

Lipid peroxidation is not the primary mechanism of bilirubin-induced neurologic dysfunction in jaundiced Gunn rat pups

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BACKGROUND: Hazardous levels of bilirubin produce oxidative stress *in vitro* and may play a role in the genesis of bilirubin-induced neurologic dysfunction (BIND). We hypothesized that the antioxidants tauroursodeoxycholic acid (TUDCA), 12S-hydroxy-1,12-pyrazolinomincycline (PMIN), and minocycline (MNC) inhibit oxidative stress and block BIND in hyperbilirubinemic j/j Gunn rat pups that were given sulfadimethoxine to induce bilirubin encephalopathy.

METHODS: At peak postnatal hyperbilirubinemia, j/j Gunn rat pups were dosed with sulfadimethoxine to induce bilirubin encephalopathy. Pups were given TUDCA, PMIN, MNC, or vehicle pretreatment (15 min before sulfadimethoxine). After 24 h, BIND was scored by using a rating scale of neurobehavior and cerebellar tissue 4-hydroxynonenal and protein carbonyl dinitrophenyl content were determined. Nonjaundiced heterozygous N/j pups served as controls.

RESULTS: Administration of sulfadimethoxine induced BIND and lipid peroxidation but not protein oxidation in hyperbilirubinemic j/j pups. TUDCA, PMIN, and MNC each reduced lipid peroxidation to basal levels observed in nonjaundiced N/j controls, but only MNC prevented BIND.

CONCLUSION: These findings show that lipid peroxidation inhibition alone is not sufficient to prevent BIND. We speculate that the neuroprotective efficacy of MNC against BIND involves action(s) independent of, or in addition to, its antioxidant effects.

A variety of pathophysiological mechanisms are postulated to contribute to the irreversible neuronal injury resulting from hazardous hyperbilirubinemia (1). Identifying mechanisms that are operative *in vivo* is essential for the development of potential pharmacologic neuroprotective therapies against bilirubin-induced neurologic dysfunction (BIND) at the organismal level. Findings from *in vitro* studies characterizing unconjugated bilirubin (UCB) effects on neurons and other cells have provided important mechanistic insights and directed much of our understanding of bilirubin-induced cytotoxicity (1). In this regard, oxidative stress observed at elevated UCB levels is a putative mechanism of bilirubin-induced cytotoxicity that has garnered considerable attention (2–4),

supported by studies demonstrating bilirubin-induced protein oxidation and lipid peroxidation *in vitro* (2–4) and lipid peroxidation *in vivo* (5). Moreover, recent *in vitro* work has shown that ursodeoxycholic acid (UDCA), a bile acid with antioxidant properties, and its conjugate derivatives glyoursodeoxycholic acid and tauroursodeoxycholic acid (TUDCA), protect cells, including neurons and astrocytes, from UCB-induced oxidative injury, apoptosis, and necrosis (3,6,7). Oxidative stress is, therefore, considered a target for therapies to curtail BIND.

To date, there is limited study *in vivo* to corroborate these *in vitro* findings and assess the functional impact of antioxidants in animal models of BIND. Study at the organismal level is necessary to firmly establish oxidative stress as a viable target for pharmacologic therapeutic intervention. We hypothesized that antioxidants inhibit oxidative stress and block BIND in the Gunn rat model of kernicterus. Three different antioxidants were administered in a prophylactic mode: TUDCA, 12S-hydroxy-1,12-pyrazolinomincycline (PMIN), a novel derivative of minocycline with potent antioxidant activity (8), and minocycline (MNC), a second-generation tetracycline with antioxidant properties (9) previously shown to prevent BIND in Gunn rat pups (10,11).

RESULTS

Total serum bilirubin (TSB) levels were significantly greater in the jaundiced j/j pups (9.0 ± 1.7 mg/dl; 154 ± 29 μ mol/l) vs. nonjaundiced N/j animals (0.1 ± 0.3 mg/dl; 1.7 ± 5.1 μ mol/l) ($P < 0.001$). Twenty-four hours following sulfa administration, TSB levels were markedly reduced in j/j pups (0.7 ± 0.5 mg/dl; 12 ± 9 μ mol/l), consistent with the displacement of UCB from albumin to tissue. TUDCA, PMIN, MNC, and vehicle when given to j/j pups in the absence of sulfa had no effect on TSB levels (data not shown), i.e., these agents did not appear to displace bilirubin from albumin to any significant degree.

