Effects of Zinc Deuteroporphyrin Bis Glycol on Newborn Mice After Heme Loading

CYNTHIA X. HE, CLAIRE M. CAMPBELL, HUI ZHAO, FLORA S. KALISH, STEPHANIE SCHULZ, HENDRIK J. VREMAN, RONALD J. WONG, AND DAVID K. STEVENSON

Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT: Infants with hemolytic diseases frequently develop hyperbilirubinemia and are treated with phototherapy, which only eliminates bilirubin after its production. A better strategy might be to directly inhibit heme oxygenase (HO), the rate-limiting enzyme in bilirubin production. Metalloporphyrins (Mps) are heme analogs that competitively inhibit HO activity *in vitro* and *in vivo* and suppress plasma bilirubin levels *in vivo*. A promising Mp, zinc deuteroporphyrin bis glycol (ZnBG), is orally absorbed and effectively inhibits HO activity at relatively low doses. We determined the I_{50} (the dose needed to inhibit HO activity by 50%) of orally administered ZnBG *in vivo* and then evaluated ZnBG's effects on *in vivo* bilirubin production, HO activity, HO protein levels, and HO-1 gene expression in newborn mice after heme loading, a model analogous to a hemolytic infant. The I_{50} of ZnBG was found to be 4.0 $\mu\text{mol/kg}$ body weight (BW). At a dose of 15 $\mu\text{mol/kg}$ BW, ZnBG reduced *in vivo* bilirubin production, inhibited heme-induced liver HO activity and spleen HO activity to and below baseline, respectively, transiently induced liver and spleen HO-1 gene transcription, and induced liver and spleen HO-1 protein levels. We conclude that ZnBG may be an attractive compound for treating severe neonatal hyperbilirubinemia caused by hemolytic disease. (*Pediatr Res* 70: 467–472, 2011)

The degradation of heme to biliverdin by heme oxygenase (HO) is the rate-limiting step in the production of bilirubin (1). HO degrades heme to produce equimolar amounts of carbon monoxide (CO), iron, and biliverdin, which is rapidly reduced to bilirubin by biliverdin reductase. Because bilirubin production is two to three times higher in a newborn compared with an adult on a body weight (BW) basis (2), and the newborn liver has an immature ability to conjugate and therefore excrete bilirubin, bilirubin can accumulate to excessive levels in the circulation and cause neonatal hyperbilirubinemia.

Infant risk factors for hyperbilirubinemia include prematurity, maternal diabetes, and hemolytic conditions such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, Rh/ABO blood incompatibilities, and closed-space bleeding (3,4). If left untreated, neonatal hyperbilirubinemia can become severe and lead to bilirubin-induced neurologic dysfunction (BIND) or, rarely, permanent irreversible brain injury called kernicterus (5).

Phototherapy and exchange transfusion remain the most common strategies for treating neonatal hyperbilirubinemia, but these approaches only eliminate bilirubin after it has been produced. Moreover, recent evidence suggests that aggressive phototherapy might increase the mortality of extremely LBW (ELBW) infants (6), further emphasizing the need for alternative treatment methods including pharmacological approaches to prevent the bilirubin production and to reduce bilirubin levels in this high-risk group.

Various compounds have been proposed as potential chemopreventive treatments for hyperbilirubinemia, but the most promising are the metalloporphyrins (Mps), which are heme analogs that contain a central metal such as tin, zinc, or chromium, and a porphyrin ring (3,7). Maines (8) and Drummond and Kappas (9) originally reported that Mps, such as zinc and tin protoporphyrins, inhibit HO activity in the liver.

We have also been investigating Mps and have concluded that besides tin mesoporphyrin (SnMP), zinc protoporphyrin, chromium mesoporphyrin (CrMP), and zinc deuteroporphyrin bis glycol (ZnBG) are promising Mps for use in treating neonates with severe hyperbilirubinemia (4,10). We believe that an ideal antihyperbilirubinemic drug should be potent, short acting, not phototoxic, not affect other important enzymes such as NOS and soluble guanylate cyclase (sGC), and not induce HO-1 gene expression (11). We have found that SnMP, which has been used in human trials, has high inhibitory potency (12) but may be photoreactive at therapeutic doses (3,7). SnMP also affects NOS and sGC and induces HO-1 expression, further limiting its clinical use (3,11,13). CrMP and ZnBG are also highly potent (12) and orally absorbable, but minimally affect NOS and sGC (14) and may be less phototoxic than SnMP at effective doses (11,15), suggesting that CrMP and ZnBG may be attractive alternative Mps to SnMP (16).

