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ARTICLES

Administration of Drugs Known to Inhibit P-Glycoprotein Increases Brain Bilirubin and Alters the Regional Distribution of Bilirubin in Rat Brain

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ABSTRACT

P-Glycoprotein (P-gp) is an ATP-dependent integral plasma membrane efflux pump, expressed in abundance in brain capillary endothelial cells and astrocytes. P-gp contributes to the blood–brain barrier in limiting the influx and retention of a variety of lipophilic compounds, including unconjugated bilirubin. Several drugs block P-gp function and thereby enhance intracellular accumulation of P-gp substrates. In this study we proposed that pretreatment with drugs known to inhibit P-gp function in clinically relevant doses would alter the uptake of bilirubin in the brain of 32- to 36-d-old rats. In the first arm of the study, the animals received pretreatment with an i.v. infusion of either propanolol, erythromycin, verapamil, ceftriaxone, rifampin, or saline, 10 min before an i.v. bolus of 50 mg/kg bilirubin was given. Except for the erythromycin-treated rats, all treatment groups had significantly higher brain-to-serum bilirubin ratios than control animals ($p < 0.05$, Welch's *t* test). In the second arm of the study, treatment with either ceftriaxone or rifampin or saline i.v. preceded a 50 mg/kg i.v. bolus of radioactive bilirubin. Analysis of seven different brain regions by scintillation counting showed that the distribution patterns differed significantly between the study groups ($p < 0.001$, ANOVA), however, not in accordance with a kernicteric staining pattern. Because of limited knowledge of expression and function of P-gp and other membrane transport proteins in the newborn, the implications of this study remain to be seen. We speculate that drugs known to inhibit P-gp function may increase the risk of bilirubin encephalopathy in the hyperbilirubinemic infant. **(*Pediatr Res* 54: 441–445, 2003)**

Abbreviations

BBB, blood–brain barrier
P-gp, P-glycoprotein
UCB, unconjugated bilirubin
MRP, non–P-gp transporter
OATp, non–P-gp transporter

Increased bilirubin entry into brain is presumably associated with increased risk of toxicity. Energy-independent passage to the CNS is restricted by the BBB and is limited to lipid-soluble substances (1). UCB, like several other lipophilic or amphipathic compounds known to cross the BBB, evinces a surprisingly low accumulation in the CNS (2, 3). This phenomenon is incompletely understood, but many of these compounds are substrates for membrane transport proteins that regulate the entry and efflux of substrates across cell membranes (2, 4). P-gp, a member of the ATP-binding cassette superfamily of membrane transporters, is the best-studied member of the membrane transport proteins. There is, however, growing evidence that non–P-gp transporters like MRP and OATp have potential for regulation of drug entry and expulsion across the BBB and the choroid plexus (5, 6).

Membrane transport proteins are expressed in a variety of normal tissues. The gene encoding for P-gp belongs to the *mdr* multigene family, consisting of two highly homologous genes, *mdr1* and *mdr2,* albeit only *mdr1* seems to be involved in the expulsion of xenobiotics (7, 8). Studies on *mdr1*-null mutant mice devoid of BBB P-gp have shown enhanced uptake of a

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variety of lipophilic xenobiotics in brain (9, 10). Watchko *et al.* (11) found that brain bilirubin content was almost 2-fold higher in these mutant mice compared with wild-type animals after an i.v. infusion of bilirubin. Brain bilirubin clearance did not differ between the groups, suggesting that the increased bilirubin content was caused by increased influx and that, hence, UCB is a substrate for P-gp at the BBB.

P-gp limits the entry of several cytotoxic drugs into cells, and is associated with multidrug resistance in cancer therapy (12, 13). Consequently, inhibition of P-gp activity to obtain higher intracellular concentrations of cytotoxic drugs has been a major issue in cancer research (12, 13). Drugs known to inhibit P-gp function, such as cortisol (14, 15) or erythromycin (16), are used in neonatal medicine. It was not known whether the administration of P-gp–inhibiting agents to hyperbilirubinemic infants influences bilirubin entry into brain. In the present study we hypothesized that treatment with drugs known to inhibit P-gp function would modify bilirubin entry into the brain of young adult rats. In the first arm of the study, whole-brain bilirubin entry was analyzed after administration of P-gp blockers followed by an i.v. infusion of bilirubin. Because of their widespread use in clinical medicine and documented inhibition of P-gp, ceftriaxone (17), erythromycin (16), rifampin (18), verapamil (19, 20), and propanolol (21, 22) were chosen. The classic bilirubin-induced brain damage, kernicterus, is localized in the basal ganglia, cerebellum, and cranial nerve nuclei (23). The mechanism(s) behind the preference for these brain regions is not known. In the second arm of the study, two of the drugs from the first study arm, rifampin and ceftriaxone, were chosen to study changes in the regional distribution pattern of bilirubin within the brain.

