

Effect of light exposure on metalloporphyrin-treated newborn mice

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BACKGROUND: Neonatal hyperbilirubinemia arises from increased bilirubin production and decreased bilirubin elimination. Although phototherapy safely and effectively reduces bilirubin levels, recent evidence shows that it has adverse effects. Therefore, alternative treatments are warranted. Metalloporphyrins, competitive inhibitors of heme oxygenase (HO), the rate-limiting enzyme in bilirubin production, effectively reduce bilirubin formation; however, many are photoreactive. Here, we investigated possible photosensitizing effects of chromium mesoporphyrin (CrMP) and zinc deuteroporphyrin bis-glycol (ZnBG).

METHODS AND RESULTS: Administration of CrMP or ZnBG to 3-d-old mouse pups (3.75–30.0 μmol/kg intraperitoneally) and exposure to cool white (F20T12CW) and blue (TL20W/52) fluorescent lights (+L) for 3 h, resulted in a dose-dependent mortality (50% lethal dose (LD50) = 21.5 and 19.5 μmol/kg, respectively). In contrast to ZnBG, there was no significant difference in survival between the CrMP+L and CrMP groups. Following 30 μmol/kg ZnBG+L, we found significant weight loss, decreased liver antioxidant capacities, and increased aspartate aminotransaminase levels. At 6-d post-light exposure, ZnBG+L-treated pups showed gross and histologic skin changes at doses >7.5 μmol/kg. No lethality was observed following treatment with 30 μmol ZnBG/kg plus exposure to blue light-emitting diodes. Phototoxicity of ZnBG was dependent on light source, emission spectrum, and irradiance.

CONCLUSION: Low doses of ZnBG (<3.75 μmol/kg) retained maximal HO inhibitory potency without photosensitizing effects, and therefore are potentially useful in treating neonatal hyperbilirubinemia.

Phototherapy is considered safe and effective for treating neonatal hyperbilirubinemia, and therefore protects infants against bilirubin-induced neurotoxicity. Since its introduction in the United States, there has been a dramatic reduction in severe hyperbilirubinemia and the need for exchange transfusions, a high-risk procedure with many possible adverse effects (1,2). Although overall reports about significant toxicity resulting from phototherapy are exceptionally rare, adverse effects have been observed in preterm newborns. Because the antioxidant capacity of preterm newborns is limited, phototherapy

can further decrease defense mechanisms against reactive oxygen species through reducing bilirubin levels and through promoting photochemical reactions. Impaired anticoagulation, re-opening of the patent ductus arteriosus, and platelet destruction have been observed in infants undergoing phototherapy (3). A NICHD trial comparing aggressive vs. conservative phototherapy indicated that extremely low birth weight infants weighing 501–750 g are more susceptible to injurious effects of phototherapy than were infants in the 751–1,000 g cohort. *Post hoc* analyses revealed a significant decrease in the overall rate of neurodevelopmental impairment with aggressive phototherapy; this reduction was offset by an increased mortality in the 501–750 g cohort, which however, was not statistically significant (2). Moreover, phototherapy can affect the immune system via alterations in cytokine production (4). Therefore, identifying alternative treatment strategies primarily targeting high bilirubin producers in extremely low birth weight infants is needed.

In 1981, using metalloporphyrins (Mps) to inhibit bilirubin production was pioneered by Drummond and Kappas (5) and Maines (6). These synthetic heme analogs competitively inhibit heme oxygenase (HO), the enzyme that degrades heme to biliverdin, carbon monoxide (CO), and free iron (Fe²⁺) (7). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase, and the iron-storage protein, ferritin, sequesters Fe²⁺ (8,9). Although many Mps have been shown to effectively inhibit bilirubin production, their clinical use has been viewed critically. Mps can affect nitric oxide synthase or soluble guanylyl cyclase (10,11), can increase gene expression and protein levels of the inducible HO-1 (12), and are potential photosensitizers (13–15). Tin protoporphyrin (SnPP) and tin mesoporphyrin (SnMP) have been studied in human neonates regarding their safety and efficacy (16,17), however, both showed photosensitizing effects in animal models (18), which led to the abandonment of SnPP. SnMP has minimal photosensitizing effects clinically, mainly because of its greater HO inhibitory potency and lower therapeutic dose (6 μmol/kg). Yet it strongly induces HO-1 gene and protein expression (19), has a long duration of action (19), passes the blood-brain barrier (unpublished data), inhibits soluble guanylyl cyclase (11), and contains a nonphysiological metal atom.

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Recently, our studies have focused on chromium mesoporphyrin (CrMP) and zinc deuteroporphyrin bis-glycol (ZnBG). Both are potent inhibitors of HO, are orally absorbable, and have negligible effects on nitric oxide synthase and soluble guanylyl cyclase (10). Moreover, CrMP does not affect brain or spleen HO activity 24 h after oral administration (unpublished data), decreases liver HO activity in a hemolytic model (unpublished data), and is not photoreactive (20). We have shown that ZnBG is highly potent and fast-acting with a short duration of action, minimally increases HO-1 gene expression and protein levels, and decreases HO activity and bilirubin production in a hemolytic mouse model (19,21). Therefore, here, we evaluated the photoreactivity of CrMP and ZnBG in a 3-d-old mouse model under various light conditions.

RESULTS

Photo- and/or Chemical Toxicity of CrMP and ZnBG

There were no significant differences between survival curves of CrMP and CrMP+L groups at any dose (Figure 1a,b). Fifty percent lethal doses (LD50s) were interpolated to be 23.0 and 21.5 $\mu\text{mol}/\text{kg}$ for CrMP and CrMP+L, respectively. Survival percentages in the ZnBG groups were 100% at all doses (Figure 1c). Following light exposure, ZnBG+L showed a concentration-dependent phototoxicity with an LD50 of 19.5 $\mu\text{mol}/\text{kg}$ (Figure 1d). Survival proportions of 15 and 30 $\mu\text{mol}/\text{kg}$ ZnBG/kg+L significantly differed from pups treated at equal doses without light exposure ($P < 0.05$ and $P < 0.001$,

respectively). Because of the observed chemical toxicity of CrMP, further studies were performed using ZnBG only.