We have previously shown that *in vivo*, successive exposures of adult mice to 30 μmol heme/kg BW effectively increase HO activity in the liver and spleen at least 2-fold (17) and thus can be used as an animal model to study the

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Correspondence: Ronald J. Wong, M.D., Stanford University School of Medicine, Division of Neonatal & Developmental Medicine, 300 Pasteur Drive, Grant Building Rm. S230, Stanford, CA 94305-5208; e-mail: rjwong@stanford.edu

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Abbreviations: BW, body weight; CrMP, chromium mesoporphyrin; FW, fresh weight; H, subcutaneous heme load; HO, heme oxygenase; I_{50} , dose needed to inhibit HO activity by 50%; MHA, methemalbumin; Mp, metalloporphyrin; OG, oral gavage; sGC, soluble guanylate cyclase; SnMP, tin mesoporphyrin; V, vehicle (saline); VeCO, total body CO excretion rate; ZnBG, zinc deuteroporphyrin bis glycol

hemolytic condition (3). In this study, we first determined the dose of ZnBG needed to inhibit *in vivo* HO activity by 50% (I_{50}) in 1-wk-old mice. We then evaluated the effects of ZnBG on bilirubin production, HO activity, HO-1 mRNA, and protein levels in a heme-loaded newborn mouse model that simulates a hemolytic infant, who has a high risk of developing hyperbilirubinemia.

MATERIALS AND METHODS

Animals. For these studies, 1-, 3-, and 5-wk-old FVB mice were used. Animal use for this study was approved by Stanford University's Institutional Animal Care and Use Committee. One-week-old mice were kept with their mothers.

Reagents. A stock solution of 4 mM ZnBG was prepared by dissolving 4.72 mg of ZnBG (Frontier Scientific, Logan, UT) in 60 μ L of 0.4 M Na_3PO_4 and adding 250 μ L deionized water. The pH was titrated to 7.4 with ~ 25 μ L 1 N HCl, and the final volume was adjusted to 1.70 mL with saline (0.9% NaCl). A 2 mM solution of ZnBG was prepared by diluting the stock solution with saline.

A 4.5 mM solution of reduced nicotinamide adenine dinucleotide phosphate (NADPH; Calbiochem, La Jolla, CA) was prepared by dissolving 3.82 mg of NADPH in 1.0 mL of 0.1 M KPO_4 .

Stock solutions of MHA—a 1.5 mM stock solution used for the HO activity assay and 4.00 mM used for s.c. injection—were prepared as previously described (3). For the HO activity assay, a working solution of 150 μ M MHA was prepared daily by diluting the stock solution with 0.1 M KPO_4 .

ZnBG at doses of 3.75, 7.5, 15, or 30 μ mol/kg BW, or an equal volume of vehicle (saline), was administered by oral gavage (OG) using a 1-mL insulin syringe attached to a 29-gauge needle covered with soft polyethylene tubing. Approximately 30 μ mol/kg BW of heme (MHA), or an equal volume of vehicle, was administered s.c. using a 0.5-mL insulin syringe fitted with a 30-gauge needle. None of the injection volumes exceeded 50 μ L.

Tissue preparation. After mice were killed, livers, brains, and spleens were harvested and rinsed with ice-cold 0.1 M KPO_4 . One hundred milligrams of each tissue was placed into a 1.5-mL microfuge tube. Liver and brain tissues were diluted 10 $\times$ with 0.1 M KPO_4 . Spleen tissues were diluted 15 $\times$ with 0.1 M KPO_4 . Tissues were sonicated at 50% power with a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY).

***In vitro* HO activity.** Because equimolar quantities of CO and bilirubin are produced during heme degradation, the production of CO in the presence of heme and NADPH in tissue sonicates can be used as an index of HO activity (18). Twenty microliters of sonicate (representing 2 mg of liver and brain or 1.33 mg of spleen) was incubated with 20 μ L of 4.5 mM NADPH and 20 μ L of 150 μ M MHA for 15 min at 37°C in septum-sealed 2 mL amber vials (18,19). Reactions were terminated with the addition of 5 μ L of 15% sulfosalicylic acid. CO generated by the reaction into the vial headspace was quantitated using gas chromatography with a Reduction Gas Analyzer (RGA-2, Peak Laboratories LLC, Mountain View, CA). HO activity was calculated as pmoles CO/h/mg fresh weight (FW) and expressed as the percent of HO activity remaining compared with age-matched control levels.

Total body CO excretion rate (VeCO). *In vivo* bilirubin production rates (or VeCO) were measured by placing the 1-wk-old mice in 15 mL acrylic chambers supplied with 10 to 15 mL of air/min for a maximum of 6 h (4). CO concentrations in the outlet air were quantified using gas chromatography. VeCO were calculated as μ L CO/h/kg BW, normalized to baseline levels, and expressed as fold change from baseline (mean $\pm$ SD).