Hyperbilirubinemic j/j Gunn rat pups first demonstrated neurobehavioral abnormalities at ~18 h postsulfa dosing, and elevated BIND scores consistent with advanced stages of bilirubin encephalopathy were evident at 24 h postsulfa dosing as contrasted with j/j animals not dosed with sulfa, that showed normal neurobehavior (Table 1). TUDCA- and PMIN-pretreated j/j

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Table 1. BIND scores of Gunn rat pups as a function of genotype and study condition

	N/j	j/j Saline	j/j Sulfa	j/j Sulfa + TUDCA	j/j Sulfa + PMIN	j/j Sulfa + MNC
BIND score (median and range)	0 (0)	0 (0)	4 (3–5)*	4 (2–5)*	4 (3–5)*	0 (0)

BIND, bilirubin-induced neurologic dysfunction; MNC, minocycline; PMIN, 12S-hydroxy-1,12-pyrazolinomincycline; TUDCA, tauroursodeoxycholic acid.

* $P < 0.001$ vs. N/j, j/j saline, and j/j sulfa + MNC.

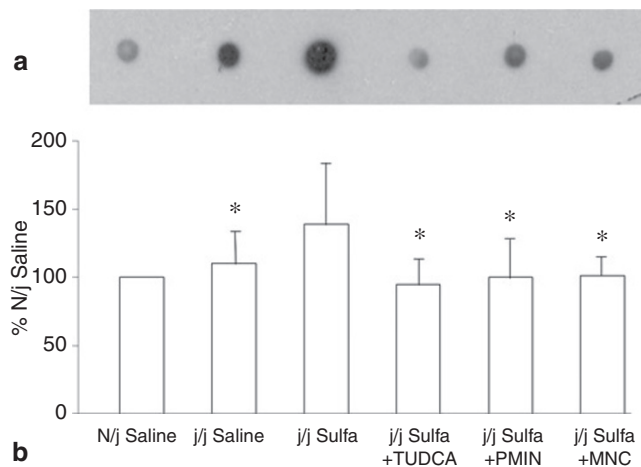


Figure 1. Cerebellar lipid peroxidation as indexed by 4-hydroxy-2-nonenal (4-HNE) levels across study conditions. (a) Representative dot blot of cerebellar 4-HNE adducts and (b) corresponding densitometric quantification of 4-HNE formation across study cohort, expressed as a percentage of that observed in N/j saline pups from N/j saline, j/j saline, j/j sulfa, j/j sulfa + TUDCA, j/j sulfa + PMIN, and j/j sulfa + MNC pups. HNE expression was significantly different across study groups (ANOVA $F = 5.93$; $P < 0.001$); *post hoc* analysis showed that HNE levels in j/j saline, j/j sulfa + TUDCA, j/j sulfa + PMIN, and j/j sulfa + MNC were each significantly lower than those of j/j sulfa animals ($*P < 0.05$) but not significantly different from N/j controls. Each bar represents the mean \pm SD. MNC, minocycline; PMIN, 12S-hydroxy-1,12-pyrazolinomincycline; TUDCA, tauroursodeoxycholic acid.

pups demonstrated significantly elevated BIND scores 24 h after sulfa dosing that were not different from j/j littermates treated with sulfa alone (Table 1). In contrast, MNC pretreatment resulted in BIND scores of 0 24 h after sulfa dosing in j/j pups (Table 1). A subgroup of MNC-pretreated j/j sulfa-dosed pups was followed 4 ($n = 7$) to 7 d ($n = 5$) after treatment, and all showed BIND scores of 0 throughout their respective observation periods. Nonjaundiced heterozygous N/j controls showed BIND scores of 0 whether dosed with sulfa or vehicle (Table 1). TUDCA, PMIN, MNC, and vehicle when administered individually to j/j pups, in absence of sulfa dosing, showed BIND scores of 0, i.e., no adverse neurobehavioral effect (data not shown).

All pups showed a basal level of cerebellar protein oxidation as indexed by dinitrophenyl (DNP) that did not differ across genotype and condition. More specifically, hyperbilirubinemic j/j pups' DNP levels did not differ from those of N/j pups irrespective of j/j sulfa dosing or antioxidant pretreatment (j/j sulfa + TUDCA; j/j sulfa + PMIN; and j/j sulfa + MNC) (ANOVA, $F = 0.276$, $P = 0.93$). In contrast, lipid peroxidation as indexed by cerebellar hydroxynonenal (HNE) levels was evident 24 h postsulfa dosing in j/j pups and significantly increased above the basal levels seen in their N/j and j/j saline littermates.