Physical Changes After ZnBG+L

Following ZnBG+L treatment, pups showed a dose-dependent weight loss, which was significant for 15 and 30 $\mu\text{mol}/\text{kg}$ ZnBG/kg (Figure 2, $P < 0.001$). Moreover, we observed discomfort during light exposure, lethargy near the end of and post-light treatment, and hypothermia, with increasing intensities for all signs listed in Table 1 associated with increasing ZnBG doses. At 15 and 30 $\mu\text{mol}/\text{kg}$ ZnBG+L doses, signs of severe systemic toxicity (i.e., ataxia, hunched back, cyanosis, and a hypersensitivity to touch) (Table 1) were observed. Survivors of different exposure setups (7.5, 15, and 30 $\mu\text{mol}/\text{kg}$) developed transient skin irritations at injection sites and on scalps, appearing 4–6-d post-light exposure, lasting for ~2 wk, and resolving 3-wk post-light exposure. Hematoxylin and eosin staining of the abdominal skin and scalp of pups treated with 30 $\mu\text{mol}/\text{kg}$ ZnBG/kg+L and exposed to 2 \times BF revealed epithelial hyperplasia with varying degrees of hyperkeratosis and parakeratosis, subepithelial expansion with severe inflammatory infiltration, and gross identifiable ulcerations 6-d post-light exposure (Figure 3).

Liver Damage and Reduced Antioxidant Capacity Following ZnBG+L

Plasma aspartate aminotransaminase (AST) levels (marker of liver function) significantly increased 1.8-fold as compared with

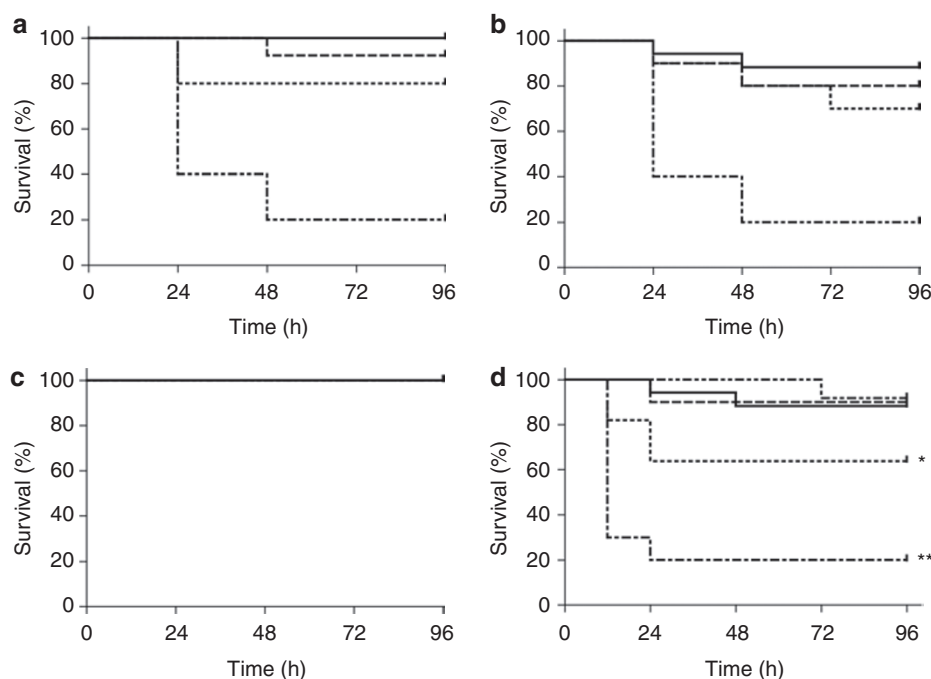


Figure 1. Survival curves of 3-d-old pups after intraperitoneal injection of vehicle (0, solid line), or 3.75 (dashed and dotted line), 7.5 (long dashed line), 15 (short dashed line), or 30 (dashed dotted dotted line) $\mu\text{mol}/\text{kg}$ of (a) chromium mesoporphyrin (CrMP) or zinc deuteroporphyrin bis-glycol (ZnBG) (c) alone and after exposure to 2 \times cool white (CW)/1 \times blue fluorescent (BF) light at $35.0 \pm 1.0 \mu\text{W}/\text{cm}^2/\text{nm}$ for 3 h (CrMP+L and ZnBG+L, (b) and (d), respectively). No significant differences were observed between CrMP and CrMP+L groups for each dose (log-rank test). In contrast, significant differences in survival were found between ZnBG and ZnBG+L groups at 15 and 30 $\mu\text{mol}/\text{kg}$. Pups given ZnBG and kept in subdued light showed no toxic effects, whereas light exposure resulted in a concentration-dependent toxicity (* $P < 0.05$, ** $P < 0.001$ vs. ZnBG at equal doses, log-rank test). $n = 10$ –17 pups from at least three different litters were used in each group.

