP) due to its efficacy and its safety profile.[23-27] Kappas *et al.* reported that preventive SnMP treatment mitigated the development of unconjugated hyperbilirubinemia in 53 infants with a positive Coombs reaction caused by ABO incompatibility, compared with 69 Coombs positive, ABO incompatible infants that did not receive SnMP prophylaxis.[23] Valaes *et al.* published the results of 5 clinical trials in a total of 517 pre-term infants.[24] This paper showed that a single prophylactic dose of 1-6 μmol/kg SnMP, administered within 24 hours after birth, ameliorated unconjugated hyperbilirubinemia dose-dependently. The maximal dose of 6 μmol/kg reduced the peak plasma bilirubin concentration by 41% and the requirement for phototherapy by 76%. In the most recent clinical trial, the therapeutic use of SnMP (6 μmol/kg) in 40 term hyperbilirubinemic infants shortened the length of hyperbilirubinemia and eliminated the need for phototherapy.[27] In a trial with newborns with glucose-6-phosphate dehydrogenase deficiency, SnMP was administered either in the first day of life (preventive use), or therapeutically after a threshold of plasma bilirubin was reached.[26] Although the bilirubin levels were not particularly elevated in any group, SnMP supplanted the need for phototherapy in both the preventive and the therapeutic group. SnMP has also been used in the management of Crigler-Najjar disease. Regular SnMP treatment in Crigler-Najjar type I patients initially

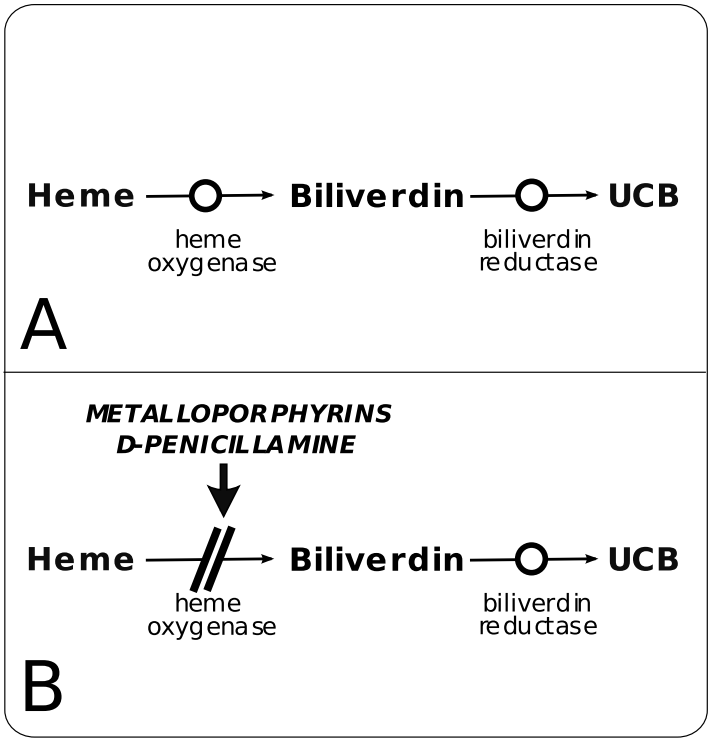

Figure 2. Decreasing UCB production is a potential strategy to treat unconjugated hyperbilirubinemia. At the end of their lifespan, red blood cells are degraded in the reticulo-endothelial system (RES). In the RES, the released heme is phagocytized by macrophages. These macrophages contain two essential enzymes for heme degradation: heme oxygenase and biliverdin reductase. The microsomal heme oxygenase catalyzes the conversion of heme into biliverdin, the first step in heme degradation. The cytosolic biliverdin reductase then converts biliverdin to UCB (A). Metalloporphyrins and D-penicillamine block the conversion of heme into biliverdin, thus decreasing UCB production (B).

decreased plasma bilirubin levels, but the effect was temporary for unknown reasons.[28,29]

Although promising, metalloporphyrins are currently not recommended for routine treatment in newborns due to insufficient evidence and unknown long-term safety.[30] The only known side effect of SnMP treatment that has been observed so far consists of a mild transient erythema due to photosensitivity.[23,24] However, some concerns have risen regarding potential adverse-effects due to the suppression of the cytoprotective effects of HO, especially in critically ill infants,[31,32] and none of the metalloporphyrins are so far available for oral administration. Therefore, the results of further clinical trials regarding safety and efficacy need to be awaited before routine clinical use can be recommended.

D-penicillamine

D-penicillamine is a chelating agent, routinely used for the treatment of Wilson's disease based on its stimulation of renal copper excretion. However, D-penicillamine also inhibits HO-activity (Fig. 2B) and has been applied for the treatment of unconjugated hyperbilirubinemia.[33] Lakatos *et al.* published the only clinical trial with D-penicillamine in 1976, which involved 120 full-term infants with ABO-hemolytic disease.[34] In this study, patients were randomized to receive D-penicillamine either within the first 24 hours, or after the third day of life. D-penicillamine decreased plasma bilirubin concentrations by ~16% and decreased the number of exchange transfusions by 91%, but only in the group treated within 24 hours.

Because D-penicillamine is a nitric oxide (NO) donor, any adverse effect related to NO, such as vasodilatation and decreased platelet aggregation, could potentially occur during treatment. Furthermore, chronic D-penicillamine use in adults is associated with serious adverse effects, such as aplastic anemia, Goodpasture's disease, and myasthenia gravis.[35] Therefore, additional clinical trials will be necessary to evaluate whether short-term administration of D-penicillamine would be an effective and safe treatment for unconjugated hyperbilirubinemia in neonates.

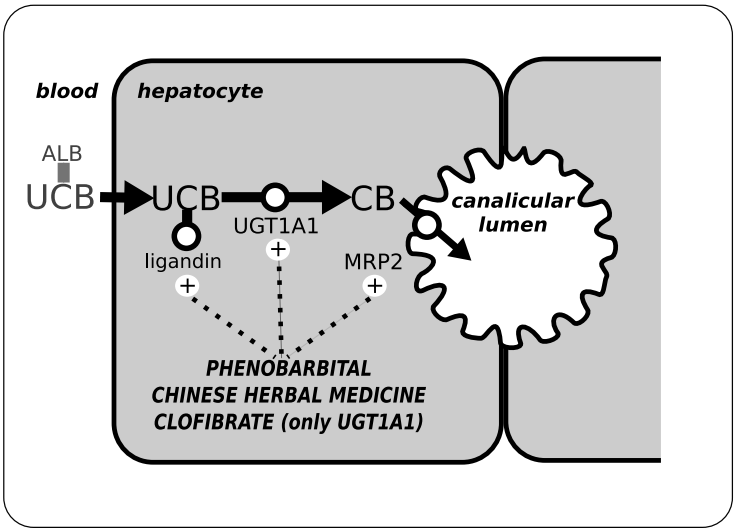

Figure 3. Increasing the hepatic UCB clearance is a potential strategy to lower plasma UCB levels. Phenobarbital, Chinese Herbal Medicine, and Clofibrate achieve this by enhancing the enzymatic steps in hepatic UCB clearance: hepatic uptake and storage *via* ligandin, hepatic conjugation *via* UGT1A1, and secretion of the bilirubin conjugates into the bile via MRP2. Alb-UCB, albumin-bound bilirubin.

2.3.2 Treatments that increase the hepatic clearance of UCB

After production in the RES, the hydrophobic UCB is transported to the liver, predominantly bound to albumin (Fig. 3). In the liver, the UCB-albumin complex enters the space of Disse *via* the endothelial fenestrae. There, UCB is dissociated from albumin and crosses the basolateral membrane *via* facilitated diffusion, involving transporters that have yet to be identified. Once in the hepatocyte, UCB is bound to ligandin (gluthathione S-transferase; Y-protein) in the cytosol.[36,37] This protein binds UCB with an affinity similar to plasma albumin and thus prevents it reflux into the space of Disse.[38] After diffusion into the cisterna of the endoplasmic reticulum, the hydrophobic UCB is conjugated with one or two glucuronic acids by the microsomal enzyme UGT1A1. Absence of UGT1A1 activity in Crigler-Najjar disease results in a permanent unconjugated hyperbilirubinemia.[6] Finally, bilirubin conjugates are efficiently excreted into the bile *via* the canalicular ATP-dependent transporter MRP2 (Fig. 3).[39] The nuclear receptor CAR (constitutive androstane receptor) is a key regulator of UCB clearance, since it enhances the activity of ligandin, UGT1A1, and MRP2.[40]

Increasing the hepatic clearance of bilirubin is a potential strategy to lower plasma UCB levels. This can be achieved by enhancing the three steps in hepatic UCB clearance that are all underactive in human neonates: (1) hepatic uptake and storage *via* ligandin, (2) hepatic conjugation *via* UGT1A1, and (3) biliary excretion of conjugated bilirubin *via* MRP2 (Fig. 3). Below we will discuss the several pharmacological treatments that enhance the biliary excretion of bilirubin by one or more of these mechanisms.

