

# Combined Treatment Strategies for Unconjugated Hyperbilirubinemia in Gunn Rats

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**ABSTRACT:** We recently demonstrated that acceleration of the gastrointestinal transit by polyethylene glycol (PEG) treats unconjugated hyperbilirubinemia in jaundiced Gunn rats. It is unclear whether acceleration of gastrointestinal transit also (partly) underlies the therapeutic effects of established hypobilirubinemic treatments or whether PEG cotreatment might enhance these effects. We treated Gunn rats with phototherapy ( $17 \mu\text{W}/\text{cm}^2/\text{nm}$ ), orlistat (200 mg/kg chow), ursodeoxycholate (5 g/kg chow), or calcium phosphate (CaP) (20 g/kg chow) either as single treatment or in combination with PEG. Three weeks of phototherapy, orlistat, ursodeoxycholic acid, or CaP treatment decreased plasma unconjugated bilirubin (UCB) levels by 47, 27, 28, and 45%, respectively (each  $p < 0.001$ ), without a significant impact on gastrointestinal transit time. PEG cotreatment accelerated the gastrointestinal transit in all treatment groups, which resulted in an additive hypobilirubinemic effect of  $-20\%$  and  $-26\%$  (final plasma UCB  $-67$  and  $-53\%$ , respectively) in phototherapy- and orlistat-treated animals. PEG cotreatment did not enhance the hypobilirubinemic effect of ursodeoxycholic acid or CaP. We conclude that phototherapy, orlistat, ursodeoxycholic acid, and CaP do not exert their hypobilirubinemic effect via acceleration of the gastrointestinal transit. PEG cotreatment enhanced the hypobilirubinemic effects of phototherapy and of orlistat treatment. Current results support a clinical trial to evaluate PEG cotreatment during phototherapy. (*Pediatr Res* 70: 560–565, 2011)

Neonatal hemolytic jaundice and Crigler-Najjar disease are characterized by severe unconjugated hyperbilirubinemia. In Crigler-Najjar patients, a genetically absent (type I) or decreased (type II) ability to conjugate bilirubin within the liver results in lifelong jaundice (1,2). Unconjugated bilirubin (UCB) is neurotoxic, and severe unconjugated hyperbilirubinemia is associated with brain damage (3). This damage occurs because nonprotein-bound plasma bilirubin ( $\text{UCB}_{\text{free}}$ ) can diffuse across the blood-brain barrier (4–9). To decrease plasma UCB levels, Crigler-Najjar disease patients rely on lifelong phototherapy, the routine treatment for unconjugated hyperbilirubinemia. Phototherapy, however, becomes less effective with age and eventually fails to prevent bilirubin-induced brain damage in up to 25% of Crigler-Najjar patients (10,11). This prompted us to develop alternative and

adjunct, treatment strategies for severe unconjugated hyperbilirubinemia.

Severe unconjugated hyperbilirubinemia allows direct transmucosal diffusion of  $\text{UCB}_{\text{free}}$  from the blood into the intestine (12–14). The efficacy of this pathway is decreased, however, by the intestines' ability to reabsorb  $\text{UCB}_{\text{free}}$  from its lumen (12,15). We recently hypothesized that acceleration of the gastrointestinal transit might interfere with this ability and thus enhance the efficacy of transmucosal bilirubin disposal. Indeed, the laxative polyethylene glycol (PEG) proved an effective treatment for unconjugated hyperbilirubinemia in Gunn rats, the animal model for Crigler-Najjar disease (16). Importantly, our experiments also revealed a strong linear relation between the gastrointestinal transit time and plasma UCB levels in these animals (16).

Our results raised the question whether other treatments also (partly) rely on acceleration of the gastrointestinal transit for their hypobilirubinemic effects. Interestingly, previous findings have suggested that phototherapy and oral treatments that enhance transmucosal UCB diffusion [*e.g.* orlistat (17,18), bile salts (13), or amorphous calcium phosphate (CaP) (19)] might indeed influence transit time (20–25). We presently show that neither phototherapy nor oral treatment with orlistat, ursodeoxycholic acid (UDCA), or CaP affects the gastrointestinal transit time in Gunn rats. We also show that addition of PEG to either phototherapy or orlistat treatment enhances their hypobilirubinemic effect.