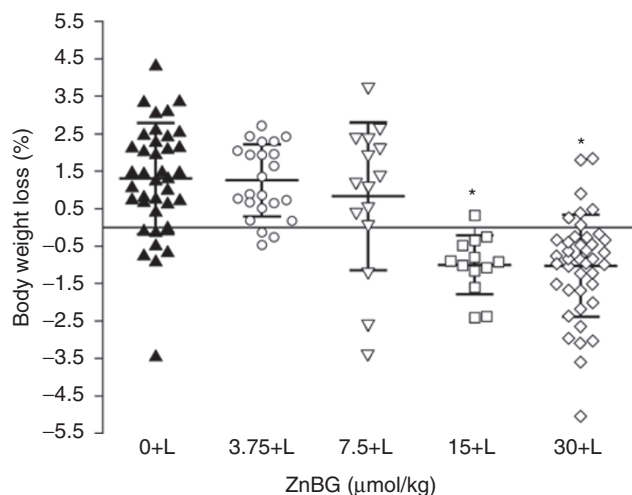


Figure 2. Weight loss after zinc deuteroporphyrin bis-glycol (ZnBG)+L treatment. 3-d-old pups treated with 15 and 30 $\mu\text{mol ZnBG/kg+L}$ ($2\times$ cool white/ $1\times$ blue fluorescent tubes) at $35.0 \pm 1.0 \mu\text{W/cm}^2/\text{nm}$ for 3 h had significant weight loss as compared with light-exposed vehicle (VEH)-treated pups (0+L). * $P < 0.001$ vs. VEH+L, one-way ANOVA, followed by Bonferroni's multiple comparison test.

vehicle-treated (VEH) pups without light treatment. Surprisingly, alkaline phosphatase (ALP) activity significantly decreased by 50% in the ZnBG+L treatment group. A total of 3.75 $\mu\text{mol/kg ZnBG+L}$ treatment did not affect AST values (Figure 4a,b). To assess the potential of liver tissue for oxidation via the Fenton reaction, CO production was measured as an index of lipid peroxidation (LP) and, therefore as an inverse index of tissue antioxidants (22). Post-light exposure, liver LP increased significantly after 30 $\mu\text{mol/kg ZnBG+L}$ ($90 \pm 64 \text{ pmol CO/h/mg fresh weight (FW)}$, $n = 28$, $P < 0.001$) as compared with VEH ($7 \pm 6 \text{ pmol CO/h/mg FW}$, $n = 22$, Figure 4c). VEH+L and 3.75 $\mu\text{mol/kg ZnBG+L}$ showed a similar, but not significant, elevation in LP ($45 \pm 42 \text{ pmol CO/h/mg FW}$, $n = 12$; $39 \pm 44 \text{ pmol CO/h/mg FW}$, $n = 9$, respectively). Of note, intramuscular administration of 10 mg Vitamin E (Vit E)/kg 24 h before the light exposure completely abolished light-mediated increases in LP of VEH+L and ZnBG+L liver tissues ($2 \pm 1 \text{ pmol CO/h/mg FW}$, $n = 3$; $6 \pm 4 \text{ pmol CO/h/mg FW}$, $n = 6$, respectively, $P < 0.01$).

Effect of Different Light Sources

Using different exposure setups (cool white (CW), blue fluorescent (BF), blue light-emitting diode (BLED), or white LED light) (Table 2), we investigated whether ZnBG-mediated phototoxicity was dependent on light source, emission spectrum, and/or irradiance. No significant differences in survival proportion were found between pups given 30 $\mu\text{mol ZnBG/kg}$ and exposed to $2\times$ BF or $2\times$ CW-low irradiance (LI) tubes ($n = 10$, Figure 5a) at $3,491 \pm 80$ and $1,991 \pm 80 \mu\text{W/cm}^2$, respectively (Figure 5b). However, survival proportions of pups exposed to $5,148 \pm 100 \mu\text{W/cm}^2$, $3\times$ CW-high irradiance (HI) were significantly decreased ($P < 0.0001$) as compared with those exposed to $2\times$ CW-LI (Figure 5a,b). Of note, 100% survival was observed after ZnBG administration and exposure to BLED ($n = 13$, Figure 5c) at an irradiance of $2,250 \pm 60 \mu\text{W/cm}^2$

Table 1. Observed physical changes in 3-d-old pups following ZnBG administration at various concentrations during and after exposure to fluorescent light ($2\times$ CW/ $1\times$ BF) at an irradiance of $35.0 \pm 1.0 \mu\text{W/cm}^2/\text{nm}$ for 3 h

ZnBG ($\mu\text{mol/kg}$)	0	0+L	3.75+L	7.5+L	15+L	30+L
Weight loss	-	-	-	+	+++	+++
Discomfort (early)	-	+	+	+	+++	+++
Lethargy (late)	-	+	+	++	+++	+++
Hunched back	-	-	-	+	++	+++
Time-to-flip from hunched back to front	Normal	Slow	Slow	Slower	Slowest	Unable
Ataxia	-	-	-	+	++	+++
Temperature loss	-	+(+)	+(+)	++	+++	+++
Cyanosis	-	-	-	++	+++	+++
Pain	-	-	-	+	++	+++
Sensitivity to touch	-	-	-	++	++	+++
Skin reactions (up to 14 d post)	-	-	-	++	ND	*ND

-, Not present; +, mildly present; ++, present; +++, dominant; BF, blue fluorescent; CW, cool white; ND, not determined; ZnBG, zinc deuteroporphyrin bis-glycol.

(Figure 5d). After exposure to white LED-LI and -HI, survival significantly decreased as compared with BLED and survival proportions were similar to those in pups exposed to $2\times$ CW-LI or $3\times$ CW-HI light.

Safety and Efficacy of ZnBG

To assess the effect of light exposure on ZnBG inhibitory potency, we measured liver HO activity 3 h after administration (HO Inhibition) and light exposure (HO Inhibition+L). Up to 3.75 $\mu\text{mol/kg}$, ZnBG appeared to have no photosensitizing effects and still retained its potency, maximally inhibiting liver HO activity to $76 \pm 4\%$ (Figure 6). However, inhibitory potency decreased $\sim 17\%$ (to $59 \pm 6\%$) after exposure to $2\times$ CW/ $1\times$ BF.