METHODS

Materials. These experiments were carried out in part in the laboratories of the Department of Anesthesia and Critical Care Medicine, University of Pittsburgh, PA, U.S.A., and in the Neurochemical Laboratory, University of Oslo, Norway. The study was approved through the appropriate review procedures for animal research at both institutions.

Sprague-Dawley rats aged 32–36 d of both sexes were obtained from Hilltop Lab Animals Inc, Scottdale, PA, U.S.A., and from Dyrlæge Møllegaards Avlslaboratorium, Ll Skensved, Denmark. [^{3}H]Bilirubin was synthesized by Moravek Biochemical, Inc, Brea, CA, U.S.A. The specific activity was greater than 50 mCi/mmol and the purity was greater than 90% as determined by HPLC by the manufacturer. Nonradioactive bilirubin and albumin were from Sigma Chemical Co, St. Louis, MO, U.S.A. Other reagents and drugs were from standard commercial suppliers.

Preparation of the reagents. Unlabeled bilirubin was dissolved in 0.1 M NaOH, stabilized with BSA (molar ratio bilirubin:albumin (B:A) = 14), and diluted with Krebs-Ringer buffer, pH 7.4, to a concentration of 3 mg/mL (final pH, 7.8). For the studies involving regional distribution radioactive bilirubin was added to give each rat 20 μCi in a bilirubin bolus of 50 mg/kg i.v. during a period of 5 min, whereas in the studies involving whole-brain uptake only unlabeled bilirubin was used. Bilirubin-containing syringes and tubing were wrapped in tin foil to reduce photodecomposition of bilirubin.

Preparation of the rat model. On the day of the experiment the rats were weighed, and then anesthetized with a s.c. injection of 1–2 mL/kg of a mixture of fentanyl, droperidol, and midazolam (0.025, 1.25, and 0.625 mg/mL, respectively) as previously described (24). Anesthesia was maintained throughout the study by supplementary injections as needed. An i.v. catheter was placed in a dorsal foot vein for infusions.

There were two arms in the study, the first involving bilirubin entry into whole brain, and the second involving regional distribution of bilirubin in brain. In both arms the study drug was administered first, followed by a 10-min interval, after which the bilirubin bolus was given for 5 min, as described above. Ten minutes after the start of the bilirubin infusion, blood samples were obtained by cardiac puncture, and the animals were killed with an i.v. dose of pentobarbital (100 mg/kg).

In the study arm involving whole-brain uptake of bilirubin the following P-gp–inhibiting drugs and doses were used: verapamil (1 mg/kg; $n = 11$), propanolol (0.15 mg/kg; $n = 7$), ceftriaxone (100 mg/kg; $n = 7$), erythromycin (10 mg/kg; $n = 6$), or rifampin (10 mg/kg; $n = 6$). Control rats ($n = 12$) were pretreated with equivalent volumes of saline. In the study arm involving regional distribution, rifampin ($n = 10$), ceftriaxone ($n = 10$), and saline ($n = 10$) were used in the same doses as in the first study arm.

Analyses in brain tissue and serum. Immediately after sacrifice of the rat the chest was opened, the ascending aorta was cannulated, the descending aorta was clamped, the jugular veins were transected, and the cerebral vasculature was flushed *in situ* with 120 mL of ice-cold saline at a rate of 25 mL/min. The brain was removed and carefully stripped of meningeal coverings and surface vessels.

In the first arm of the study the brain was then weighed, and the bilirubin content was determined by acid chloroform extraction (25). In the second arm of the study the brains were dissected into seven regions as described by Glowinski and Iversen (26). Each region was then weighed, and the content of bilirubin was determined by scintillation counting as described previously (24). Serum bilirubin (obtained at sacrifice) was analyzed by the diazo method (27). Serum unbound bilirubin was analyzed with the peroxidase method (28).