Phenobarbital

Phenobarbital, an anti-epileptic drug, is a CAR agonist that enhances the three steps in hepatic UCB clearance; uptake and storage in the liver, hepatic conjugation, and hepatic excretion of bilirubin. *Net* uptake and storage is enhanced *via* an increased concentration of ligandin, conjugation is enhanced *via* induction of UGT1A1, and biliary secretion is enhanced *via* induction of Mrp2 (Fig. 3).[40-42]

Phenobarbital has been used to treat neonatal jaundice since the 1960s. Numerous clinical trials have shown that both administration of phenobarbital to pregnant mothers before delivery and phenobarbital administration to neonates after delivery limits the severity of unconjugated hyperbilirubinemia and the need for exchange transfusion (for a complete review [43]). In one of the largest clinical trials, daily administration of 1 g phenobarbital to 310 mothers in the last week of pregnancy decreased the incidence of severe hyperbilirubinemia (bilirubin > 274 μmol/l) and lowered the need for exchange transfusion by 85%.[44]

Nonetheless, phenobarbital is not routinely used to treat neonatal unconjugated hyperbilirubinemia due to several reasons.[45] First, phototherapy is more effective compared with either phenobarbital alone or with phenobarbitone combined with phototherapy.[46] In addition, the bilirubin lowering effect of phenobarbital is not evident until a few days after administration, in contrast to some of the adverse effects, *i.e.* sedation of the newborn infant.[43] Furthermore, phenobarbital diminishes the oxidative metabolism of UCB in the rat brain, which could lead to an increased neurotoxicity of the pigment.[47] Phenobarbital is useful to distinguish between type I and type II Crigler-Najjar disease. Phenobarbital is not effective in patients with Crigler-Najjar type I because there is no UGT1A1 to induce, and conjugation is essential for the biliary excretion of UCB. In patients with Crigler-Najjar type II disease, who have about 5% of normal baseline UGT1A1 activity, phenobarbital treatment reduces plasma UCB concentrations by 30% or more.[48]

Clofibrate

Clofibrate, an activator of peroxisome proliferator-activated receptors (PPARS), is a lipid-lowering drug used in patients with hypercholesterolemia or hyperlipoproteinemia.[49] Clofibrate treatment also increases the hepatic conjugation of UCB *via* induction of UGT1A1 (Fig. 3).[50,51] Clofibrate treatment in Sprague-Dawley rats increased Ugt1A1 activity and resulted in an 84% increase in the hepatic clearance of IV-administered UCB.[50,52]

In 1981 Lindenbaum *et al.* published the first randomized placebo-controlled trial with clofibrate, involving 93 full-term neonates with physiological jaundice. Of these 93 neonates, 47 received one preventive oral dose of clofibrate, which significantly lowered plasma UCB levels from the 16th hour after administration and curtailed the duration of jaundice.[53] A similar study in 89 preterm neonates showed comparable results, including indications for a dose-response relationship: the hypobilirubinemic effect appeared to correlate with the plasma concentration of clofibric acid.[54] Three randomized controlled trials, each involving 60 full-term neonates, compared the use of clofibrate and phototherapy with the use of phototherapy alone.[55-57] Clofibrate, as add-on treatment, accelerated the decrease in serum total bilirubin concentrations and decreased the duration of phototherapy in all of these trials. The clinical utility of these results, however, can be disputed based on the methodology: enrollment in these studies was generally 5-8 days postpartum, and therefore after the period in which the peak of neonatal jaundice would normally occur.

Long-term clofibrate treatment has been associated with serious adverse effects. In animals, clofibrate treatment is carcinogenic.[58] Although this effect has not been confirmed in adult humans, it is not known whether long-term carcinogenesis could occur after neonatal treatment.[59] Human clofibrate treatment for hypercholesterolemia or hyperlipoproteinemia has also been associated with an overall increase in non-cardiovascular mortality.[59] In theory, the clofibric acid metabolite p-chlorophenoxyisobutyric acid could displace UCB from albumin, but this has not been confirmed *in vitro*.[60] Other known side effects of clofibrate include vomiting, diarrhea, increased incidence of gallstones, and muscular myopathy.[61,62] None of these side effects have been reported in the short-term studies in infants described above. Nevertheless, long-term safety issues need to be clarified before its clinical use can be considered, despite the indication that clofibrate is an effective (add-on) therapy for unconjugated hyperbilirubinemia.

Traditional herbal medicine

Various herbs are used in the treatment of neonatal jaundice in traditional medicine throughout Asia. One of the most commonly used concoctions is Yin Zhi Huang, a mixture of four different plant extracts.[63] By now, several studies have elucidated the mechanism underlying its hypobilirubinemic effect (Fig. 3). Administration of Yin Zhi Huang to rats increased the hepatic clearance of IV-administered UCB and induced the enzyme activity of ligandin and Ugt1A1.[64] Yin Zhi Huang seems to act as a CAR agonist, since treatment induced the expression of ligandin, Ugt1A1, and Mrp2 in normal, but not in CAR-knockout mice.[65] Coumarin 6,7-dimethylesculetin (Scoparone), a component of Artesemia capillaris (Yin Chin), which is one of the four plant extracts in Yin Zhi Huang, appeared to be the largest contributor to the effect of Yin Zhi Huang.[65]

The clinical efficacy of Yin Zhi Huang for unconjugated hyperbilirubinemia has not been tested in randomized, controlled clinical trials. Also, there is a legitimate tendency to develop treatments based on individual molecules, rather than mixtures, to guarantee reproducibility and minimize the risk of side effects. Indeed, some herbal concoctions have been able to displace bilirubin from albumin, thereby increasing the concentration of unbound unconjugated bilirubin.[66] Contamination of herbal medicines with heavy metals, *i.e.* lead and mercury, has been reported.[67] Finally, Yin Zhi Huang treatment is associated with hemolysis in glucose-6-phosphate dehydrogenase-deficient patients.[63] Therefore, clinical use of traditional herbal medicine during neonatal jaundice is not recommended.

2.3.3 Treatments that interrupt UCB's enterohepatic circulation

In the intestine, conjugated bilirubin is extensively hydrolyzed by β-glucuronidases to UCB, which can be converted to urobilinoids or can be reabsorbed into the enterohepatic circulation (Fig. 4A).[68,69] This reabsorption is especially important in human newborns, which do not develop the anaerobic colonic microflora that converts UCB to urobilinoids until 2-6 weeks postnatal.[70] Reabsorption of UCB from the intestinal lumen seems to occur, at least in part, *via* a passive mode of transport.[71] Intestinal accumulation of UCB, for instance during a delayed passage of meconium or during starvation,[4,72] increases its intestinal reabsorption into the portal venous blood.[3] Because the liver extracts only 30% of the UCB load, this will directly contribute to the pathogenesis of unconjugated hyperbilirubinemia.[73] However, if the plasma UCB concentrations are very high, the concentration gradient will favor UCB diffusion from the blood into the intestinal lumen.[74,75] In Gunn rats, the well-

established animal model for Crigler-Najjar disease type 1, the majority of the total bilirubin disposal occurred *via* this excretory pathway.[74,75]

The transport of UCB across the intestinal membrane thus seems to be predominantly passive, bi-directional, and driven by the concentration gradient between (unbound) plasma UCB and UCB in the intestinal lumen. Theoretically, capturing UCB in the intestinal lumen could enhance the diffusion of UCB from the blood and limit its reabsorption into the enterohepatic circulation. Several capturing strategies that influence the gradient towards *net* excretion have been tested. (Fig. 4B). Some involve oral administration of insoluble, poorly absorbable solids, or of detergents, that bind UCB. Others work by decreasing the intestinal absorption of endogenous compounds that bind bilirubin. Alternatively, enhancing the transit and fecal elimination of intestinal contents, for example by frequent feeding or laxatives, can also be applied to decrease plasma UCB concentrations (Fig. 4C). Below, we discuss the effectiveness, applicability and future perspectives of the several classes of agents that diminish the enterohepatic circulation of UCB.