Western blots. Following standard laboratory procedure, 50 μ g of liver, spleen, or brain protein was mixed with equal volumes of 2 $\times$ loading buffer (3). Samples were applied to a 12% polyacrylamide gel, separated using electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). HO-1 and HO-2 proteins were detected, immunocomplexes were visualized, and signals were quantified as previously described (3). Results were expressed as fold change (mean $\pm$ SD) from protein levels of age-matched vehicle-treated control animals.

RT-PCR. Approximately, 5 $\times$ 5 $\times$ 1 mm pieces of liver, spleen, and brain were taken immediately after sacrifice, placed in RNeasy lysis buffer (Qiagen, Valencia, CA), and stored at -80°C until use. Total RNA was extracted and HO-1 mRNA was measured by RT-PCR according to standard laboratory procedure, using the RNeasy Mini Kit (Qiagen) and a Mx-3005 Quantitative PCR

System (Stratagene, Cedar Creek, TX) (3). All results were normalized to 5 β -actin mRNA levels in the same tissues and expressed as fold change (mean $\pm$ SD) from baseline levels.

Statistical analyses. Statistical significance was calculated using unpaired, two-tailed *t* tests, with differences deemed significant when $p \leq 0.05$.

Determination of I_{50} . One-week-old mice were given vehicle, 3.75, 7.5, 15, or 30 μ mol/kg BW of ZnBG by OG. Because we have previously reported that maximum inhibition of HO activity by 30 μ mol ZnBG/kg BW occurs 3 h after oral administration (3), HO activity was quantitated in liver, spleen, and brain at this time point. The I_{50} was determined using interpolation.

Determination of ZnBG effectiveness after heme loading. Although ZnBG is most effective at 3 h after administration (3), hemolysis in infants can be unpredictable or ongoing, and so a single dose of ZnBG should ideally mitigate hemolytic insults occurring more than 3 h after ZnBG administration. As such, to model a more persistent hemolytic condition and extend the efficacy of ZnBG, we administered a higher dose (15 μ mol ZnBG/kg BW) between two heme loads and measured HO activity 24 h after the second heme load, *i.e.* 48 h after ZnBG administration (Fig. 1).

At $t = -24$ h, baseline *in vivo* bilirubin production was measured. Mice were randomly assigned to the following three treatment groups:

- Vehicle-treated controls (V-V-V): vehicle given at $t = 0, 24$, and 48 h.
- Heme controls (H-V-H): heme given at $t = 0$ h, vehicle at $t = 24$ h, and heme at $t = 48$ h.
- ZnBG-treated (H-Zn-H): heme given at $t = 0$ h, 15 μ mol ZnBG/kg BW at $t = 24$ h, and heme at $t = 48$ h.

At $t = 0$ h, 30 μ mol heme/kg BW or vehicle was administered *via* s.c., and VeCO was monitored for up to 6 h to assess the effect of a single heme load on bilirubin production. At $t = 24$ h, 15 μ mol ZnBG/kg BW or vehicle was administered *via* OG. At $t = 48$ h, a second 30 μ mol heme/kg BW dose or vehicle was administered *via* s.c., and VeCO was monitored for up to 6 h to assess the effect of the ZnBG on bilirubin production after a second heme load. A separate set of animals treated with V-V-V, H-V-H, or H-Zn-H was used to assess HO-1 mRNA levels in the liver and spleen at $t = 54$ h, or 6 h after the second heme load. At $t = 72$ h, VeCO was again measured. Animals were then killed, and liver, spleen, and brain tissues were harvested for *in vitro* assays of HO activity, HO-1 mRNA levels, and HO protein levels.

RESULTS

Determination of ZnBG I_{50} . At 3 h after administration of vehicle, liver HO activity was 186 ± 25 pmol CO/h/mg FW

Groups:

Figure 1. Heme-loaded study design. After administration of the first heme load or vehicle at $t = 0$ h, VeCO was monitored for up to 6 h to assess the effect of a single heme load on *in vivo* bilirubin production. At $t = 24$ h, 15 μ mol ZnBG/kg BW or vehicle was administered OG. At $t = 48$ h, a second heme load or vehicle was administered, and VeCO was again monitored for up to 6 h. An additional set of liver and spleen tissues from mice treated with the V-V-V or H-Zn-H regimen was harvested at $t = 54$ h for measurements of HO-1 mRNA levels. At $t = 72$ h, VeCO was again measured. Mice were then killed, and liver, spleen, and brain tissues were harvested for HO activity, HO-1 mRNA, and HO protein levels.

Figure 2. HO activity after treatment with various doses of ZnBG. Liver (A), spleen (B), and brain (C) HO activity was measured 3 h after oral administration with 3.75, 7.5, 15, and 30 μmol ZnBG/kg BW to 1-wk-old mice. Data are expressed as %HO activity (mean $\pm$ SD), $n \geq 3$ mice for each treatment group. * $p \leq 0.05$ compared with the control levels.