DISCUSSION

During the past three decades, the potential use of Mps for preventing neonatal hyperbilirubinemia has been evaluated. Clinical studies of SnPP and SnMP have shown their efficacy, yet there is still no Mp available for human use. Here, we studied the photosensitizing effects of CrMP and ZnBG in 3-d-old pups. We found that CrMP shows no phototoxicity but does show chemical toxicity, with an $\text{LD}_{50} = 23 \mu\text{mol/kg}$. In contrast, ZnBG is phototoxic only, with an $\text{LD}_{50} = 19.5 \mu\text{mol/kg}$. ZnBG's phototoxicity was dependent on dose, light source emission spectrum, and irradiance used, and was characterized by significant weight loss, decreased liver antioxidant capacity,

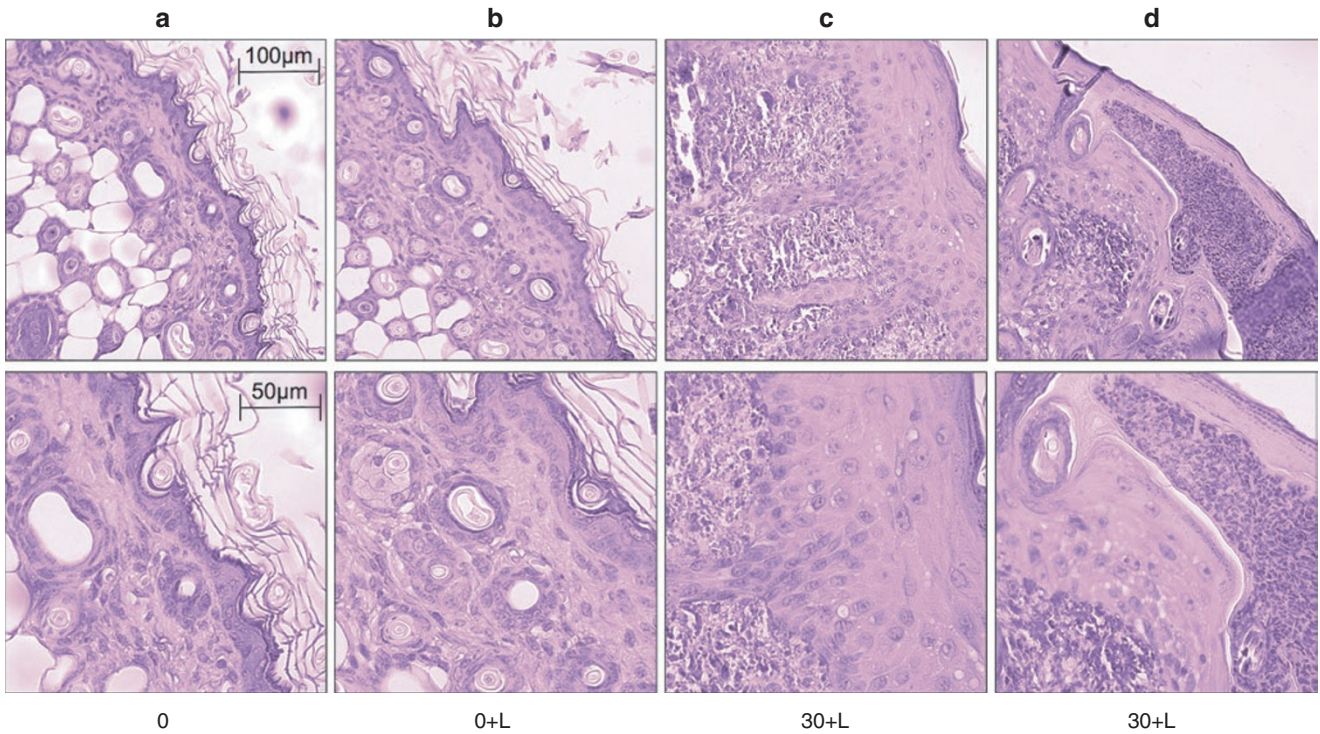


Figure 3. Histological changes in skin after zinc deuteroporphyrin bis-glycol (ZnBG)+L treatment. (a, b) Skin of 3-d-old vehicle (VEH)-treated pups, exposed to 2xblue fluorescent (BF) tubes at $35.0 \pm 1.0 \mu\text{W}/\text{cm}^2/\text{nm}$ for 3 h shows mild hyperplasia of the epidermal squamous epithelium (b, 0+L) compared to VEH-treated no light controls (a, 0). (c, d) Abdominal skin (c, 30+L) and scalp (d, 30+L) of 30 μmol ZnBG/kg-treated pups exposed to BF light for 3 h shows severe damage 6 d post-light exposure. (c) Abdominal skin reveals epithelial hyperplasia with irregular cord-like growths of epithelial cells into the dermis and varying degrees of hyperkeratosis and parakeratosis. (d) Scalp shows predominant inflammatory infiltration and ulceration. Upper and lower panels show sections at 200x and 400x original magnifications, respectively.