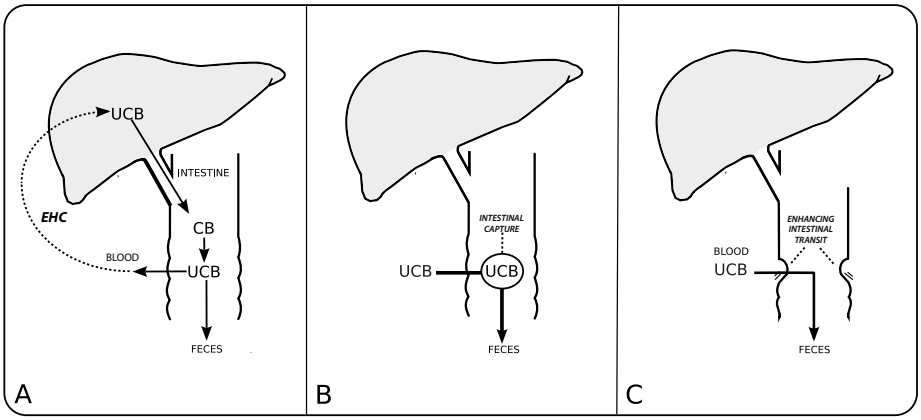

Figure 4. Interruption of the enterohepatic circulation of UCB decreases plasma UCB levels. In the intestinal lumen, conjugated bilirubin (CB) is partly hydrolyzed to UCB, which can be reabsorbed from the intestine. Reabsorbed UCB is transported back to the liver via the vena porta. In the liver, this UCB is re-conjugated and excreted via the bile, thus constituting an enterohepatic circulation (EHC) (A). Reabsorption occurs via a passive mode of transport and is, consequently, bi-directional and driven by the UCB concentration gradient between the plasma and the intestinal lumen. Capturing UCB in the lumen with cholestyramine, charcoal, agar, calcium, zinc, and fat limits intestinal reabsorption of the pigment. This induces a shift of UCB from the blood into the intestinal lumen, from where UCB can then be excreted with the feces (B). Accelerating intestinal transit by frequent meals may result in a lower intestinal UCB concentration, which also enhances UCB diffusion from the blood into the intestinal lumen (C).

Cholestyramine

Cholestyramine is an insoluble, quaternary ammonium compound and anion exchanger, known to bind bile salts in the small intestine. In 1962 Lester *et al.* hypothesized that cholestyramine could lower plasma UCB levels by binding the UCB in the intestinal lumen, thus preventing its reabsorption (Fig. 4B). To prove this hypothesis, four adult Gunn rats were supplemented with dietary cholestyramine, which resulted in a 30-45% drop in plasma UCB levels.[76] Enteral administration of cholestyramine to jaundiced preterm infants, however, did not significantly decrease plasma bilirubin levels compared with controls.[77] Schmid *et al.* speculated that the discrepancy between animal and human data could be due to a higher affinity between UCB and albumin in the human blood, compared with Gunn rats. This could limit the diffusion of UCB from the blood into the intestinal lumen.[77] Subsequent trials have reported either for,[78-80] or against [81] the effectiveness of cholestyramine as add-on treatment to phototherapy. Side effects of cholestyramine therapy include: hyperchloremic metabolic acidosis, constipation, and diarrhea.[77,79-81] Because its safety and effectiveness remain questionable, treatment of unconjugated hyperbilirubinemia with cholestyramine is not recommended.

Charcoal

In 1964, Ulstrom *et al.* observed that activated charcoal removed bilirubin from human bile more avidly than did cholestyramine.[82] In that same year, Künzer *et al.* independently showed that activated charcoal removed nearly all the bilirubin content from human duodenal fluids.[83] Both groups hypothesized that activated charcoal could trap bilirubin in the intestinal tract, prevent its enterohepatic circulation and decrease plasma bilirubin levels in neonates (Fig. 4B). To investigate this hypothesis, Ulstrom *et al.* administered 11-15 doses of activated charcoal in 30 full-term neonates. Treatment started either 4 hours (n=15) or 12 hours (n=15) after birth. Interestingly, UCB levels decreased, but only in the group that received the first charcoal administration at 4 hours postpartum.[82] Künzer *et al.* daily administered activated charcoal from the second day after birth in 25 premature neonates, but found no effect on plasma bilirubin levels.[83] Ulstrom *et al.* concluded that charcoal should be administered soon after birth, possibly because it thus could prevent the intestinal reabsorption of meconial UCB.[82] After these initial reports, the effect of oral charcoal on plasma bilirubin has been investigated in three Gunn rat studies and in one prospective study involving jaundiced newborns.[84-86] In Gunn rats, activated charcoal decreased plasma unconjugated bilirubin levels as effectively as phototherapy.[84-86] In jaundiced neonates, charcoal treatment, started within the first day of life, combined with phototherapy decreased plasma bilirubin levels more effectively compared with phototherapy alone.[87]

Side effects of activated charcoal are relatively rare, but include vomiting and dehydration.[84,88] Although activated charcoal seems to have some merit in the treatment of neonatal unconjugated hyperbilirubinemia, more research regarding its safety and effectiveness (*e.g.* timing of postnatal administration) is necessary before clinical use can be considered. Also, it is unclear whether binding by charcoal limits the intestinal absorption of essential nutrients and other potentially beneficial compounds.

Agar

Agar is a gelatinous substance that is derived from seaweed. In the 1970s, Poland *et al.* showed that UCB binds to dried agar and that agar thus could act as a trapping agent for the UCB in the intestinal lumen (Fig. 4B).[89,90] In Gunn rats, agar exhibited a protective effect against bilirubin-induced nephropathy.[91] The results of agar in hyperbilirubinemic newborns, however, have been conflicting. Poland *et al.* initially reported favorable results in term infants.[89] Yet, subsequent papers showed either beneficial effects,[92,93] or no effects[94-99] of agar treatment on decreasing UCB levels or shortening of the duration of phototherapy. However, most of these studies failed to assess the binding affinity for UCB of the ingested agar. Also, many of the studied populations were small and/or heterogeneous.[100] Consequently, although oral agar has appeared to be free from serious side effects in these studies, its effectiveness remains unproven. Thus, treatment of unconjugated hyperbilirubinemia with agar is not recommended currently.

Calcium phosphate

In pigment gallstones there is a strong interaction between calcium and UCB, which is present in the stones as the insoluble salt $Ca(HB\text{-})_2$.[101,102] Van der Veere *et al.* showed that amorphous calcium phosphate rapidly precipitates UCB *in vitro* in a dose-dependent fashion.[103] The UCB almost exclusively associated with insoluble amorphous calcium phosphate and not with free calcium ions.[103] Van der Veere *et al.* subsequently showed that dietary treatment of Gunn rats with calcium phosphate decreased plasma UCB levels by ~35% and transiently increased fecal UCB excretion, in accordance with intestinal capture (Fig. 4B).[104]

Following these promising results, a placebo-controlled double blind cross-over trial was performed in 11 patients with Crigler-Najjar disease.[105] Patients received calcium phosphate supplementation during 3 weeks as adjunct to their usual phototherapy regimen. Calcium phosphate supplementation decreased plasma UCB concentrations by 18% in patients with type I Crigler-Najjar disease, but had no effect in patients with type II disease.[105] Although its

hypobilirubinemic effect is only moderate, calcium phosphate is currently used by a number of Dutch Crigler-Najjar type 1 patients as an adjunct to phototherapy when plasma UCB concentrations reach dangerously high levels (personal communication Dr. M. Sinaasappel, Rotterdam).

The adverse effects of calcium phosphate treatment seem limited. No side effects have been observed in the animal or patient studies, or during routine treatment of patients. However, there are some concerns that prolonged treatment with high doses of calcium phosphate might cause calcium depositions in the kidneys.