Data handling. Data for each experimental group in the first study arm were compared with control animals using unpaired *t* test. Because of heterogeneity of variance, Welch's *t* test was chosen. The serum bilirubin values differed significantly from those of control rats in some of the treatment groups in the first arm of the study. As this might conceivably have influenced brain bilirubin content, a brain-to-serum bilirubin ratio ($\times$1000) was calculated for each animal and used in the subsequent calculations as previously described (29, 30). In the second arm of the study data regarding regional distribution were compared with ANOVA, followed by the Tukey-Kramer multiple comparisons test. The level of significance of $p < 0.05$ was chosen. Data are reported as mean $\pm$ SD.

RESULTS

Effects of pretreatment with drugs known to inhibit P-gp function on bilirubin entry into whole brain. The mean serum bilirubin values in the first arm of the study are shown in Table 1. The mean serum bilirubin value for the verapamil-treated rats was significantly higher than for the control rats and the ceftriaxone-treated animals. This phenomenon was most likely related to serendipitous experimental variability. Unbound serum bilirubin values did not differ among the groups (overall mean ± SD, 36 ± 18 nmol/L). To eliminate a possible systematic error a brain-to-serum bilirubin ratio was calculated. These ratios are shown in Figure 1. With the exception of the group that received erythromycin, all the groups had significantly higher brain-to-serum bilirubin ratios ranging from 143% (verapamil) to 236% (ceftriaxone) compared with control rats.

Effects of pretreatment with drugs known to inhibit P-gp function on bilirubin entry into brain regions. The mechanism(s) behind the preference for basal ganglia, cerebellum, and cranial nerve nuclei as seen in kernicterus is not known. In the second arm of the study, two of the drugs from the first study arm, rifampin and ceftriaxone, were chosen to elucidate possible changes in the regional distribution pattern of bilirubin within the brain. In this arm of the study serum bilirubin values did not differ significantly among the groups (Table 2). Therefore the brain bilirubin concentrations were compared directly using ANOVA. The concentrations differed significantly among brain regions in all three study groups ($p < 0.0001$; Fig. 2). Although the pattern of distribution was similar in the control rats *versus* the ceftriaxone-treated rats, in that both groups had a significantly higher bilirubin concentration in the cerebellum, the rifampin-treated rats differed in having a significantly lower bilirubin concentration in the cerebral cortex.

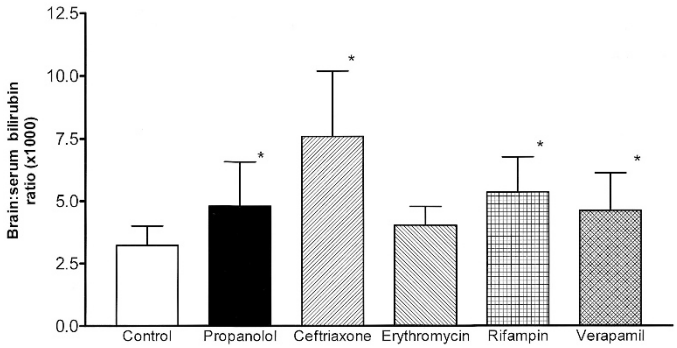

Figure 1. Effect of P-gp–inhibiting drugs on bilirubin entry into whole brain of young adult rats. Immediately after sacrifice of the animals the entire brain was removed and weighed, and the bilirubin content was determined by acid chloroform extraction. For each animal a brain-to-serum bilirubin ratio was calculated. Data are given as brain-to-serum bilirubin ratio ×1000, and represent mean ± SD, $*p < 0.05$ *vs* control. The data were analyzed by Welch's *t* test.

Table 2. *Serum bilirubin values at sacrifice in rats examined for the regional distribution of bilirubin in brain regions**

	Control ($n = 10$)	Ceftriaxone ($n = 10$)	Rifampin ($n = 10$)
Mean ± SD	618 ± 73	535 ± 43	586 ± 104

* The study drug was administered i.v., followed by a 10-min interval, after which labeled bilirubin was given for 5 min. Blood samples were obtained by cardiac puncture immediately before sacrifice of the animals 10 min later. Serum bilirubin was analyzed by diazo method and reported as μmol/L. The groups were compared using ANOVA (NS).