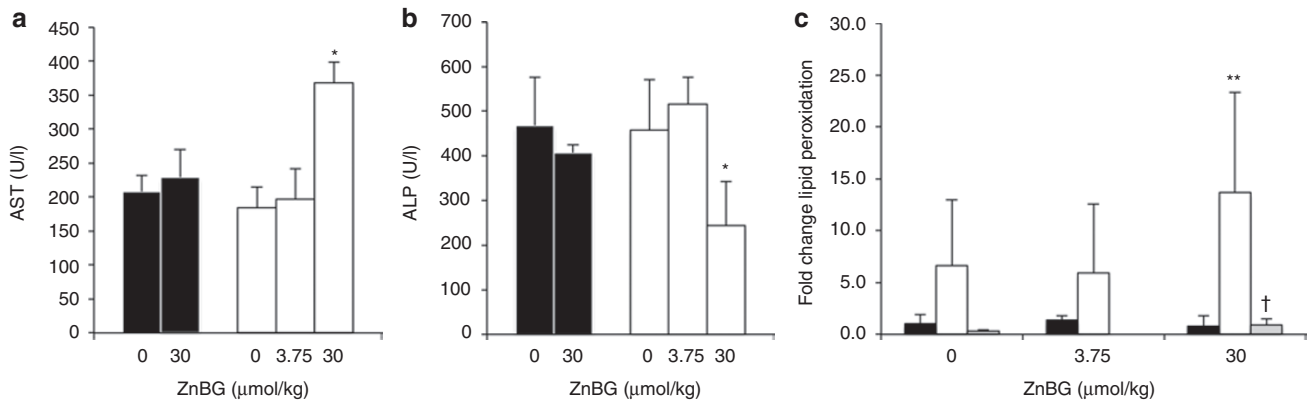


Figure 4. Markers of tissue injury after zinc deuteroporphyrin bis-glycol (ZnBG)+L treatment. (a) Aspartate aminotransaminase (AST) and (b) alkaline phosphatase (ALP) activities after vehicle (VEH) (0) and ZnBG treatment of 3-d-old pups kept in subduced light (black bars) or exposed to 2xcool white (CW)/1xblue fluorescent (BF) (white bars). Pups treated with 30 μmol ZnBG/kg+L showed significant increases in AST levels, and decreases in ALP levels, as compared with VEH-treated mice, whereas pups treated with 3.75 μmol ZnBG/kg+L showed similar AST, and ALP levels to those of VEH-treated pups. Each data point represents pooled tissue ($n = 3$) in the same treatment group with 3–14 data points for each treatment group. * $P < 0.01$ vs. VEH, one-way ANOVA followed by Bonferroni's multiple comparison test. (c) Antioxidant capacity of liver tissues after VEH (0), ZnBG, and VEH or ZnBG treatment plus pre-treatment with 10 mg vitamin E (Vit E)/kg (gray bars) of 3-d-old pups in subduced light (black bars), or exposed to 2xCW/1xBF light (white bars). Lipid peroxidation (LP) significantly increased in liver tissues of pups treated with 30 μmol ZnBG/kg+L as compared with VEH. Administration of 10 mg Vit E/kg intramuscularly, 24 h before ZnBG injection (ZnBG+L+Vit E), decreased LP and therefore increased liver antioxidant capacity up to VEH values. $n \geq 12$ with and without ZnBG administration, $n \geq 3$ for Vit E-treated groups. ** $P < 0.001$ vs. VEH; † $P < 0.01$ vs. ZnBG+L, one-way ANOVA followed by Bonferroni's multiple comparison test.

liver injury, skin reactions, and a decreased HO inhibitory potency. However, ZnBG at doses $\leq 3.75 \mu\text{mol}/\text{kg}$ did not appear to be photosensitizing, but was still highly effective toward inhibiting HO.

These results confirm earlier *in vitro* observations describing CrMP as not photoreactive (20). Although we found no photosensitizing effects of CrMP *in vivo*, its safe use in neonates is concerning because of its chemical toxicity, which

was previously shown in rabbits (23). Because of its high HO inhibitory potency (24), CrMP's toxicity may occur only beyond therapeutic doses. However, we still found 30% inhibition of liver HO activity 5 wk after a single intraperitoneal dose of 7.5 $\mu\text{mol/kg}$ (data not shown), suggesting a long duration of action and poor elimination. Furthermore, accumulation in tissues to toxic levels after multiple applications is conceivable. Scheingraber *et al.* (25) demonstrated that 40 μmol CrMP/kg intravenously decreases mean arterial pressure, sinusoidal diameter, and blood flow in rats. Moreover, it induced hemolysis, marked inflammatory responses, and AST levels. It is also possible that the trivalent chromium (Cr^{3+}), which alters liver structures (26), is released from the porphyrin ring. Whether these effects of CrMP and/or Cr^{3+} are responsible for its toxicity observed in this study is uncertain.

As compared with SnPP (LD50 = 11.7 $\mu\text{mol/kg}$), ZnBG is less phototoxic, but has a higher toxicity than SnMP (42% mortality at 30 $\mu\text{mol/kg}$) (18). Our determined LD50 for ZnBG+L is consistent with earlier reports in 1-d-old Wistar rats (LD50 = 23 $\mu\text{mol/kg}$) (27). Although species, light exposure time, and experimental setup were different, the LD50s are relatively similar. In general, Zn porphyrins are weak photosensitizers as compared with Sn analogs (28,29), leading us to assume that the higher photosensitivity of ZnBG as compared with SnMP could be attributed to its BG structure. As compared with deuteroporphyrins with various central metal atoms, deuteroporphyrin BG derivatives (except FeBG) showed significant greater photoreactivity *in vitro* (30). In aqueous solutions, ZnBG is expected to be mainly monomeric because of its more hydrophilic side chains. Some speculate that the aggregation of porphyrins is a major factor determining the quantum yield of singlet oxygen production and that monomeric porphyrins produce singlet oxygen in a greater yield than those aggregated (31).

One striking finding of our study was the absence of ZnBG phototoxicity after BLED exposure. Delaney *et al.* (32) demonstrated that the triplet lifetime of SnPP decreases ~95% when excited at 450 nm. Like SnPP, ZnBG shows similar absorptions peaks at 407, 538, and 575 nm. The narrow BLED emission spectrum (spectral width: 425–525 nm, Table 2, Figure 5d) may just minimally react with the absorption peaks of ZnBG

and therefore does not excite the molecule to a significant degree. In contrast, BF light (Table 2, Figure 5b) has a wider emission spectrum (spectral width: 400–560 nm), including several mercury lines at 403, 435, 545, and 575 nm, which may be responsible, in addition to the higher total irradiance measured with the Ocean Optics spectroradiometer (OOS), for the significant higher mortality in pups exposed to BF light. An increase in irradiance appeared to directly correlate with higher mortality. Regarding the spectral qualities of the light source, full-spectrum visible light, independent of the source, appeared to be the most harmful to ZnBG-treated pups. White LED spectrum shows two distinct emission ranges, 405–475 and 475–740 nm (Figure 5D). Because the shorter wavelength emission range is equal to BLEDs, the higher toxicity may be because of the longer wavelength emission. Further studies are needed to determine if the ZnBG absorption peaks at 538 and 575 nm may have a higher impact on the photoreactivity than the absorption at the Soret peak or if the harm of longer wavelengths is because of a deeper penetration into the skin.