Zinc salts

In 2001, Mendez-Sanchez *et al.* demonstrated that UCB rapidly, and dose-dependently, precipitates with zinc salts *in vitro*.[106] UCB was hypothesized to associate both with the insoluble zinc salts and with the free zinc cations, but the study did not discriminate between these two possible mechanisms.[107] In the same paper, they showed that dietary administration of zinc sulfate decreased the biliary excretion of UCB in hamsters.[107] These observations suggested that zinc salts might trap UCB in the intestinal lumen (Fig. 4B). To further explore this hypothesis, Mendez-Sanchez *et al.* administered a single oral dose of zinc sulfate to 10 patients with Gilbert's syndrome. This inherited condition is characterized by a chronic, mild, unconjugated hyperbilirubinemia related to diminished hepatic UGT1A1 expression. Zinc sulfate enhanced fecal UCB excretion and, moderately (12%-18%), decreased plasma UCB concentrations in these patients.[106] Vitek *et al.* administered zinc sulfate and zinc methacrylate to Gunn rats, after demonstrating that both zinc salts could precipitate with UCB *in vitro*. Both zinc sulfate (-36%) and zinc methacrylate (-26%) treatment decreased plasma UCB concentrations.[108] In all three studies zinc sulfate, but not zinc methacrylate, administration increased plasma zinc levels.[106-108] Because elevated plasma zinc levels are associated with diarrhea, vomiting and, eventually, anemia, zinc methacrylate would be the agent of choice for future clinical studies involving zinc salts.[109]

Orlistat / Fat

Gollan *et al.* had shown in the 1970s that lipid withdrawal from the diet aggravated unconjugated hyperbilirubinemia in Gunn rats and in patients with Gilbert's disease.[110,111] The underlying mechanism, however, remained unknown. In 2002, Verkade hypothesized that fat, due to its ability to form a hydrophobic association with the pigment, could act as an intestinal trapping agent for UCB (Fig. 4B).[112] In that same year, McDonagh demonstrated *in vitro* that UCB admixed in buffer completely partitions into an oil phase upon vigorous shaking.[113] In accordance with Verkade's hypothesis, Nishioka *et al.* showed

that treatment with the lipase inhibitor orlistat increased the fecal excretion of both fat and UCB in Gunn rats, while decreasing their plasma UCB levels by ~40%.[114] Orlistat was used because it decreases the intestinal hydrolysis, and thereby the subsequent absorption, of dietary triglycerides.[114] The resulting increase in fecal fat excretion was strongly, negatively, correlated with plasma UCB levels.[115,116] Orlistat treatment was as effective as phototherapy for reducing plasma UCB levels in Gunn rats, and a combination of orlistat and phototherapy was superior to either treatment alone.[116] In the same study, Hafkamp *et al.* showed that switching Gunn rats from a low-fat to a high-fat diet, increased fecal fat excretion, and decreased plasma UCB levels by 46%. Using steady-state ^{3}H-UCB kinetics, Hafkamp *et al.* demonstrated that orlistat treatment induced *net* transmucosal excretion of UCB into the intestinal lumen, compatible with the hypothesis that fat could act as an intestinal trapping agent for UCB.[117] Still, the presently available data do not clarify whether UCB actually associates with unabsorbed fat and if so, with which class of unabsorbed fat (*e.g.* partially hydrolyzed triglycerides, fatty acids, phospholipids). *In vitro* experiments will be needed to characterize the exact mechanism.[117]

The effects of orlistat treatment of Crigler-Najjar patients were less pronounced than observed in Gunn rats. Hafkamp *et al.* conducted a randomized placebo-controlled double blind cross-over trial in 16 Crigler-Najjar patients.[117] Orlistat was tested during 4-6 weeks as an adjunct treatment to their regular phototherapy and/or phenobarbital regimen. Orlistat treatment decreased plasma UCB concentrations in the whole group by 9%, but the magnitude of the effect was not considered clinically relevant. Interestingly, orlistat treatment decreased plasma UCB by 21% in a subgroup of patients. This clinically relevant response to orlistat treatment appeared to correlate with a relatively low dietary fat intake.[118] It was suggested that apart from dietary fat intake, the responsiveness of Crigler-Najjar patients to orlistat treatment was probably determined by several other factors, which may include gastrointestinal lipase activity levels and the bacterial flora of the patients. Until these factors have been identified, orlistat cannot be recommended as a routine adjunct to conventional treatment for Crigler-Najjar disease. Although some patients reported mild gastrointestinal discomfort, no serious adverse effects were observed in the animal and patient studies. However, orlistat treatment decreased vitamin E levels in Crigler-Najjar patients, indicating that vitamin E supplementation would be required with long-term treatment.

Bile salts

Bile salts are the major organic constituents of the bile. Bile salt administration could lower plasma UCB concentrations for several reasons. First, bile salt administration increased the biliary disposal of organic anions, including UCB, in

rats.[119,120] Secondly, Einarsson *et al.* showed that treatment in healthy volunteers with the bile salt ursodeoxycholic acid (UDCA) decreased the expiration of $^{14}CO_2$ from triolein and suggested that UDCA therapy mildly decreased the absorption of fat.[121] This mild fat malabsorption may well decrease plasma bilirubin levels in a similar fashion as orlistat treatment. Finally, Rege and Ostrow showed *in vitro* that bile salts associate with UCB. Bile salt administration could therefore capture UCB in the intestinal lumen and enhance the fecal excretion of UCB-bile salt complexes (Fig. 4B)[122,123]. The treatment of unconjugated hyperbilirubinemia with bile salts is discussed in chapter 3.

Accelerating intestinal transit

Caloric deprivation, which often occurs in neonates during the initiation of breast-feeding,[4] is associated with unconjugated hyperbilirubinemia.[7] Kotal *et al.* demonstrated in fasted Gunn rats that this unconjugated hyperbilirubinemia was due to a delayed intestinal transit of stools.[3] This delay resulted in intestinal UCB accumulation and enhanced enterohepatic circulation of UCB. Kotal *et al.* also showed that feeding non-absorbable bulk to the fasted Gunn rats normalized intestinal transit time and prevented the increase in plasma UCB concentrations.[3] Kotal's observations are supported by the fact that clinical causes of a delayed intestinal transit, such as a delayed passage of meconium,[72] pyloric stenosis,[124] and Hirschprung's disease,[125] are also associated with unconjugated hyperbilirubinemia.

Based on these observations, it can be hypothesized that accelerating the intestinal transit time decreases plasma UCB concentrations, presumably by decreasing UCB reabsorption (Fig. 4C). This hypothesis is supported by several publications. In 1966 Wennberg *et al.* and in 1967 Wu *et al.* showed that neonates fed within 2-6 hours after birth passed meconium earlier, better maintained their body weights, and had lower plasma bilirubin levels compared with infants initially fed within 24-36 hours after birth.[126,127] In 1982, De Carvalho *et al.* showed that frequent breast feedings were associated with lower plasma UCB levels.[128] In 1984, Cottrell *et al.* showed that accelerating meconium passage with rectal stimulation also decreased plasma UCB concentrations.[129] Frequent and early feedings of neonates thus seem to be effective in lowering plasma UCB concentrations by enhancing its fecal elimination. In this thesis we attempted to lower plasma bilirubin in Gunn rats by accelerating the gastrointestinal transit time. The results of these experiments are discussed in chapter 4.