Moreover, TUDCA, PMIN, and MNC pretreatment in sulfa-dosed j/j pups were each associated with low levels of cerebellar HNE, significantly lower than those observed in j/j sulfa counterparts and not statistically different from those seen in N/j controls and j/j saline pups (Figure 1).

DISCUSSION

The novel observations of this investigation relate to the disparate neuroprotective effects of the three antioxidants against BIND. TUDCA, PMIN, and MNC pretreatment reduced lipid peroxidation during sulfa-induced bilirubin encephalopathy to basal levels observed in nonjaundiced N/j controls consistent with their reported antioxidant activities (8,9,12). However, only MNC was neuroprotective against BIND. The comparable lipid peroxidation inhibition profiles of these three antioxidants, coupled with their disparate effects on BIND, show that lipid peroxidation inhibition alone is not sufficient to prevent BIND and strongly suggest that oxidative stress is not an important mechanism of bilirubin toxicity because oxidation is reduced without reducing encephalopathy.

MNC is a potent antioxidant with free-radical scavenging capacity similar to that of vitamin E (9). Its antioxidant effects are posited to contribute to its broad neuroprotective efficacy across several central nervous system (CNS) injury models (9). In our study, MNC was neuroprotective against BIND, consistent with prior investigations in the Gunn rat model (10,11), and showed antioxidant effects by reducing 4-HNE levels in sulfa-dosed j/j pups to those observed in N/j controls. MNC, however, has protean activities (13), and its neuroprotective efficacy in this instance appears to require more than an antioxidant effect. Further study is needed to determine which MNC properties are necessary and sufficient to prevent BIND.

In this regard, PMIN, a novel MNC derivative synthesized via the reaction of MNC with hydrazine hydrate (8), was studied and also inhibited lipid peroxidation during sulfa-induced BIND in j/j pups. This is not surprising as a previous study reported that PMIN is a more potent antioxidant than MNC at least as indexed by three commonly used assays, 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), and superoxide scavenging *in vitro* (8). The higher antioxidant activity of PMIN relative to MNC *in vitro* is attributed to the presence of an extra heterocyclic ring and supplementary $>NH$ and $-OH$ functions (8). PMIN, however, despite its effective inhibition of lipid peroxidation, did not provide neuroprotection against BIND. This strongly suggests that some nonantioxidant property of the parent compound MNC is lost in the chemical modification producing PMIN. In fact, PMIN as contrasted with MNC is nonchelating, failing to bind Mg^{2+} (8), Zn^{2+} (8), or Ca^{2+} (14), and as a result differs from MNC in being

both nonbactericidal and devoid of matrix metalloproteinase inhibitory activity (8,14). The former would not be expected to impact BIND risk whereas the latter might (13) and therefore merits further study. Given the broad array of enzymes dependent on Ca^{2+} , Mg^{2+} , and/or Zn^{2+} , it is likely that other functions of MNC are not evidenced by PMIN. PMIN is, however, able to chelate Cu^{2+} , a catalyst of free-radical formation and contributor to oxidative stress (8). PMIN also reportedly shows anti-inflammatory effects similar to those of MNC (14).

TUDCA, a taurine conjugate of UDCA and potent antioxidant, also inhibited cerebellar lipid peroxidation during sulfa-induced BIND in j/j Gunn rat pups. This finding is consistent with prior work demonstrating TUDCA's antioxidant effects (12,15,16). TUDCA, however, like PMIN, did not provide neuroprotection against BIND. Prior *in vitro* work reported that UDCA and its conjugate derivatives glycooursodeoxycholic acid and TUDCA protect cells, including neurons and astrocytes, from UCB-induced oxidative injury, apoptosis, and necrosis (3,6,7). In this study, TUDCA did effectively reduce lipid peroxidation in the CNS during bilirubin encephalopathy but did not prevent bilirubin-induced neurotoxicity, as reflected by elevated BIND scores. How can we reconcile these discrepant effects against UCB-induced neurotoxicity *in vitro* and *in vivo*? Monotypic cell cultures are valuable in identifying operative mechanisms of cell injury but by design limit the potential modifying influence of other cell types and cell-cell interactions whose interplay is likely critical to tissue homeostasis and overall CNS function. Indeed, neurons and glial cells respond differently to the toxicity of UCB (17) and the latter cell type may impact the vulnerability of neurons to injury (18). We speculate that other cell-cell interactions and changes to the metabolic milieu induced by UCB and modified by MNC are likely involved as well. Our data show this must be the case with hazardous hyperbilirubinemia and underscore the importance of complementing *in vitro* monotypic cell study with *in vivo* organismal approaches in the investigation of bilirubin-induced neurologic dysfunction and in the testing of agents designed to provide pharmacologic neuroprotection against BIND.