## ANIMALS, MATERIALS, AND METHODS

**Animals.** Homozygous male Gunn rats (RHA/jj; 244–375 g;  $n = 60$ ) were obtained from our breeding colony at the University Medical Center Groningen (UMCG, The Netherlands). Animals, housed individually in an environmentally controlled facility with a diurnal (12/12 h) light cycle, were fed *ad libitum* and had free access to water. The Ethics Committee for Animal Experiments of the UMCG approved the experimental protocols for this study.

**Materials. Diets.** Hope Farms BV (Woerden, The Netherlands) produced all diets. The semisynthetic control diet (code 4063.02) contained 13% energy fat (17). Supplemented diets were identical to the control diet, except for the

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**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; CaP, calcium phosphate; Creat, creatinine; PEG, polyethylene glycol; UCB, unconjugated bilirubin;  $\text{UCB}_{\text{free}}$ , free unconjugated bilirubin; UDCA, ursodeoxycholic acid; Ur, urea

supplementation of orlistat (200 mg/kg chow), UDCA (5 g/kg chow), or CaP (20 g/kg chow). Gunn rats were fed the semisynthetic purified control diet (4063.02) during a 6-wk run-in period before the experiments (13).

**Chemicals.** PEG 4000 (Colofort) was obtained from Ipsen Farmaceutica B.V. (Hoofddorp, The Netherlands). Colofort contained per sachet (74 g): 64 g PEG, 5.7 g sodium sulfate (anhydric), 1.68 g sodium bicarbonate, 1.46 g sodium chloride, and 0.75 g potassium chloride. We dissolved one sachet of PEG 4000 (Colofort) in 900 mL water to obtain the PEG solution we used in our experiments (drinking water solution and gavage solution). Orlistat (Xenical) was obtained from Roche Nederland B.V. (Woerden, The Netherlands). UDCA was a generous gift from Dr. Falk Pharma GmbH (Freiburg, Germany). Carmine red dye was obtained from Macro-imPulse Saveur Ltd. (Stadtdendorf, Germany).

**Phototherapy lamps.** Phototherapy devices were designed according to the prototype by Ostrow (26). Two phototherapy lamps (Philips TL-20 W/03 T) were suspended in a reflective framework 20 cm above the bottom of the cage. The light intensity of phototherapy was measured by a Dale 40 phototherapy radiometer (Dale Technologies, Carson City, NV) at 20 cm distance from the light source and was set to 17  $\mu\text{W}/\text{cm}^2/\text{nm}$ . Gunn rats, shaven on their backs and flanks every 7 d, received continuous phototherapy (27).

**Methods. Long-term experiment.** Male Gunn rats were randomized ( $n = 5-6$  per group) to receive no treatment (controls) or treatment with phototherapy (17  $\mu\text{W}/\text{cm}^2/\text{nm}$ ), orlistat (200 mg/kg chow), UDCA (5 g/kg chow), or CaP (20 g/kg chow). Body weight was determined weekly during the experiments. After a 3-wk treatment period, which ensured the presence of steady-state conditions (13,16-18), we determined the gastrointestinal transit time in untreated (controls) and treated Gunn rats by measuring the interval between intragastrical administration of carmine red and its appearance in the feces (13). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before (baseline) and 3 wk after randomization to determine steady-state plasma bilirubin concentrations. After 3 wk, these treatments were combined with PEG, both via drinking water and via intragastrical gavage (2.5 mL, every 12 h). The gastrointestinal transit time was determined again 6 wk after randomization (3 wk after the addition of PEG). A final blood sample was then obtained, as described above, for the determination of bilirubin, urea (Ur), creatinine (Creat), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

**Short-term experiment.** Male Gunn rats were randomized ( $n = 6-7$  per group) to receive no treatment (controls), phototherapy (17  $\mu\text{W}/\text{cm}^2/\text{nm}$ ), phototherapy (17  $\mu\text{W}/\text{cm}^2/\text{nm}$ ) combined with PEG (via drinking water and via 2.5 mL gavage), orlistat (200 mg/kg chow), or orlistat (200 mg/kg chow) combined with PEG (via drinking water and via 2.5 mL gavage). Body weight was determined daily during the experiments. Carmine red was administered directly after the start of PEG administration to determine the gastrointestinal transit as described above. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia every 12 h for the first 48 h for the determination of bilirubin, Ur, Creat, AST, and ALT. A final blood sample was obtained 14 d after randomization.