2.3.4 Other pharmacological interventions

Increasing UCB oxidation

Oxidation converts the hydrophobic UCB into more polar derivatives that can be excreted into the bile without conjugation. When conjugation of UCB is deficient, as in Crigler-Najjar disease or in the Gunn rat model, oxidation can thus serve as an alternate route for UCB excretion.[130,131] The microsomal mixed-function oxidases CYP1A1 and CYP1A2, which catalyze UCB oxidation, were indeed found to be markedly upregulated in the liver of young Gunn rats.[132] Bilirubin can also be oxidized by the constitutive, non-inducible enzyme bilirubin oxidase, found in the mitochondria of the liver, intestine, and kidney of guinea pigs and rats.[133-135]

In 1978, Kapitulnik *et al.* treated Gunn rats with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent inducer of microsomal P450-1A1. Although this treatment reduced plasma UCB levels by 60% in Gunn rats, clinical treatment with TCDD was not possible due to its toxicity.[136] Jorritsma *et al.* showed that induction of CYP1A1 with the natural compound indole-3-carbinol, which is abundant in cruciferous vegetables, decreased plasma UCB levels up to 21% in Gunn rats.[137] The safety of long-term administration of this inexpensive compound to humans has been demonstrated in trials of its prevention of breast cancer in women, but its effects in human neonates are not known. Exogenous bilirubin oxidase from plants has been studied in several publications. In one study, the passage of human or rat blood through an extracorporeal filter containing immobilized bilirubin oxidase degraded more than 90% of the bilirubin in a single pass.[138] In another study, a single dose of bilirubin oxidase, coupled to polyethylene glycol in order to increase its half-life in the blood, was administered intravenously to Gunn rats. This resulted in normalization of plasma UCB levels for a period of 12-48 hours.[139] Although bilirubin oxidase treatment was not associated with adverse effects in animals, it has not yet been applied in clinical trials, human neonates, or Crigler-Najjar patients.

2.4 Future perspectives

Conventional treatment for unconjugated hyperbilirubinemia involves phototherapy and exchange transfusion. These treatment options have several disadvantages and are not always available in developing countries. Various pharmacological alternatives have been evaluated. Ideally, an alternative pharmacological intervention should be less invasive, simpler, and at least as effective and safe as phototherapy. However, many of the experimental therapies presently available do not (yet) meet these criteria. Phototherapy currently remains the preferred treatment strategy for unconjugated hyperbilirubinemia.

Nevertheless, several treatment options, such as metalloporhyrins, clofibrate, bile salts, laxatives, indole-3-carbinol, and bilirubin oxidase deserve further investigation to determine their clinical applicability in the near future. Because the harm from unconjugated hyperbilirubinemia is due to its deposition in the central nervous system, future pharmacological treatment strategies should aim more directly at preventing neurological damage. One possible strategy could involve the induction of the ATP-Binding Cassette (ABC) proteins MRP1 and MDR1, which protect the central nervous system by exporting UCB across the blood-brain barrier.[39,140-144] Another strategy could involve the prevention of UCB-induced apoptosis by enhancement of the neurocellular (anti-caspase) defense mechanism against unconjugated hyperbilirubinemia.[145,146] A focus on neurological damage, rather than plasma bilirubin levels, could induce a paradigm shift in the development of new treatment strategies for unconjugated hyperbilirubinemia.

References

1. **Maisels MJ**, Kring E. The contribution of hemolysis to early jaundice in normal newborns. Pediatrics. 2006;**118**:276–279.

2. **Kawade N**, Onishi S. The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. Biochem. J. 1981;**196**:257–260.

3. **Kotal P**, Vitek L, Fevery J. Fasting-related hyperbilirubinemia in rats: the effect of decreased intestinal motility. Gastroenterology. 1996;**111**:217–223.

4. **Bertini G**, Dani C, Tronchin M, Rubaltelli FF. Is breastfeeding really favoring early neonatal jaundice? Pediatrics. 2001;**107**:41–45.

5. **Stocker R**, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science. 1987;**235**:1043–1046.

6. **Crigler JF**, Najjar VA. Congenital familial nonhemolytic jaundice with kernicterus. Pediatrics. 1952;**10**:169–180.

7. **Gilbert A**, Herscher M. Sur les variations de la cholemie physiologique. Presse Med. 1906;**14**:209–211.

8. **Shapiro SM**. Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction (BIND). J Perinatol. 2005;**25**:54–59.

9. **Ostrow JD**. Photocatabolism of labeled bilirubin in the congenitally jaundiced (Gunn) rat. J. Clin. Invest. 1971;**50**:707–718.

10. **Van der Veere CN**, Sinaasappel M, McDonagh AF, Rosenthal P, Labrune P, Odievre M, et al. Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. Hepatology. 1996;**24**:311–315.

11. **Yohannan MD**, Terry HJ, Littlewood JM. Long term phototherapy in Crigler-Najjar syndrome. Arch Dis Child. 1983;**58**:460–462.

12. **Keenan WJ**, Novak KK, Sutherland JM, Bryla DA, Fetterly KL. Morbidity and mortality associated with exchange transfusion. Pediatrics. 1985;**75**:417–421.

13. **Dennery PA**. Metalloporphyrins for the treatment of neonatal jaundice. Curr. Opin. Pediatr. 2005;**17**:167–169.

14. **McDonagh AF**, Palma LA, Schmid R. Reduction of biliverdin and placental transfer of bilirubin and biliverdin in the pregnant guinea pig. Biochem. J. 1981;**194**:273–282.

15. **Kappas A**, Simionatto CS, Drummond GS, Sassa S, Anderson KE. The liver excretes large amounts of heme into bile when heme oxygenase is inhibited competitively by Sn-protoporphyrin. Proc. Natl. Acad. Sci. U.S.A. 1985;**82**:896–900.

16. **Stevenson DK**, Rodgers PA, Vreman HJ. The use of metalloporphyrins for the chemoprevention of neonatal jaundice. Am J Dis Child. 1989;**143**:353–356.

17. **Maines MD**, Kappas A. Enzymatic oxidation of cobalt protoporphyrin IX: observations on the mechanism of heme oxygenase action. Biochemistry. 1977;**16**:419–423.

18. **Drummond GS**, Kappas A. Chemoprevention of severe neonatal hyperbilirubinemia. Semin Perinatol. 2004;**28**:365–368.

19. **Fang J, Sawa T**, Akaike T, Akuta T, Sahoo SK, Khaled G, et al. In vivo antitumor activity of pegylated zinc protoporphyrin: targeted inhibition of heme oxygenase in solid tumor. Cancer Res. 2003;**63**:3567–3574.

20. **Akins RJ**, McLaughlin T, Boyce R, Gilmour L, Gratton K. Exogenous metalloporphyrins alter the organization and function of cultured neonatal rat heart cells via modulation of heme oxygenase activity. J Cell Physiol. 2004;**201**:26–34.

21. **Park RM**, Bena JF, Stayner LT, Smith RJ, Gibb HJ, Lees PS. Hexavalent chromium and lung cancer in the chromate industry: a quantitative risk assessment. Risk Anal. 2004;**24**:1099–1108.

22. **Beri R**, Chandra R. Chemistry and biology of heme. Effect of metal salts, organometals, and metalloporphyrins on heme synthesis and catabolism, with special reference to clinical implications and interactions with cytochrome P-450. Drug Metab Rev. 1993;**25**:49–152.

23. **Kappas A**, Drummond GS, Manola T, Petmezaki S, Valaes T. Sn-protoporphyrin use in the management of hyperbilirubinemia in term newborns with direct Coombs-positive ABO incompatibility. Pediatrics. 1988;**81**:485–497.

24. **Valaes T**, Petmezaki S, Henschke C, Drummond GS, Kappas A. Control of jaundice in preterm newborns by an inhibitor of bilirubin production: studies with tin-mesoporphyrin. Pediatrics. 1994;**93**:1–11.

25. **Kappas A**, Drummond GS, Henschke C, Valaes T. Direct comparison of Sn-mesoporphyrin, an inhibitor of bilirubin production, and phototherapy in controlling hyperbilirubinemia in term and near-term newborns. Pediatrics. 1995;**95**:468–474.

26. **Valaes T**, Drummond GS, Kappas A. Control of hyperbilirubinemia in glucose-6-phosphate dehydrogenase-deficient newborns using an inhibitor of bilirubin production, Sn-mesoporphyrin. Pediatrics. 1998;**101**:E1.

27. **Martinez JC**, Garcia HO, Otheguy LE, Drummond GS, Kappas A. Control of severe hyperbilirubinemia in full-term newborns with the inhibitor of bilirubin production Sn-mesoporphyrin. Pediatrics. 1999;**103**:1–5.

28. **Rubaltelli FF**, Guerrini P, Reddi E, Jori G. Tin-protoporphyrin in the management of children with Crigler-Najjar disease. Pediatrics. 1989;**84**:728–731.

29. **Galbraith RA**, Drummond GS, Kappas A. Suppression of bilirubin production in the Crigler-Najjar type I syndrome: studies with the heme oxygenase inhibitor tin-mesoporphyrin. Pediatrics. 1992;**89**:175–182.