We also found a significant increase in LP in livers after 30 μmol ZnBG/kg+L. There may be a causal association between increased peroxidation with elevated ASTs and the weight loss of ZnBG+L-treated pups. Other studies documented a positive correlation between LP and increased ASTs (33,34). Weight loss may be due to LP tissue damage, leading to alterations of membrane integrity. Also, skin and respiratory water loss as underlying mechanisms have been shown to play a role (18). The significant decreases in ALP levels for the ZnBG+L group were unexpected. Simonatto *et al.* (35) reported that SnPP has no effect on bile flow in bile duct-cannulated rats. Therefore, we assume that ZnBG did not decrease ALP through an increased excretion into the bile. ALP is a zinc-dependent metallo-enzyme, and there is evidence that high plasma Zn^{2+} ion levels can depress ALP activity (36). However, whether light exposure and subsequent photobleaching of ZnBG lead to a release of Zn^{2+} from the porphyrin ring is speculative.

Confirming that phototherapy can decrease antioxidant capacity, we found elevated liver LP and elevated AST activities in VEH+L pups, although these were not statistically significant. In contrast, ZnBG-treated pups had LP and AST levels equal to those of VEH pups. Histological examination

Table 2. Light exposure setups with corresponding light sources and irradiances

Exposure setup	Light source	$I_{400-520}$ BB-II ($\mu\text{W}/\text{cm}^2/\text{nm}$)	TI OOS ($\mu\text{W}/\text{cm}^2$)
2×CW/1×BF	2 F20T12/CW fluorescent tubes (Sylvania, Danvers, MA), 1 TL20W/52 BF tube (Philips, Andover, MA)	35.0 ± 1.0	5,175 ± 100
2×BF	2 TL20W/52 BF tubes (Philips)	35.0 ± 1.0	3,491 ± 80
2×CW-LI	2 F20T12/CW tubes (Sylvania)	5.0 ± 0.8	1,991 ± 80
3×CW-HI	3 F20T12/CW tubes (Sylvania)	13.2 ± 0.8	5,148 ± 100
BLED	Blue LED (neoBLUE, Natus Medical Inc., San Carlos, CA)	35 ± 0.8	2,250 ± 60
WLED-LI	Wrap, 30 × 30 × 0.3 cm, containing 576 equally squared white LEDs (Bright Light LED, Canoga Park, CA)	13.1 ± 0.6	2,106 ± 80
WLED-HI	Wrap, 30 × 30 × 0.3 cm, containing 576 equally squared white LEDs (Bright Light)	24.2 ± 0.8	5,200 ± 100

BB-II, BiliBlanket Meter II; BF, blue fluorescent; BLED, blue light-emitting diode; CW, cool white; HI, high irradiance; LI, low irradiance; TI, total irradiance; WLED, white light-emitting diode.