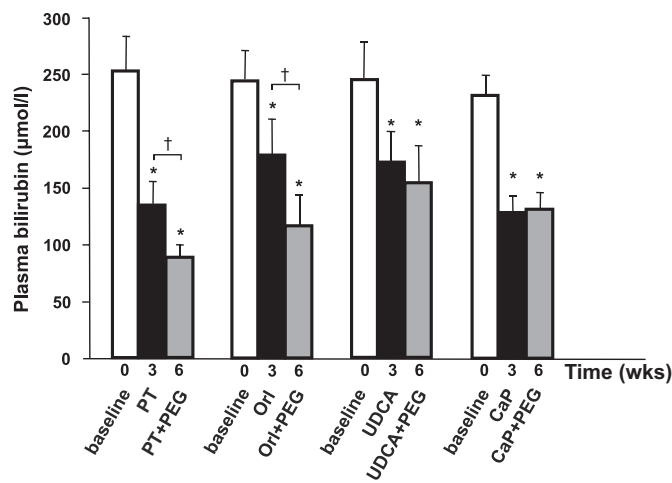
**Plasma analysis.** Blood samples were protected from light and processed immediately. Bilirubin, Ur, Creat, AST, and ALT were determined by routine spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). We previously found that the total bilirubin concentration in Gunn rat plasma, measured by spectrophotometry, equaled the total UCB concentration, measured by high-liquid performance chromatography after chloroform extraction (17,27).

**Statistical analysis.** All data were normally distributed, displayed homogeneity of variance by calculation of Levene's statistic, and were expressed as mean  $\pm$  SD. ANOVA with *post hoc* Bonferroni correction was performed for comparison between groups. *t* test was used for comparison of paired data within groups. The level of significance was set at  $p < 0.05$ . Analyses were performed using SPSS 16.0 for Mac (SPSS Inc., Chicago, IL).

## RESULTS

### Long-term experiment.

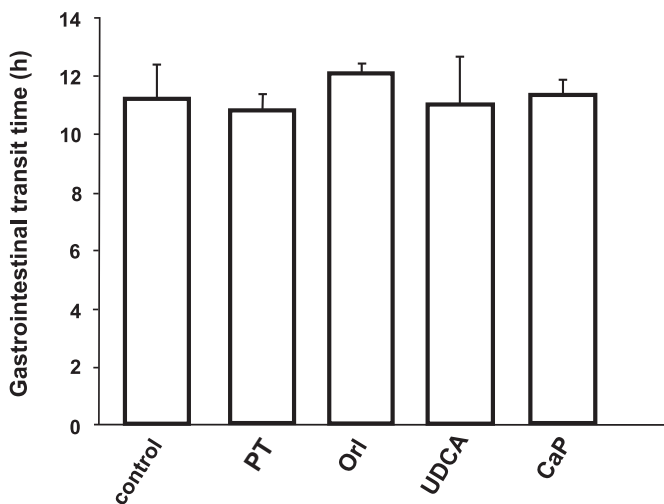
**Neither phototherapy nor oral treatments affect gastrointestinal transit time.** Because acceleration of gastrointestinal transit decreases plasma bilirubin concentrations in Gunn rats, we assessed whether phototherapy and known oral treatments exert their hypobilirubinemic effects, either partly or exclusively, by acceleration of the gastrointestinal transit. Figure 1 shows that 3 wk of phototherapy, orlistat, UDCA, or CaP decreased plasma bilirubin concentrations by 47, 27, 28,