30. **Suresh GK**, Martin CL, Soll RF. Metalloporphyrins for treatment of unconjugated hyperbilirubinemia in neonates. Cochrane.Database.Syst.Rev. 2003;:CD004207.

31. **Dani C**, Masini E, Bertini G, di Felice AM, Pezzati M, Ciofini S, et al. Role of heme oxygenase and bilirubin in oxidative stress in preterm infants. Pediatr. Res. 2004;**56**:873–877.

32. **Willis D**, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med. 1996;**2**:87–90.

33. **Oroszlan G**, Lakatos L, Szabo L, Matkovics B, Karmazsin L. Heme oxygenase activity is decreased by D-penicillamine in neonates. Experientia. 1983;**39**:888–889.

34. **Lakatos L**, Kover B, Oroszlan G, Vekerdy Z. D-penicillamine therapy in ABO hemolytic disease of the newborn infant. Eur J Pediatr. 1976;**123**:133–137.

35. **Munro R**, Capell HA. Penicillamine. Br J Rheumatol. 1997;**36**:104–109.

36. **Levi AJ**, Gatmaitan Z, Arias IM. Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein, and other anions. J. Clin. Invest. 1969;**48**:2156–2167.

37. **Habig WH**, Pabst MJ, Fleischner G, Gatmaitan Z, Arias IM, Jakoby WB. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proc. Natl. Acad. Sci. U.S.A. 1974;**71**:3879–3882.

38. **Wolkoff AW**, Goresky CA, Sellin J, Gatmaitan Z, Arias IM. Role of ligandin in transfer of bilirubin from plasma into liver. Am J Physiol. 1979;**236**:E638–48.

39. **Paulusma CC**, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, et al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science. 1996;**271**:1126–1128.

40. **Wagner M**, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, et al. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. Hepatology. 2005;**42**:420–430.

41. **Yaffe SJ**, Levy G, Matsuzawa T, Baliah T. Enhancement of glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital. N. Engl. J. Med. 1966;**275**:1461.

42. **Catz C**, Yaffe SJ. Barbiturate enhancement of bilirubin conjugation and excretion in young and adult animals. Pediatr. Res. 1968;**2**:361.

43. **Valaes TN**, Harvey-Wilkes K. Pharmacologic approaches to the prevention and treatment of neonatal hyperbilirubinemia. Clin Perinatol. 1990;**17**:245–273.

44. **Valaes T**, Kipouros K, Petmezaki S, Solman M, Doxiadis SA. Effectiveness and safety of prenatal phenobarbital for the prevention of neonatal jaundice. Pediatr. Res. 1980;**14**:947–952.

45. **Thomas JT**, Muller P, Wilkinson C. Antenatal phenobarbital for reducing neonatal jaundice after red cell isoimmunization. Cochrane.Database.Syst.Rev. 2007;:CD005541.

46. **Valdes OS**, Maurer HM, Shumway CN, Draper DA, Hossaini AA. Controlled clinical trial of phenobarbital and-or light in reducing neonatal hyperbilirubinemia in a predominantly Negro population. J. Pediatr. 1971;**79**:1015–1017.

47. **Hansen TW**, Tommarello S. Effect of phenobarbital on bilirubin metabolism in rat brain. Biol Neonate. 1998;**73**:106–111.

48. **Sinaasappel M**, Jansen PL. The differential diagnosis of Crigler-Najjar disease, types 1 and 2, by bile pigment analysis. Gastroenterology. 1991;**100**:783–789.

49. **Despres JP**, Lemieux I, Robins SJ. Role of fibric acid derivatives in the management of risk factors for coronary heart disease. Drugs. 2004;**64**:2177–2198.

50. **Foliot A**, Drocourt JL, Etienne JP, Housset E, Fiessinger JN, Christoforov B. Increase in the hepatic glucuronidation and clearance of

bilirubin in clofibrate-treated rats. Biochem. Pharmacol. 1977;**26**:547–549.

51. **Jemnitz K**, Lengyel G, Vereczkey L. In vitro induction of bilirubin conjugation in primary rat hepatocyte culture. Biochem Biophys Res Commun. 2002;**291**:29–33.

52. **Jean F**, Foliot A, Celier C, Housset E, Etienne JP. Influence of clofibrate on hepatic transport of bilirubin and bromosulfophthalein in rats. Biochem Biophys Res Commun. 1979;**86**:1154–1160.

53. **Lindenbaum A**, Hernandorena X, Vial M, Benattar C, Janaud JC, Dehan M, et al. [Clofibrate for the treatment of hyperbilirubinemia in neonates born at term: a double blind controlled study (author's transl)]. Arch. Fr. Pediatr. 1981;**38** Suppl 1:867–873.

54. **Lindenbaum A**, Delaporte B, Benattar C, Dehan M, Magny JF, Gerbet D, et al. [Preventive treatment of jaundice in premature newborn infants with clofibrate. Double-blind controlled therapeutic trial]. Arch. Fr. Pediatr. 1985;**42**:759–763.

55. **Mohammadzadeh A**, Farhat A, Iranpour R. Effect of clofibrate in jaundiced term newborns. Indian J Pediatr. 2005;**72**:123–126.

56. **Eghbalian F**, Pourhossein A, Zandevakili H. Effect of clofibrate in non-hemolytic indirect hyperbilirubinemia in full term neonates. Indian J Pediatr. 2007;**74**:1003–1006.

57. **Zahedpasha Y**, Ahmadpour-Kacho M, Hajiahmadi M, Naderi S. Effect of clofibrate in jaundiced full-term infants:a randomized clinical trial. Arch Iran Med. 2007;**10**:349–353.

58. **Reddy JK**, Qureshi SA. Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-alpha-p-chlorophenoxyisobutyrate (clofibrate) in rats. Br J Cancer. 1979;**40**:476–482.

59. WHO cooperative trial on primary prevention of ischaemic heart disease with clofibrate to lower serum cholesterol: final mortality follow-up. Report of the Committee of Principal Investigators. Lancet. 1984;**324**:600–604.

60. **Erkul I**, Yavuz H, Ozel A. Clofibrate treatment of neonatal jaundice. Pediatrics. 1991;**88**:1292–1294.

61. **Steiner A**, Weisser B, Vetter W. A comparative review of the adverse effects of treatments for hyperlipidaemia. Drug Saf. 1991;**6**:118–130.

62. **Rush P**, Baron M, Kapusta M. Clofibrate myopathy: a case report and a review of the literature. Semin Arthritis Rheum. 1986;**15**:226–229.

63. **Fok TF**. Neonatal jaundice--traditional Chinese medicine approach. J Perinatol. 2001;**21** Suppl 1:S98–S100; discussion S104–7.

64. **Yin J**, Miller M, Wennberg RP. Induction of hepatic bilirubin-metabolizing enzymes by the traditional Chinese medicine yin zhi huang. Dev Pharmacol Ther. 1991;**16**:176–184.

65. **Huang W**, Zhang J, Moore DD. A traditional herbal medicine enhances bilirubin clearance by activating the nuclear receptor CAR. J. Clin. Invest. 2004;**113**:137–143.

66. **Yeung CY**, Leung CS, Chen YZ. An old traditional herbal remedy for neonatal jaundice with a newly identified risk. J Paediatr Child Health. 1993;**29**:292–294.

67. **Chan TY**. The prevalence use and harmful potential of some Chinese herbal medicines in babies and children. Vet Hum Toxicol.

1994;**36**:238–240.

68. **Lester R**, Schmid R. Intestinal absorption of bile pigments. I. The enterohepatic circulation of bilirubin in the rat. J. Clin. Invest. 1963;**42**:736–746.

69. **Lester R**, Schmid R. Intestinal absorption of bile pigments. II. Bilirubin absorption in man. N. Engl. J. Med. 1963;**269**:178–182.

70. **Vitek L**, Kotal P, Jirsa M, Malina J, Cerna M, Chmelar D, et al. Intestinal colonization leading to fecal urobilinoid excretion may play a role in the pathogenesis of neonatal jaundice. J Pediatr Gastroenterol Nutr. 2000;**30**:294–298.

71. **Zucker SD**, Goessling W, Hoppin AG. Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and native hepatocyte membranes. J. Biol. Chem. 1999;**274**:10852–10862.