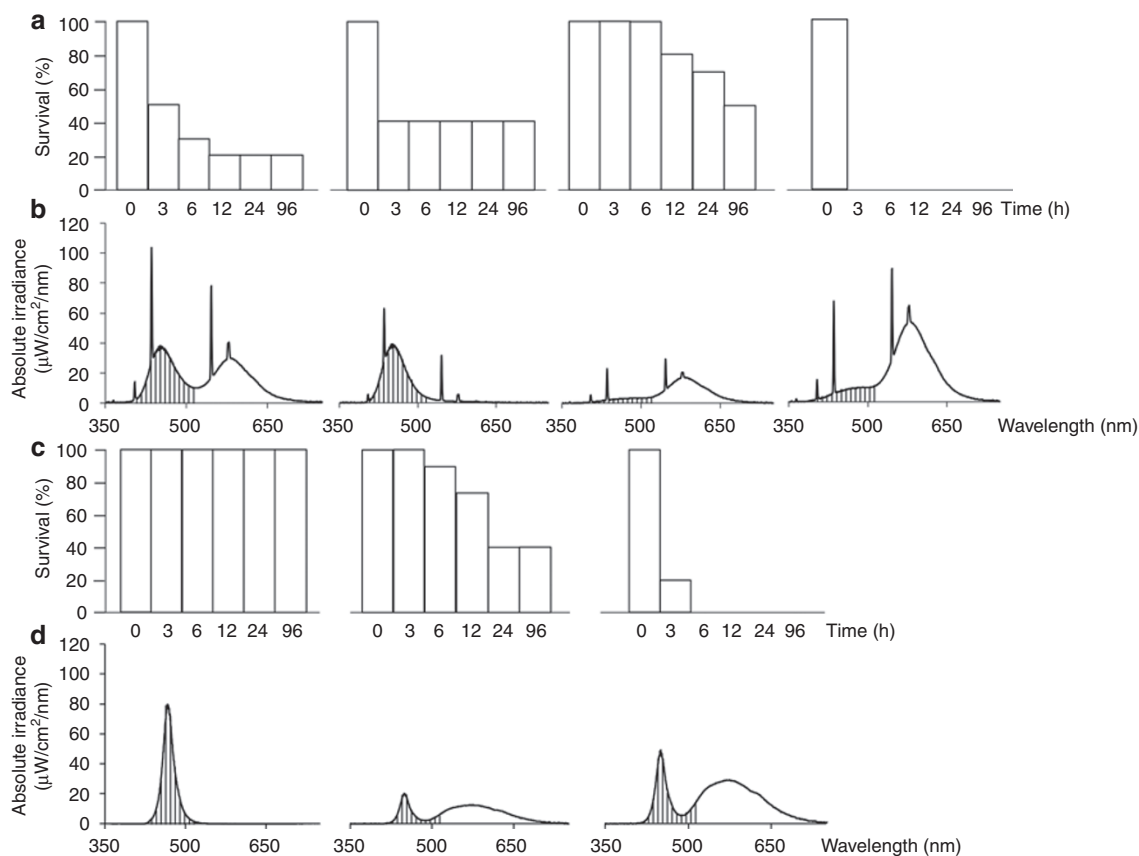


Figure 5. Survival proportions after zinc deuteroporphyrin bis-glycol (ZnBG) treatment and light exposure to different fluorescent and light-emitting diode (LED) light setups. (a) Survival proportions of 3-d-old pups treated with 30 μmol ZnBG/kg and exposed to fluorescent light. Spectra of light sources taken with an Ocean Optics spectroradiometer (OOS) are shown under each respective survival plot in (b). Corresponding total irradiance measured with the OOS and the irradiance in the sensitivity range of the BiliBlanket-II (BB-II) meter from 400–520 nm (represented as the gray striped area or $I_{400-520}$) are listed in Table 2. Percentage survival and spectra of 2×cool white (CW)/1×blue fluorescent (BF), 2×BF, 2×CW, and 3×CW are displayed from left to right. After ZnBG administration mortality was 100% following exposure to 3×CW tubes at high irradiance (HI). Mortality decreased following exposure to 2×BF and 2×CW lights at low irradiance (LI). (c) Survival proportions of 3-d-old pups treated with 30 μmol ZnBG/kg and exposed to LEDs, with the corresponding spectra shown under each respective survival plot in (d). Percentage survival and spectra of blue LED (BLED), white LED (WLED)-LI, and WLED-HI are displayed from left to right. No mortality was observed after 30 μmol ZnBG/kg treatment and exposure to BLEDs. In contrast, mortality significantly increased after exposure to WLED-HI as compared with BLED ($P < 0.0001$) and WLED-LI exposure ($P < 0.0001$).

of liver sections taken from ZnBG+L treated pups, 24 h and 6 d after treatment, showed no morphological changes as compared with livers of VEH pups (data not shown). These findings suggest that the use of ZnBG in a safe treatment strategy, such as protection from intense light exposure, appears less harmful than phototherapy itself. We also performed a pilot experiment treating 3-d-old pups ($n = 6$) with 30 μmol ZnBG/kg and initiating light exposure 3 h post-ZnBG treatment. No mortality was observed (data not shown). Moreover, antioxidant capacity and AST levels of 3.75 μmol/kg ZnBG+L did not differ from VEH+L treatment. Injection of 10 mg Vit E/kg intramuscularly 24 h before 30 μmol/kg ZnBG+L protected livers against LP. Although no reduction in mortality was seen (data not shown), the use of antioxidants could be protective, at least in part, against ZnBG+L-mediated tissue damage. This, in particular, is important in a human neonates, because β-carotene, glutathione, Vit E, and catalase are at very low levels and therefore the antioxidant capacity of tissues is limited (37).

The results of this study characterize CrMP as a compound with no phototoxicity, but one with chemical toxicity. Although its LD50 may be beyond therapeutic doses, its potential clinical use is concerning because of its long duration and risk of accumulation in target tissues. In contrast, ZnBG is photosensitizing but appears to be safe under light-restricted conditions or in low doses. We observed no photosensitizing effects at 3.75 μmol ZnBG/kg+L. The ZnBG-mediated phototoxicity was dependent primarily on its dose but also on the light source emission spectrum and irradiance. Considering its high inhibitory potency and short duration of action, low doses of ZnBG may be a safe therapeutic option in the treatment of neonatal hyperbilirubinemia.

MATERIALS AND METHODS

Reagents

Mps. ZnBG (5.6 mg) and CrMP (5.2 mg) (Frontier Scientific, Logan, UT) were prepared as previously described (19,21) to yield 4 mM stock solutions. VEH was prepared similarly without the addition of Mps. All solutions were prepared in subdued light and stored at 4 °C in the dark until use.

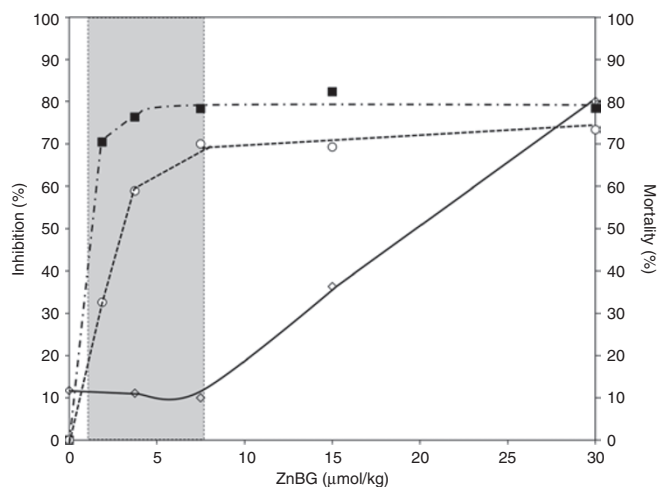


Figure 6. Safety and efficacy of zinc deuteroporphyrin bis-glycol (ZnBG). Treatment of 3-d-old pups with ZnBG up to 7.5 μmol/kg appeared to have no toxic effects (white diamonds) and still maximally inhibited heme oxygenase (HO) activity to 80% (black bars). The inhibitory potency was strongly decreased following fluorescent light exposure (2×cool white/1×blue fluorescent) (white circles). The gray box indicates the estimated therapeutic window for ZnBG.