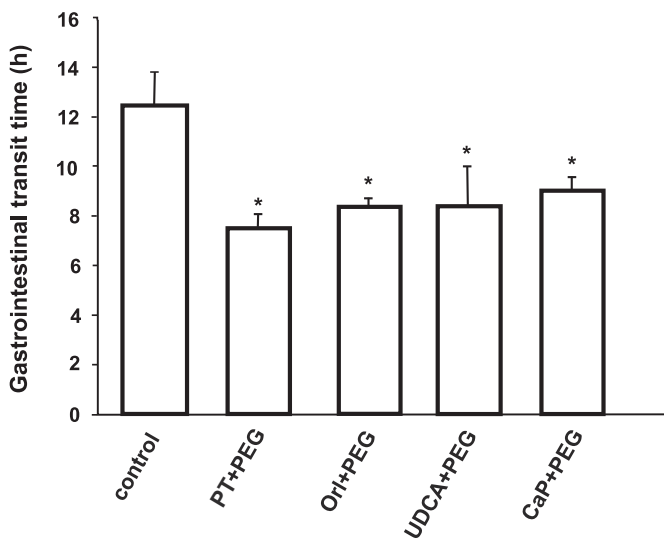
**Figure 1.** Treatment with continuous phototherapy (PT), or oral treatment with orlistat (Orl), UDCA, or CaP significantly decreased plasma bilirubin concentrations after 3 wk and PEG induced an additive therapeutic effect in phototherapy- and orlistat-treated animals. Gunn rats ( $n = 5-6$  per group) received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After 3 wk, these treatments were combined with PEG administration for an additional 3 wk. \* $p < 0.001$ , compared with baseline values. † $p < 0.01$ , compared with single treatment.

and 45%, respectively, compared with baseline values ( $p < 0.001$ ). Figure 2 shows that, after 3 wk of treatment, neither phototherapy nor oral treatments with orlistat, UDCA, or CaP significantly affected the gastrointestinal transit time, compared with controls.

**Additive therapeutic effect of PEG administration on phototherapy and orlistat treatment.** Because neither phototherapy nor any of the oral treatments accelerated the gastrointestinal transit, we assessed whether adjunct PEG treatment could increase their hypobilirubinemic effect. Figure 3 shows that combining phototherapy, orlistat, UDCA, and CaP with PEG administration for 3 wk accelerated the gastrointestinal transit by 40, 33, 33, and 28%, respectively, compared with controls ( $p < 0.001$ ; each). Figure 1 shows that 3 wk of combined treatment of PEG with phototherapy, orlistat, UDCA, and CaP decreased plasma bilirubin concentrations by 67, 53, 30, and 45%, respectively, ( $p < 0.001$ ). Combining phototherapy or orlistat treatment with PEG thus induced an additive therapeutic effect of 20 and 26%, respectively, compared with single treatment ( $p < 0.01$ ). The hypobilirubinemic effect of PEG + phototherapy is in line with our previous results, which showed a 62% decrease in plasma bilirubin levels after 2 wk of phototherapy + PEG treatment (16). Table 1 shows effect of PEG cotreatment on renal function, liver function, and growth rate in the various treatment groups. Addition of PEG to phototherapy, orlistat, UDCA, or CaP did not affect plasma levels of urea, compared with controls, which pleaded against clinically relevant dehydration during PEG cotreatment. PEG + orlistat treatment resulted in a mild increase in plasma Creat levels compared with the control group, although still within the reference range for Gunn rats (28). UDCA + PEG-treated animals showed a 5% decrease in growth rate during the entire experiment, compared with a 2.5% increase in control animals ( $p < 0.01$ ; Table 1).



**Figure 2.** Three weeks of continuous phototherapy (PT) or oral treatment with orlistat (Ori), UDCA, or CaP did not significantly affect the gastrointestinal transit time. Gunn rats ( $n = 5-6$  per group) received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After 3 wk, we determined the gastrointestinal transit time by measuring the interval between intragastrical administration of carmine red and its appearance in the feces.



**Figure 3.** Combining phototherapy (PT), orlistat (Ori), UDCA, and CaP with PEG administration significantly accelerated the gastrointestinal transit. Gunn rats ( $n = 5-6$  per group) received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After 3 wk, these treatments were combined with PEG administration for an additional 3 wk. After 6 wk, we determined the gastrointestinal transit time as described in Fig. 2. \* $p < 0.001$ , compared with controls.

### Short-term experiment.

**Immediate PEG cotreatment with orlistat is effective within days.** To further explore its clinical potential, we subsequently determined whether immediate PEG cotreatment could accelerate the hypobilirubinemic effect of phototherapy or orlistat during a short-term period of treatment. Figure 4A shows that phototherapy alone decreased plasma bilirubin concentrations by 35% within 48 h ( $p < 0.001$ ), compared with control animals. Immediate cotreatment with PEG accelerated the gastrointestinal transit by 28% ( $p < 0.001$ ; Fig. 4B) and decreased plasma bilirubin concentrations by 37% ( $p <$

0.001; Fig. 4A), compared with controls. Figure 4C shows that orlistat alone decreased plasma bilirubin concentrations by 10% (NS), and that cotreatment with PEG decreased plasma bilirubin by 24% ( $p < 0.001$ ), compared with control animals. PEG thus did not enhance the rapid hypobilirubinemic effect of phototherapy but did increase that of orlistat treatment by 14% ( $p < 0.05$ ). Continuation of treatment in the PEG + phototherapy-treated animals for 2 wk, however, did result in a final decrease of 67%, which was virtually identical to the decrease in the long-term experiment. Table 2 shows that there were no differences renal function, liver function, or growth rates between the various treatment groups.