Unconjugated bilirubin at low concentrations demonstrates potent antioxidant properties that may represent a physiologically relevant defense against oxidative stress (19). However, at higher hazardous concentrations bilirubin induces oxidative stress. Indeed, this study demonstrated increased levels of cerebellar lipid peroxidation in hyperbilirubinemic j/j Gunn rat pups during sulfa-induced BIND, a finding consistent with previous reports of bilirubin-induced oxidation of lipids *in vitro* (2,3). The latter investigations used synaptosomes (2) or primary monotypic neuronal cultures (3) and showed modest but significant increases in 4-HNE levels in both systems (2,3). Corresponding prior *in vivo* study is limited to the work of Park *et al.* (5), who observed significantly elevated levels of conjugated dienes, a marker of lipid peroxidation, in the CNSs of 3-d-old piglets following 4 h of hazardous hyperbilirubinemia (TSB ~27 mg/dl) artificially induced by an intravenous load followed by a continuous infusion of bilirubin, coupled

with sulfa dosing to promote the transfer of free bilirubin into the brain. The significantly increased formation of 4-HNE during sulfa-induced BIND in hyperbilirubinemic j/j Gunn rat pups was essentially identical in degree to the aforementioned *in vitro* and *in vivo* studies (2,3,5).

It is noteworthy that enhanced lipid peroxidation (4-HNE), but not protein oxidation (protein carbonyls), was observed in our study during BIND. This contrasts with prior *in vitro* work in which UCB-induced protein oxidation was observed in conjunction with lipid peroxidation (2,3). The piglet study by Park *et al.* (5) did not assay for protein oxidation. The discordant nature of lipid peroxidation and protein oxidation during BIND in j/j Gunn rat pups may relate to the lipophilic nature of UCB, which might preferentially affect lipid membranes and oxidation of lipid moieties. In addition, an important pathway of HNE biotransformation and clearance is conjugation with glutathione (GSH) (20). Lower GSH concentrations enhance HNE levels, and UCB is reported to cause a dose-dependent depletion of intracellular GSH stores via inhibition of GSH synthesis (2,3). Thus, hazardous UCB levels would be predicted to enhance HNE formation and impair HNE clearance, additive effects resulting in increased HNE levels. The latter is particularly evident at higher UCB concentrations *in vitro* (~1,000 nmol/l) (6), levels predicted to occur in the CNS of j/j Gunn rat pups during sulfadimethoxine-induced BIND (21).

METHODS

The Gunn rat was used to model BIND and the institutional animal care and use committee of the University of Pittsburgh and Magee-Womens Research Institute approved the study protocol. Neonatal hyperbilirubinemia in homozygous j/j Gunn rat pups develops spontaneously as a result of bilirubin conjugating enzyme uridine-diphosphate-glucuronosyl transferase (UGT1) deficiency (22); heterozygous N/j littermates have reduced UGT1 at ~50% of wild-type, do not develop neonatal hyperbilirubinemia or kernicterus, and served as controls (22). Pups were studied between 14 and 18 d of life when postnatal TSB levels naturally peak in j/j animals (23). Litters were the product of homozygous j/j male mating with heterozygous N/j females. Twenty-four hours before being killed, pups were injected with sulfadimethoxine (sulfa) (200 mg/kg i.p.) (Sigma Chemical, St Louis, MO), a long-acting sulfonamide to induce BIND, or an equal volume of vehicle (control). Sulfa acutely displaces bilirubin from albumin (24,25), increases brain bilirubin content (26), and induces characteristic neurobehavioral abnormalities of acute bilirubin encephalopathy (24,25,27). Pups were killed with pentobarbital sodium (50 mg/kg i.p.), and the cerebellum was rapidly removed, snap-frozen in liquid nitrogen, and stored at -80°C while the pups were in a deeply anesthetized state. The cerebellum was the focus of study as this CNS region is the most adversely affected in the Gunn rat model (10,25,28), exhibits the highest brain bilirubin content (26), and is the site of neuropathology underlying much of the Gunn rat neurobehavioral phenotype (ataxia, gait abnormality, and movement disorder) during BIND (27).