Methalbumin. Hemin and bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) were dissolved as previously described (19,21) to yield a stock solution of methalbumin (1.5 mM heme/0.15 mM BSA).

Nicotinamide adenine dinucleotide phosphate-oxidase. A 4.5 mM solution was made by dissolving 3.8 mg nicotinamide adenine dinucleotide phosphate, reduced (Calbiochem, San Diego, CA) in 1 ml buffer (0.1M K_2HPO_4/KH_2PO_4 , pH 7.4).

Butylated hydroxytoluene. Butylated hydroxytoluene (10 mM) was dissolved in 95% ethanol.

Vit E. Vit E (50 mg/ml, VIPRIMOL, Hofmann-La Roche, Nutley, NJ) was diluted to 1 mg/ml with saline. A total of 10 μl was injected intramuscularly to reach a final dose of 10 mg/kg.

Materials

For most experiments, two CW (F20T12CW; Sylvania, Danvers, MA.) flanking one BF tube (TL20W/52; Philips, Andover, MA) were placed overhead 20 ± 1.0 cm from the pups, which delivered a spectral irradiance of 35.0 ± 1.0 μW/cm²/nm. In addition, other exposure setups were used (see Table 2). All fluorescent tubes were fitted with reflectors to improve light distribution. Unless indicated differently, irradiance was measured using a BiliBlanket Meter II (BB-II, Ohmeda, GE Healthcare, Fairfield, CT), which measures irradiance in the range of 400–520 nm (peak sensitivity = 450 ± 10 nm, bandwidth = 60 nm). The BB-II is a commonly used meter and its manufacturer claims that it can measure irradiance of fluorescent, halogen, and fiberoptic light sources (15,38). Lamp emission spectra and irradiance was also measured using a flat-response fiberoptic spectrometer (OOS, Ocean Optics S2000, Dunedin, FL), having a measurement range of 200–1100 nm. For each light source, light emission distance to the pup was adjusted to achieve a total irradiance of 2,115 ± 200 μW/cm², defined as LI, and 5,175 ± 125 μW/cm², defined as HI.

Animals

FVB breeders were obtained from Charles River Laboratories (Wilmington, MA). Post-partum, pups were kept with the dam and maintained in the Department of Comparative Medicine (Stanford University, Stanford, CA) until 3-d-old. We used 3-d-old pups because they have no fur (hair) and very thin skin, comparable with the immature translucent skin of the human preterm newborn. The study was approved by Stanford University's Institutional Animal Care and Use Committee.

Experimental Protocol

Pups were randomly given VEH, CrMP, or ZnBG (3.75–30.0 μmol/kg) intraperitoneally, immediately placed in an open plastic tray (8 × 11.5 × 2.5 cm) lined with wet paper towels, and exposed to overhead light for 3 h (VEH+L; CrMP+L; and ZnBG+L). The temperature measured at pup level was 27 ± 1.0°C. Pups were misted with water every 30 min for hydration. Age-matched pups, with and without ZnBG or CrMP, were returned to mothers and kept under subdued light (VEH; CrMP; and ZnBG). Unless otherwise indicated, all experiments were performed using the 2×CW/1×BF light setup.

Histological Staining

At 6 d post-light exposure, skin samples were harvested and placed in 10% formalin, embedded in paraffin after 24 h according to standard protocols, and sectioned (6 μm) using a microtome. Deparaffinized sections were stained with hematoxylin and eosin (American Master*Tech Scientific, Lodi, CA).

Serum Biochemistry

Immediately post-light exposure, pups and controls were killed and blood was collected in lithium-heparin gel tubes (Capiject, Terumo Medical, Somerset, NJ) and centrifuged at 13,000 g × 1 min to separate red blood cells. Blood from three pups was pooled for measurements of AST and ALP levels (Diagnostic Laboratories, Department of Comparative Medicine, Stanford, CA).

Tissue Preparation

After animals were killed, harvested tissues were immediately rinsed with ice-cold buffer, prepared as previously described (19,21), and kept on ice until HO activity or LP was measured.

Lipid Peroxidation

LP measurements were performed as previously described (22). The standard reaction mixture, containing 6 μM $FeSO_4$ (Fe^{2+}), 100 μM potassium ascorbate in buffer (80 μl), tissue sonicate (20 μl = 2 mg FW), and 1 μl butylated hydroxytoluene (100 μM, $n = 2$ blank reactions) was pipetted into $n = 5$ amber glass vials. Capped vials were purged with CO-free air and incubated at 37°C for 30 min. Reactions were terminated by adding 10 μl sulfosalicylic acid (15%, w/v) and placing vials in wet ice. CO released into the headspace was measured by gas chromatography, calculated as pmol CO/h/mg FW, and expressed as fold change from VEH.

HO Activity

A total of 20 μl sonicate was incubated for 15 min at 37°C in CO-free septum-sealed glass vials containing 20 μl 150 μM/15 μM methalbumin and 20 μl 4.5 mM β nicotinamide adenine dinucleotide phosphate, reduced as previously described (39). Blank reaction vials contained buffer instead of β nicotinamide adenine dinucleotide phosphate, reduced. Reactions were terminated by adding 5 μl sulfosalicylic acid and placing the vials in wet ice. CO released into the headspace was quantitated by gas chromatography. HO activity was calculated as pmol CO/h/mg FW and expressed as % inhibition of HO activity.

Data Analyses

Data are presented as mean ± SD. Kaplan–Meier plots were used to determine survival over time, and compared using the log-rank test. One-way ANOVA, followed by Bonferroni's multiple comparison test, was used to compare groups. Differences were considered significant when $P \leq 0.05$.

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