## DISCUSSION

In this study, we demonstrate that neither routine phototherapy nor experimental oral treatments (e.g. orlistat, UDCA, or CaP) exert their hypobilirubinemic effect via acceleration of the gastrointestinal transit in Gunn rats. We also show that acceleration of the gastrointestinal transit by PEG enhances the therapeutic effects of both phototherapy and orlistat in these animals.

Several studies have suggested a relationship between gastrointestinal transit and plasma bilirubin levels. Conditions that delay the gastrointestinal transit, such as Hirschsprungs disease (29) or fasting (30,31), have been associated with an exaggeration of neonatal jaundice. Conditions that accelerate transit, such as prebiotic supplementation of infant formula, seem to mitigate jaundice in neonates (32,33). We recently demonstrated that acceleration of the gastrointestinal transit by PEG effectively treated unconjugated hyperbilirubinemia in jaundiced Gunn rats (16). The strong relationship between the gastrointestinal transit and plasma UCB concentrations in that study prompted us to evaluate the mechanistic contribution of the transit time in phototherapy and in experimental oral treatments. The hypobilirubinemic effects of these treatments have traditionally been linked to different underlying mechanisms. Phototherapy, the routine treatment for neonatal jaundice, has been shown to exert its hypobilirubinemic effect by conversion of UCB into photoisomers (26). Experimental oral treatments, such as orlistat, UDCA, or CaP, are thought to bind UCB within the gut lumen, thereby preventing its reabsorption and interrupting its enterohepatic circulation (13,27,34). Interestingly, both phototherapy and oral treatments have also been suggested to accelerate the gastrointestinal transit. Phototherapy, especially intensive phototherapy, can induce diarrhea. Diarrhea is also a well-known potential side effect of orlistat and UDCA treatment (35,36). Orlistat has been shown to accelerate gastric emptying (22), whereas UDCA treatment accelerated the gastrointestinal transit in dogs and can improve gastrointestinal motility defects in bile stone patients (20,37). Theoretically, phototherapy or oral treatments could thus well exert (part of) their hypobilirubinemic effect by acceleration of the gastrointestinal transit. Our Gunn rat model, however, allowed us to demonstrate that neither routine phototherapy nor experimental oral treatments exert their hypobilirubinemic effect via acceleration of the gastrointestinal transit. These findings prompted us to inves-