Pharmacologic Interventions

MNC and PMIN were solubilized in 20% dimethylsulfoxide and administered 15 min before sulfadimethoxine. MNC dosing (50 mg/kg i.p.) (Sigma Chemical) was based on a previously reported dose-response curve that demonstrated 50 mg/kg i.p. was optimal in terms of neuroprotection against BIND in Gunn rat pups (11). PMIN was synthesized as previously described (8) and provided to the study by Yasuo Konishi and Jean-Manuel Cloarec of the Biotechnology Research Institute, National Research Council of Canada (Montreal). PMIN was studied at 45 mg/kg i.p., a dose

equimolar to 50 mg/kg of MNC (14). This dosage was shown to be safe and to exhibit antinociceptive and anti-inflammatory activities *in vivo* in mice (14). Administration of PMIN and MNC alone in *j/j* pups *i.p.* was not associated with any neurobehavioral abnormalities, change in total serum bilirubin, and/or brain bilirubin content as compared with saline-treated *j/j* counterparts. High-performance liquid chromatography chromatograms of PMIN and MNC demonstrate that PMIN is as hydrophobic as MNC (Y. Konishi, personal communication) and thereby predictive of PMIN CNS penetration on par with the substantial uptake exhibited by MNC (29).

TUDCA (EMD Chemicals, Gibbstown, NJ) is a taurine conjugate of UDCA that crosses the blood-brain barrier (15,30), where it demonstrates antioxidant properties (31) and affords neuroprotection across several murine models of CNS injury (15,30,32). *In vitro*, TUDCA prevents the production of reactive oxygen species (12,16) and limits neuronal apoptosis (30), UCB-induced mitochondrial permeabilization, and cytochrome *c* release (33). The sodium salt of TUDCA (200 mg/kg *s.c.*) or vehicle (0.15 mol/l NaHCO₃) was administered as a single dose 15 min before administration of sulfa. This regimen was based on preliminary studies showing effective lipid peroxidation inhibition at 200 mg/kg in sulfa-dosed *j/j* pups, reports of TUDCA neuroprotection in other murine CNS injury models at doses ranging from 50 to 500 mg/kg (15,30,32), and pilot studies showing toxicity (death) in both *N/j* and nonsulfa-dosed *j/j* pups at doses ≥ 400 mg/kg. Administration of TUDCA (200 mg/kg) alone in *j/j* pups was not associated with any neurobehavioral abnormalities, change in total serum bilirubin, and/or brain bilirubin content as compared with saline treatment in *j/j* counterparts.

Neurobehavioral Testing

Pups were monitored for signs of neuromotor dysfunction 24 h postsulfa dosing immediately before being killed and were assigned a BIND score using a previously described numeric rating scale that quantifies gait abnormalities and dystonia on a scale of 0–5 based on the following findings: 0 = normal; 1 = mildly abnormal with slight hindlimb ataxia, dystonia, and gait abnormality; 2 = mild hindlimb ataxia, dystonia, and gait abnormality with impaired righting reflex; 3 = abnormal as in 2, but with a more severe movement disorder and prolonged righting reflex; 4 = severe failure of locomotion, general lack of spontaneous movement with occasional bursts of hyperactivity and no righting reflex; and 5 = moribund including seizures and/or agonal respirations (27). The evaluator was not blinded to genotype as *j/j* animals are overtly jaundiced but was blinded to treatment assignment (sulfa, vehicle, MNC, PMIN, and TUDCA).

Bilirubin Measurements

TSB levels were determined by the diazo method (34) and reported as mg/dl ($\mu\text{mol/l}$). Care was taken to protect serum samples from ambient light to reduce the photodecomposition of bilirubin. As in previous studies (25,26), the TSB measured in saline-injected littermate controls was taken as the pre-sulfa TSB for their respective sulfa-treated *j/j* counterparts.

Oxidative Stress

Markers for oxidative stress were assessed using cerebellar synaptosomes isolated from study pups and prepared with minor modifications as previously described (2). Briefly, ~ 50 mg of cerebellar tissue was homogenized in 1 ml of buffer containing 0.32 mol/l sucrose, 20 mmol/l HEPES, pH 7.4, 2 mmol/l EDTA, 2 mmol/l EGTA, 0.2 mmol/l phenylmethylsulfonyl fluoride (Sigma Chemical), and 2 μl of protease inhibitor cocktail (Sigma Chemical) using 12 passes of a Potter-Elvehjem homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged (Eppendorf tabletop centrifuge – Model 5403; Fisher Scientific) at 1500g at 4°C for 10 min and the pellet discarded. The supernatant was centrifuged at 20,000g for another 10 min. The final pellet was resuspended in 100 μl of the buffer and the Lowry protein determination was performed. Samples were diluted to 1 mg/ml.