72. **Rosta J**, Makoi Z, Kertesz A. Delayed meconium passage and hyperbilirubinaemia. Lancet. 1968;**292**:1138.

73. **Tiribelli C**, Ostrow JD. Intestinal flora and bilirubin. J. Hepatol. 2005;**42**:170.

74. **Kotal P**, Van der Veere CN, Sinaasappel M, Elferink RO, Vitek L, Brodanova M, et al. Intestinal excretion of unconjugated bilirubin in man and rats with inherited unconjugated hyperbilirubinemia. Pediatr. Res. 1997;**42**:195–200.

75. **Schmid R**, Hammaker L. Metabolism and disposition of C14-bilirubin in congenital nonhemolytic jaundice. J. Clin. Invest. 1963;**42**:1720–1734.

76. **Lester R**, Hammaker L, Schmid R. A new therapeutic approach to unconjugated hyperbilirubinaemia. Lancet. 1962;**280**:1257.

77. **Schmid R**, Forbes A, Rosenthal IM, Lester R. Lack of Effect of Cholestyramine Resin on Hyperbilirubinaemia of Premature Infants. Lancet. 1963;**282**:938–939.

78. **Arrowsmith WA**, Payne RB, Littlewood JM. Comparison of treatments for congenital nonobstructive nonhaemolytic hyperbilirubinaemia. Arch Dis Child. 1975;**50**:197–201.

79. **Nicolopoulos D**, Hadjigeorgiou E, Malamitsi A, Kalpoyannis N, Karli I, Papadakis D. Combined treatment of neonatal jaundice with cholestyramine and phototherapy. J. Pediatr. 1978;**93**:684–688.

80. **Malamitsi-Puchner A**, Hadjigeorgiou E, Papadakis D, Kalpoyannis N, Nicolopoulos D. Combined treatment of neonatal jaundice with phototherapy, cholestyramine, and bicarbonate. J. Pediatr. 1981;**99**:324–325.

81. **Tan KL**, Jacob E, Liew DS, Karim SM. Cholestyramine and phototherapy for neonatal jaundice. J. Pediatr. 1984;**104**:284–286.

82. **Ulstrom RA**, Eisenklam E. The Enterohepatic Shunting of Bilirubin in the Newborn Infant. I. Use of Oral Activated Charcoal to Reduce Normal Serum Bilirubin Values. J. Pediatr. 1964;**65**:27–37.

83. **Kuenzer W**, Vahlenkamp H, Jarre W, Fuss W. [on the Treatment of Jaundice in the Newborn with Charcoal.]. Ann Paediatr. 1964;**203**:247–255.

84. **Davis DR**, Yeary RA. Activated charcoal as an adjunct to phototherapy for neonatal jaundice. Dev Pharmacol Ther. 1987;**10**:12–20.

85. **Davis DR**, Yeary RA, Lee K.

Improved embryonic survival in the jaundiced female rat fed activated charcoal. Pediatr Pharmacol (New York). 1983;**3**:79–85.

86. **Davis DR**, Yeary RA, Lee K. Activated charcoal decreases plasma bilirubin levels in the hyperbilirubinemic rat. Pediatr. Res. 1983;**17**:208–209.

87. **Amitai Y**, Regev M, Arad I, Peleg O, Boehnert M. Treatment of neonatal hyperbilirubinemia with repetitive oral activated charcoal as an adjunct to phototherapy. J Perinat Med. 1993;**21**:189–194.

88. **James LP**, Nichols MH, King WD. A comparison of cathartics in pediatric ingestions. Pediatrics. 1995;**96**:235–238.

89. **Poland RL**, Odell GB. Physiologic jaundice: the enterohepatic circulation of bilirubin. N. Engl. J. Med. 1971;**284**:1–6.

90. **Poland RL**, Odell GB. The binding of bilirubin to agar. Proc Soc Exp Biol Med. 1974;**146**:1114–1118.

91. **Odell GB**, Bolen JL, Poland RL, Seungdambong S, Cukier JO. Protection from bilirubin nephropathy in jaundiced Gunn rats. Gastroenterology. 1974;**66**:1218–1224.

92. **Odell GB**, Gutcher GR, Whitington PF, Yang G. Enteral administration of agar as an effective adjunct to phototherapy of neonatal hyperbilirubinemia. Pediatr. Res. 1983;**17**:810–814.

93. **Caglayan S**, Candemir H, Aksit S, Kansoy S, Asik S, Yaprak I. Superiority of oral agar and phototherapy combination in the treatment of neonatal hyperbilirubinemia. Pediatrics. 1993;**92**:86–89.

94. **Maurer HM**, Shumway CN, Draper DA, Hossaini AA. Controlled trial comparing agar, intermittent phototherapy, and continuous phototherapy for reducing neonatal hyperbilirubinemia. J. Pediatr. 1973;**82**:73–76.

95. **Romagnoli C**, Polidori G, Foschini M, Cataldi L, De Turris P, Tortorolo G, et al. Agar in the management of hyperbilirubinaemia in the premature baby. Arch Dis Child. 1975;**50**:202–204.

96. **Windorfer AJ**, Kunzer W, Bolze H, Ascher K, Wilcken F, Hoehne K. Studies on the effect of orally administered agar on the serum bilirubin level of premature infants and mature newborns. Acta Paediatr Scand. 1975;**64**:699–702.

97. **Ebbesen F**, Moller J. Agar ingestion combined with phototherapy in jaundiced newborn infants. Biol Neonate. 1977;**31**:7–9.

98. **Blum D**, Etienne J. Agar in control of hyperbilirubinemia. J. Pediatr. 1973;**83**:345.

99. **Moller J**. Agar ingestion and serum bilirubin values in newborn infants. Acta Obstet Gynecol Scand Suppl. 1974;**29**:61–63.

100. **Kemper K**, Horwitz RI, McCarthy P. Decreased neonatal serum bilirubin with plain agar: a meta-analysis. Pediatrics. 1988;**82**:631–638.

101. **Moore EW**. Biliary calcium and gallstone formation. Hepatology. 1990;**12**:206S–214S; discussion 214S–218S.

102. **Ostrow JD**. Unconjugated bilirubin and cholesterol gallstone formation. Hepatology. 1990;**12**:219S–224S; discussion 224S–226S.

103. **Van der Veere CN**, Schoemaker B, Van Der Meer R, Groen AK, Jansen

PL, Oude Elferink RP. Rapid association of unconjugated bilirubin with amorphous calcium phosphate. J. Lipid Res. 1995;**36**:1697–1707.

104. **Van der Veere CN**, Schoemaker B, Bakker C, Van Der Meer R, Jansen PL, Elferink RP. Influence of dietary calcium phosphate on the disposition of bilirubin in rats with unconjugated hyperbilirubinemia. Hepatology. 1996;**24**:620–626.

105. **Van der Veere CN**, Jansen PL, Sinaasappel M, Van Der Meer R, Van der Sijs H, Rammeloo JA, et al. Oral calcium phosphate: a new therapy for Crigler-Najjar disease? Gastroenterology. 1997;**112**:455–462.

106. **Mendez-Sanchez N**, Martinez M, Gonzalez V, Roldan-Valadez E, Flores MA, Uribe M. Zinc sulfate inhibits the enterohepatic cycling of unconjugated bilirubin in subjects with Gilbert's syndrome. Ann Hepatol. 2002;**1**:40–43.

107. **Mendez-Sanchez N**, Roldan-Valadez E, Flores MA, Cardenas-Vazquez R, Uribe M. Zinc salts precipitate unconjugated bilirubin in vitro and inhibit enterohepatic cycling of bilirubin in hamsters. Eur J Clin Invest. 2001;**31**:773–780.

108. **Vitek L**, Muchova L, Zelenka J, Zadinova M, Malina J. The effect of zinc salts on serum bilirubin levels in hyperbilirubinemic rats. J Pediatr Gastroenterol Nutr. 2005;**40**:135–140.

109. **Roney N**, Osier M, Paikoff SJ, Smith CV, Williams M, De Rosa CT. ATSDR evaluation of the health effects of zinc and relevance to public health. Toxicol Ind Health. 2006;**22**:423–493.

110. **Gollan JL**, Bateman C, Billing BH. Effect of dietary composition on the unconjugated hyperbilirubinaemia of Gilbert's syndrome. Gut. 1976;**17**:335–340.