DISCUSSION

The principal findings of this study are that therapeutic doses of several drugs known to inhibit P-gp function *1*) enhance bilirubin entry into the brain and *2*) may impact on the mechanism for localization of bilirubin in rat brain. P-gp–inhibiting drugs appear to have a similar effect on brain bilirubin entry as that previously found in *mdr1a* (−/−) P-gp–deficient mice. Other studies have also found that inhibition of P-gp increases the brain accumulation of P-gp substrates. In one study the P-gp inhibitor cyclosporin A administered intraperitoneally increased brain concentrations of ivermectin 2.5-fold in adult female rats (31). In an *in vivo* microdialysis study in freely moving young adult rats, cyclosporin A induced a 3-fold increase in the cortical concentration of rhodamine 123 (32).

In the 1950s sulfisoxazole was blamed for numerous cases of kernicterus (33). This was subsequently shown to be caused by the ability of sulfisoxazole to displace bilirubin from its binding to albumin. Ceftriaxone is both a P-gp inhibitor and a highly potent displacer of bilirubin. As the brain-to-serum ratio bilirubin in ceftriaxone-treated animals increased relatively more than in former studies on *mdr1*-null mutant mice devoid of P-gp (11), the bilirubin-displacing property of the drug apparently had an additive impact on bilirubin entry into brain. However, pretreatment with agents without a significant bilirubin-displacing effect increased bilirubin accumulation in whole brain by 24% (erythromycin, NS) to 66% (rifampin) (34). This increase corresponds to the increase in brain bilirubin induced by other known risk factors such as hyperosmolarity or hypercapnia. In one study on a comparable group of young adult rats induction of hyperosmolarity or hypercapnia increased bilirubin uptake in whole brain by 39 and 70%, respectively (30).

The mechanism behind the preference for the basal ganglia, cerebellum, and cranial nerve nuclei in kernicterus is not

Table 1. *Serum bilirubin values at sacrifice in rats examined for the uptake of bilirubin in whole brain**

	Control ($n = 12$)	Verapamil ($n = 11$)	Propanolol ($n = 7$)	Ceftriaxone ($n = 7$)	Erythromycin ($n = 6$)	Rifampin ($n = 6$)
Mean ± SD	495 ± 62	609 ± 57	537 ± 108	497 ± 75	597 ± 37	585 ± 74
p value	N/A	0.0002	0.37	0.95	0.0006	0.03

* The study drug was administered i.v., followed by a 10-min interval, after which bilirubin (50 mg/kg, i.v.) was given for 5 min. Blood samples were obtained by cardiac puncture immediately before sacrifice of the animals 10 min later. Serum bilirubin was analyzed by the diazo method, and expressed as μmol/L. The data were analyzed by Welch's *t* test, and each group was contrasted separately with controls.

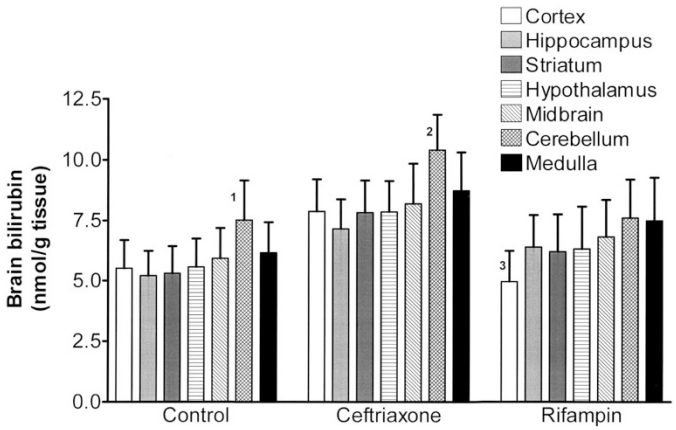

Figure 2. Effect of P-gp–inhibiting drugs on bilirubin entry into brain regions of young adult rats. Immediately after sacrifice of the animals the brains were dissected into seven regions, each region was then weighed, and the content of labeled bilirubin was determined by scintillation counting. Data are expressed as nanomoles of bilirubin per gram of brain tissue and represent mean ± SD. The data were analyzed by ANOVA. Between groups $p < 0.0001$; within groups $p < 0.0001$; 1, $p < 0.05$ *vs* all regions except midbrain and medulla; 2, $p < 0.05$ *vs* all regions except medulla; 3, $p < 0.01$ *vs* cerebellum and medulla.