Protein oxidation was measured using the OxiSelect Protein Carbonyl Immunoblot Kit (Cell Biolabs, San Diego, CA) per the supplier's directions. One microgram of each synaptosome preparation was applied to a standard dot blot on polyvinylidene fluoride membrane (Sigma Chemical). Carbonyl groups were derivatized with dinitrophenylhydrazine and the blot incubated overnight at 4°C in rabbit anti-DNP antibody (1:2,000). Secondary goat antirabbit immunoglobulin G-horseradish peroxidase conjugated antibody (1:1,000) (Jackson ImmunoResearch, West Grove, PA) was applied for 1 h at room temperature and enhanced chemiluminescence performed (Western Lighting Plus – ECL kit; Perkin-Elmer, Waltham, MA). An *N/j* sample was run with each dot blot as a control. Films were scanned using Hewlett Packard PrecisionScan Pro 2.52 software, quantitated using SigmaScan Pro Image Analysis software 5.0 (Systat Software, Point Richmond, CA) and the results were reported as percentage *N/j*.

Lipid peroxidation was indexed by levels of 4-HNE, a major end product of free-radical attack on polyunsaturated fatty acids and specific marker of oxidative stress (35). HNE formation has been demonstrated across a range of acute and chronic CNS disorders (36) and may itself be neurotoxic (37). Immobilon-P transfer membrane (Millipore, Temecula, CA) was immersed in methanol for 15 s and washed with water for 2 min with a subsequent 5-min wash in Tris-buffered saline before application of sample. Samples were subjected to dot blot analysis by spotting 1 μl volume (1 μg) to the prewet membrane and then allowed to dry. Lipid peroxidation was detected using a mouse HNE antibody (1:500, R&D Systems, Minneapolis, MN) overnight at 4°C. Rabbit antimouse horseradish peroxidase-labeled secondary antibody (1:1,000, Jackson Immuno Research, West Grove, PA) was applied for 1 h at room temperature and enhanced chemiluminescence was used to detect HNE expression. Quantification was done using densitometry as described earlier.

A total of 74 Gunn rat pups comprising hyperbilirubinemic *j/j* pups across five conditions (saline, sulfa, TUDCA-sulfadimethoxine (TUDCA-sulfa), PMIN-sulfadimethoxine (PMIN-sulfa), or minocycline-sulfa (MNC-sulfa)) and nonjaundiced *N/j* controls were studied and served as the basis for group comparisons. Study groups included (i) *j/j* saline ($n = 12$; age 15.3 ± 1.0 d; weight (wt) 28.8 ± 4.2 g); (ii) *j/j* sulfa ($n = 18$; age 16.2 ± 1.2 d; wt 30.8 ± 5.5 g); (iii) *j/j* TUDCA-sulfa ($n = 11$; age 16.5 ± 1.2 d; wt 32.5 ± 3.3 g); (iv) *j/j* PMIN-sulfa ($n = 8$; age 15.8 ± 1.3 d; wt 29.7 ± 5 g); (v) *j/j* MNC-sulfa ($n = 6$; age 16 ± 0.9 d; wt 30.2 ± 4.9 g), and (vi) *N/j* saline ($n = 19$; age 15.8 ± 1.2 ; wt 32.6 ± 4.2 g). *N/j* sulfa-treated pups showed no neuromotor abnormalities and their brain bilirubin contents were low and not different from those of their saline-treated counterparts. Across all study groups, pups did not differ significantly in postnatal age ($F = 1.50$; $P = 0.20$) or weight ($F = 1.42$; $P = 0.23$) at study.

All data were analyzed as a function of genotype (*j/j* vs. *N/j*) and study condition (*N/j*, *j/j* vehicle, *j/j* sulfa, *j/j* TUDCA-sulfa, *j/j* PMIN-sulfa, and *j/j* MNC-sulfa) using ANOVA. In the event of a significant ANOVA, *post hoc* analysis using the Tukey-Kramer multiple comparison test was performed. Data are reported as mean \pm SD.

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