111. **Gollan JL**, Hatt KJ, Billing BH. The influence of diet on unconjugated hyperbilirubinaemia in the Gunn rat. Clin Sci Mol Med. 1975;**49**:229–235.

112. **Verkade HJ**. A novel hypothesis on the pathophysiology of neonatal jaundice. J. Pediatr. 2002;**141**:594–595.

113. **McDonagh AF**. Lyophilic properties of protoporphyrin and bilirubin. Hepatology. 2002;**36**:1028–1029.

114. **Guerciolini R**. Mode of action of orlistat. Int J Obes Relat Metab Disord. 1997;**21** Suppl 3:S12–23.

115. **Nishioka T**, Hafkamp AM, Havinga R, vn Lierop PP, Velvis H, Verkade HJ. Orlistat treatment increases fecal bilirubin excretion and decreases plasma bilirubin concentrations in hyperbilirubinemic Gunn rats. J. Pediatr. 2003;**143**:327–334.

116. **Hafkamp AM**, Havinga R, Sinaasappel M, Verkade HJ. Effective oral treatment of unconjugated hyperbilirubinemia in Gunn rats. Hepatology. 2005;**41**:526–534.

117. **Hafkamp AM**, Havinga R, Ostrow JD, Tiribelli C, Pascolo L, Sinaasappel M, et al. Novel kinetic insights into treatment of unconjugated hyperbilirubinemia: phototherapy and orlistat treatment in Gunn rats. Pediatr. Res. 2006;**59**:506–512.

118. **Hafkamp AM**, Nelisse-Haak R, Sinaasappel M, Oude Elferink RPJ, Verkade HJ. Orlistat treatment of unconjugated hyperbilirubinemia in Crigler-Najjar disease: a randomized controlled trial. Pediatr. Res. 2007;**62**:725–730.

119. **Vonk RJ**, Jekel P, Meijer DK. Choleresis and hepatic transport mechanisms. II. Influence of bile salt choleresis and biliary micelle binding on biliary excretion of various organic anions. Naunyn Schmiedebergs Arch

Pharmacol. 1975;**290**:375–387.

120. **Ostrow JD**. Regulation by bile salts of the excretion of conjugated and unconjugated bilirubin in bile. In: Fromm H, Leuschner U, eds. Proceedings of the falk symposium No. 84, held in Berlin, Germany, June 9-10, 1995. Kluwer Academic Publishers, 1996.

121. **Einarsson K**, Bjorkhem I, Eklof R, Ewerth S, Nilsell K, Blomstrand R. Effect of ursodeoxycholic acid treatment on intestinal absorption of triglycerides in man. Scand. J. Gastroenterol. 1984;**19**:283–288.

122. **Ostrow JD**, Celic L, Mukerjee P. Molecular and micellar associations in the pH-dependent stable and metastable dissolution of unconjugated bilirubin by bile salts. J. Lipid Res. 1988;**29**:335–348.

123. **Rege RV**, Webster CC, Ostrow JD. Interactions of unconjugated bilirubin with bile salts. J. Lipid Res. 1988;**29**:1289–1296.

124. **Etzioni A**, Shoshani G, Diamond E, Zinder O, Bar-Maor JA. Unconjugated hyperbilirubinaemia in hypertrophic pyloric stenosis, an enigma. Z Kinderchir. 1986;**41**:272–274.

125. **Gartner LM**. Breastfeeding and jaundice. J Perinatol. 2001;**21** Suppl 1:S25–9; discussion S35–9.

126. **Wennberg RP**, Schwartz R, Sweet AY. Early versus delayed feeding of low birth weight infants: effects on physiologic jaundice. J. Pediatr. 1966;**68**:860–866.

127. **Wu PY**, Teilmann P, Gabler M, Vaughan M, Metcoff J. "Early" versus "late" feeding of low birth weight neonates: effect on serum bilirubin, blood sugar, and responses to glucagon and epinephrine tolerance tests. Pediatrics. 1967;**39**:733–739.

128. **De Carvalho M**, Klaus MH, Merkatz RB. Frequency of breast-feeding and serum bilirubin concentration. Am J Dis Child. 1982;**136**:737–738.

129. **Cottrell BH**, Anderson GC. Rectal or axillary temperature measurement: effect on plasma bilirubin and intestinal transit of meconium. J Pediatr Gastroenterol Nutr. 1984;**3**:734–739.

130. **Berry CS**, Zarembo JE, Ostrow JD. Evidence for conversion of bilirubin to dihydroxyl derivatives in the gunn rat. Biochem Biophys Res Commun. 1972;**49**:1366–1375.

131. **Blanckaert N**, Fevery J, Heirwegh KP, Compernolle F. Characterization of the major diazo-positive pigments in bile of homozygous Gunn rats. Biochem. J. 1977;**164**:237–249.

132. **Kapitulnik J**, Gonzalez FJ. Marked endogenous activation of the CYP1A1 and CYP1A2 genes in the congenitally jaundiced Gunn rat. Mol.Pharmacol. 1993;**43**:722–725.

133. **Cardenas-Vazquez R**, Yokosuka O, Billing BH. Enzymic oxidation of unconjugated bilirubin by rat liver. Biochem. J. 1986;**236**:625–633.

134. **Brodersen R**, Bartels P. Enzymatic oxidation of bilirubin. Eur J Biochem. 1969;**10**:468–473.

135. **Yokosuka O**, Billing B. Enzymatic oxidation of bilirubin by intestinal mucosa. Biochim Biophys Acta. 1987;**923**:268–274.

136. **Kapitulnik J**, Ostrow JD. Stimulation of bilirubin catabolism in jaundiced Gunn rats by an induced of microsomal mixed-function monooxygenases. Proc. Natl. Acad. Sci. U.S.A. 1978;**75**:682–685.

137. **Jorritsma U**, Schrader E, Klaunick G, Kapitulnik J, Hirsch-Ernst KI,

Kahl GF, et al. Monitoring of cytochrome P-450 1A activity by determination of the urinary pattern of caffeine metabolites in Wistar and hyperbilirubinemic Gunn rats. Toxicology. 2000;**144**:229–236.

138. **Lavin A**, Sung C, Klibanov AM, Langer R. Enzymatic removal of bilirubin from blood: a potential treatment for neonatal jaundice. Science. 1985;**230**:543–545.

139. **Sugi K**, Inoue M, Morino Y. Degradation of plasma bilirubin by a bilirubin oxidase derivative which has a relatively long half-life in the circulation. Biochim Biophys Acta. 1989;**991**:405–409.

140. **Watchko JF**, Daood MJ, Hansen TW. Brain bilirubin content is increased in P-glycoprotein-deficient transgenic null mutant mice. Pediatr. Res. 1998;**44**:763–766.

141. **Hankø E**, Tommarello S, Watchko JF, Hansen TWR. Administration of drugs known to inhibit P-glycoprotein increases brain bilirubin and alters the regional distribution of bilirubin in rat brain. Pediatr. Res. 2003;**54**:441–445.

142. **Rigato I**, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C. The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. Biochem. J. 2004;**383**:335–341.

143. **Gennuso F**, Fernetti C, Tirolo C, Testa N, L'Episcopo F, Caniglia S, et al. Bilirubin protects astrocytes from its own toxicity by inducing up-regulation and translocation of multidrug resistance-associated protein 1 (Mrp1). Proc. Natl. Acad. Sci. U.S.A. 2004;**101**:2470–2475.

144. **Falcao AS**, Bellarosa C, Fernandes A, Brito MA, Silva RF, Tiribelli C, et al. Role of multidrug resistance-associated protein 1 expression in the in vitro susceptibility of rat nerve cell to unconjugated bilirubin. Neuroscience. 2007;**144**:878–888.

145. **Lin S**, Wei X, Bales KR, Paul AB, Ma Z, Yan G, et al. Minocycline blocks bilirubin neurotoxicity and prevents hyperbilirubinemia-induced cerebellar hypoplasia in the Gunn rat. Eur J Neurosci. 2005;**22**:21–27.

146. **Cesaratto L**, Calligaris SD, Vascotto C, Deganuto M, Bellarosa C, Quadrifoglio F, et al. Bilirubin-induced cell toxicity involves PTEN activation through an APE1/Ref-1-dependent pathway. J Mol Med. 2007;**85**:1099–1112