known (23). In the present study significant differences in the regional entry of bilirubin were found for the cerebellum in the control rats and ceftriaxone-treated rats and for the cortex cerebri in the rifampin-treated rats. For all other areas of the brain bilirubin content was equally enhanced. Few studies have investigated changes in distribution patterns within the brain as a result of P-gp–inhibiting drugs. In two studies by Drion *et al.* (35, 36) pretreatment with the second-generation P-gp blocker PSC-833 or verapamil increased the distribution volume of the cytostatic drug colchicine 8- and 4-fold, respectively, in eight gray areas of the brain, but distribution volumes were similarly enhanced in all brain areas. A possible explanation for the modest impact of P-gp–inhibiting drugs may be that P-gp was unequally saturated in the different areas of the brain. The significance of the altered distribution in the present study is uncertain. Factors other than P-gp may play a role as regards the distribution of bilirubin within the brain, but the specific nature of these factors is unknown. Thus, neither hypercapnia nor hyperosmolarity induced regional differences in bilirubin uptake in young adult rats (30). Also, no differences in the clearance of bilirubin from different brain regions could be detected in young adult rats (24).

The relative contribution of P-gp and non–P-gp processes to drug efflux is largely unknown. This is mainly because of limited knowledge of the distribution and function of membrane transporter proteins at the BBB. Furthermore, there seems to be a considerable overlap in substrate specificity among different transporters and a lack of selective inhibitors. There is, for example, evidence that the quinolone antibacterial agent HSR-903 is a substrate both for P-gp and an anion-sensitive efflux transport mechanism at the BBB (37). Further, both P-gp and OATp2 in rodents and OATpA in the human brain have proven to be involved in the transport of opioid peptides across the BBB (10, 38). UCB is a substrate for MRP in human choriocarcinoma cell lines (39), and OATp2 in liver cells (40). However, transport of UCB by these transporters at the BBB has to our knowledge not been studied. Of the P-gp inhibitors used in the present study, both verapamil and rifampin have been shown to interfere with MRP function (41, 42). More studies of the substrate properties of OATp and MRP at the BBB, and the identification of other membrane transport proteins for which UCB may be a substrate at the BBB, are eagerly awaited.

Although little is known about the ontogeny of MRP and OATp in the brain, brain P-gp expression has been shown to be limited in early fetal life and seems to undergo a consistent up-regulation perinatally. In the human brain P-gp is expressed in capillary endothelial cells, astrocytes, and choroid plexus epithelium by 23 wk gestation (43). At term the distribution pattern was similar to the one found in adult brain, although with markedly less density. In mice, brain P-gp is expressed from embryonic d 16, and adult levels were reached by postnatal d 21 (44). In a recent study in the rat brain P-gp was not detected until postnatal d 7 and was then found mainly in brain capillary endothelial cells in the cerebellum, the hippocampus, and the frontal cortex, reaching adult levels from postnatal d 28 (45). Consequently, the animals of the present study (age, 32–36 d) had all reached adult levels of P-gp expression in the brain. Caution should be taken in extrapolating the findings of the present study to newborns. The functional impact of developmental changes of P-gp and other membrane transport proteins at the BBB in the first weeks of life are not known.

CONCLUSIONS

In conclusion we have shown that administration of therapeutic doses of drugs with modest P-gp–inhibiting properties enhances bilirubin entry into the brain of young adult rats and may have an impact on the distribution pattern of bilirubin in the brain. The finding that drugs without significant bilirubin-displacing properties may enhance bilirubin entry into brain is novel. Because of the limited knowledge of the ontogeny of membrane transport proteins, the overlap in substrate specificity between different transporters, and a lack of selective inhibitors, it is at present not clear whether the drugs used in the present study inhibited P-gp exclusively. Further studies are warranted to clarify the implications of our findings in newborns.

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