**Table 1.** Renal and liver parameters after 6 wk of treatment

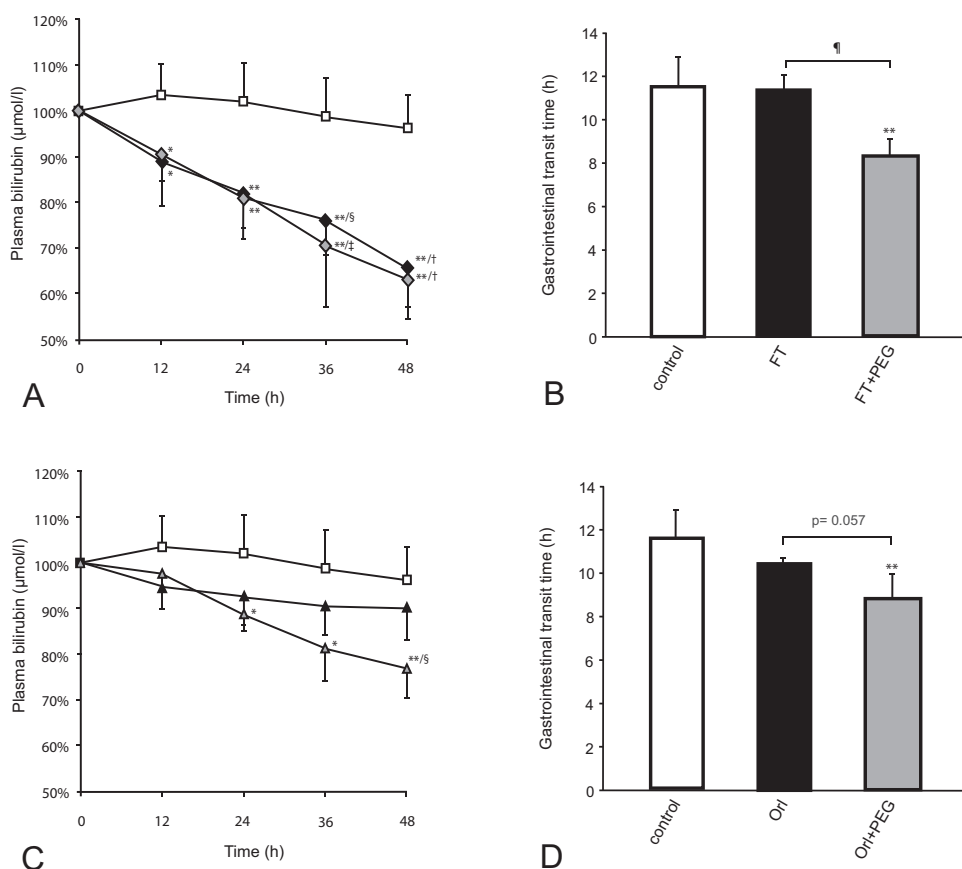
	Controls	PT + PEG	Orl + PEG	UDCA + PEG	Ca + PEG
Renal parameters					
Creat (mmol/L)	17 ± 9	23 ± 12	31 ± 11*	11 ± 3	11 ± 4
Urea (mmol/L)	7.7 ± 2.0	6.0 ± 1.3	7.1 ± 0.9	6.1 ± 1.2	6.9 ± 0.9
Liver parameters					
AST (U/L)	96 ± 37	74 ± 11	86 ± 17	120 ± 37	98 ± 29
ALT (U/L)	35 ± 25	21 ± 7	21 ± 10	29 ± 9	28 ± 6
Growth parameters					
Growth rate (% , compared with $T = 0$ wk)	2.5 ± 4.7	-1.9 ± 4.5	-2.0 ± 2.7	-5.3 ± 1.4†	-1.3 ± 2.2

For experimental setup, please refer to Figure 1. Plasma urea, creatinine, AST, ALT concentrations, and growth rate were determined after 6 wk of treatment. Data represent mean ± SD.

\*  $p < 0.05$ , compared with controls.

†  $p < 0.01$ , compared with controls.

PT, phototherapy; Orl, Orlistat.



**Figure 4.** PEG cotreatment induces an additive hypobilirubinemic effect during orlistat (Orl) treatment (A and C) and accelerates the gastrointestinal transit in all treatment groups (B and D). Gunn rats ( $n = 6-7$  per group) received either no treatment or treatment with phototherapy, phototherapy + PEG, orlistat, or orlistat + PEG. The gastrointestinal transit was determined by administration of carmine red directly after the start of treatment as in Fig. 2. \* $p < 0.05$ ; \*\* $p < 0.001$ , compared with controls. † $p < 0.001$ , compared with single phototherapy treatment. § $p < 0.05$ ; ‡ $p < 0.01$ ; † $p < 0.001$ , compared with single orlistat treatment. □: Controls; ◆: phototherapy (PT); ◇: phototherapy + PEG; ▲: orlistat (Orl); △: orlistat + PEG.

tigate whether PEG cotreatment might induce an additional decrease in plasma bilirubin levels during these treatments. We added PEG cotreatment only after 3 wk to ensure steady-state conditions, which prevented any interference of non-steady-state plasma bilirubin fluctuations (13,16–18).

PEG accelerated the gastrointestinal transit in all treatment groups and induced an additive therapeutic effect in phototherapy- and orlistat-treated animals. The most impressive effect was observed in the phototherapy + PEG treatment group, which concurred with the hypobilirubinemic result of adding phototherapy to PEG treatment alone in a previous study by us (16). The additive therapeutic effect (~20%) thus occurred irrespective of whether phototherapy was added to PEG treatment (previous study) or *vice versa* (present study),

indicating that both therapies truly complement each other (16). The efficacy of phototherapy + PEG treatment, which resulted in a final 67% decrease in plasma bilirubin levels, might well result from the distinct mechanisms by which phototherapy and PEG decrease plasma bilirubin levels. Phototherapy predominantly decreases plasma bilirubin levels by enhancing its disposal via the bile (26), whereas oral treatments, including PEG, selectively enhance net bilirubin diffusion from the blood into the intestinal lumen (13,27). We and others have demonstrated in  $^3\text{H}$ -labeled bilirubin studies that a maximal therapeutic effect often results from combining therapies that enhance distinct pathways for bilirubin disposal (13,26,27). The complementary effect of PEG and phototherapy might thus well result from the combination of their

**Table 2.** Renal and liver parameters after 2 wk of treatment

	Controls	PT	PT + PEG	Orl	Orl + PEG
Renal parameters					
Creat (mmol/L)	16 ± 3	13 ± 2	13 ± 2	19 ± 4	16 ± 4
Urea (mmol/L)	8.6 ± 1.8	5.5 ± 0.7*	6.9 ± 1.5	10 ± 2	5.8 ± 0.9†
Liver parameters					
AST (U/L)	95 ± 26	95 ± 17	88 ± 25	125 ± 45	95 ± 13
ALT (U/L)	55 ± 21	38 ± 7	43 ± 7	73 ± 12	60 ± 12
Growth parameters					
Growth rate (% compared with <i>T</i> = 0 wk)	0.4 ± 2.4	-0.1 ± 0.0	3.7 ± 2.0	4.4 ± 1.4	0.3 ± 2.5

For experimental setup, please refer to Figure 3. Plasma urea, creatinine, AST, and ALT concentrations, and growth rate were determined after 2 wk of treatment. Data represent mean ± SD.

\* *p* < 0.01, compared with controls.

† *p* < 0.05, compared with controls.

PT, phototherapy; Orl, Orlistat.

distinct hypobilirubinemic mechanisms. The additive effect of PEG was not observed during the first days of phototherapy treatment. We speculate that this occurred because short-term phototherapy extracted bilirubin at a maximal rate from the body. This rate could thus not be further increased by PEG cotreatment. Short-term orlistat treatment, however, was notably less effective compared with phototherapy. In agreement with our postulate above, PEG was a useful adjunct strategy in this treatment group.

PEG cotreatment did not induce an additive therapeutic effect in UDCA- or CaP-treated animals. This lack of additive effect cannot be attributed to an insufficient acceleration of the gastrointestinal transit, which was comparable between all PEG-treated groups. PEG administration, however, has several effects on the intestinal *milieu* that might counter the UDCA or CaP-induced decrease in plasma UCB levels. First, PEG decreases the amount of bile salts in the feces (13). This, evidently, would counteract the effect of simultaneous UDCA feeding. Second, PEG decreases the formation of secondary bile salts, possibly by affecting the microflora that promotes bile salt metabolism. This microflora, however, is also involved in the irreversible degradation of intestinal bilirubin into urobilinoids, which is promoted by UDCA feeding (13,16). PEG might thus well interfere with the apparent ability of UDCA to promote intestinal bilirubin degradation. Finally, PEG increases the amount of intestinal water and accelerates the gastrointestinal transit. This might well prevent the precipitation of amorphous CaP with intestinal bilirubin during CaP treatment (34).

Animals that were treated with UDCA + PEG showed a small (-5%), but significant, decrease in growth rate compared with control animals. Although this was not observed in other groups, it should be emphasized that diarrhea and malabsorption of vitamins (A, D, E, K) have been reported during orlistat, UDCA, and CaP treatment (18,35,36,38). Regular assessments of the nutritional (vitamin) status and/or vitamin supplementation would be indicated if one would combine these treatments with a laxative such as PEG in patient studies. These precautions may not apply for the combination of PEG with phototherapy, which seems safe and deserves further exploration in hyperbilirubinemic patient studies.

In conclusion, our data show that that neither phototherapy nor orlistat, UDCA, or CaP exerts its hypobilirubinemic effect

via acceleration of the gastrointestinal transit. Combining PEG-induced acceleration of the gastrointestinal transit with phototherapy decreased plasma bilirubin levels by 67% in Gunn rats. Our results support the evaluation of PEG cotreatment during phototherapy in clinical trials, *e.g.* in Crigler-Najjar patients.

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