Figure 3. Effects of heme and heme/ZnBG on bilirubin production. *In vivo* bilirubin production rates of control (◇), (V-V-V), heme-loaded (▲), (H-V-H), and heme-loaded and ZnBG-treated (■) (H-Zn-H, 15 μmol ZnBG/kg BW) mice were measured after a second heme load, with peak levels measured in H-Zn-H-treated mice averaged and compared with the average peak level of all H-V-H-treated mice. Data are expressed as fold change from baseline (mean $\pm$ SD), $n \geq 5$ mice for each treatment group. * $p \leq 0.05$.

($n = 16$) (Fig. 2A). Administration of 3.75-, 7.5-, 15-, or 30-μmol ZnBG/kg BW by OG resulted in significant inhibition of HO activity to 97 ± 1 (48% inhibition), 73 ± 2 (61%), 71 ± 9 (62%), and 61 ± 8 (67%) pmol CO/h/mg FW,

respectively ($n = 3$ at each ZnBG dose). Interpolation of these results revealed that the I_{50} was ~ 4.0 μmol/kg BW. No significant inhibition on spleen (Fig. 2B) or brain (Fig. 2C) HO activity was found at any dose.

Effectiveness of ZnBG after heme loading

Bilirubin production. No change in the rates of total body CO production, an index of *in vivo* bilirubin production, was observed in the V-V-V group (Fig. 3). After a second heme load (H-V-H), bilirubin production significantly increased to a maximum of 4.5-fold in 2.5 h. In mice treated with 15 μmol ZnBG/kg BW (H-Zn-H), the peak heme-induced fold change was significantly reduced by 19% ($p \leq 0.05$). For all groups, bilirubin production rates returned to baseline by the next day.

HO activity. When HO activity was measured at 24 h after a second heme load, liver HO activity in the V-V-V group was found to be 220 ± 19 pmol CO/h/mg FW ($n = 5$). Liver HO activity in the H-V-H group was significantly increased by 45% (319 ± 35 pmol CO/h/mg FW, $n = 5$) over control levels, as expected (Fig. 4A). In mice treated with 15 μmol ZnBG/kg BW (H-Zn-H), the heme-induced increase in liver HO activity was completely suppressed to 232 ± 9 pmol CO/h/mg FW ($n = 5$), or 105% of control levels.

In the spleen, there was no significant increase in HO activity in the H-V-H group (Fig. 4B), with an H-V-H activity level of 446 ± 30 pmol CO/h/mg FW ($n = 5$) compared with the V-V-V level of 401 ± 82 pmol CO/h/mg FW ($n = 5$).

Figure 4. Effects of heme and heme/ZnBG on HO activity. HO activity (mean $\pm$ SD) in the liver (A), spleen (B), and brain (C) of control (V-V-V), heme-loaded (H-V-H), and heme-loaded and ZnBG-treated (H-Zn-H, 15 μmol ZnBG/kg BW) mice was measured 24 h after a second heme load. Data are expressed as %HO activity (mean $\pm$ SD), $n \geq 5$ mice for each treatment group. * $p \leq 0.05$ compared with V-V-V levels; † $p \leq 0.05$ compared with H-V-H levels.

After ZnBG treatment (H-Zn-H), HO activity was significantly inhibited by 35% (compared with H-V-H) to 288 ± 44 pmol CO/h/mg FW ($n = 5$) or 70% of control (V-V-V) levels. No change in HO activity was found in the brain as a result of either heme loading (H-V-H) or ZnBG treatment (H-Zn-H) (Fig. 4C).

HO-1 protein levels. After measuring HO-1 protein levels at 24 h after a second heme load, we found no significant difference in liver protein levels in the H-V-H group, compared with the V-V-V group (Fig. 5A). In the H-Zn-H group, treatment with ZnBG increased liver HO-1 protein to 1.6-fold. In the spleen, protein levels also did not significantly increase after the second heme load (H-V-H) compared with the V-V-V group (Fig. 5B), whereas after treatment with ZnBG (H-Zn-H), protein levels significantly increased to 1.2-fold. In the brain, protein levels did not significantly change after repeated heme loads (H-V-H) or ZnBG treatment (H-Zn-H) (Fig. 5C). When HO-2 protein levels in the same tissue samples were measured, there were no significant changes found in any tissue or any group.

HO-1 mRNA levels. Because HO-1 protein levels were increased in the H-Zn-H group, we assessed HO-1 mRNA levels in liver and spleen samples from V-V-V, H-V-H, and H-Zn-H animals at 6 h after a second heme load. In the liver, HO-1 mRNA levels did not significantly increase after the second heme load (H-V-H) compared with the V-V-V group but did significantly increase to 2.3-fold after ZnBG treatment (H-Zn-H) (Fig. 6A). In the spleen, HO-1 mRNA levels significantly increased to 2.1-fold after repeated heme loads (H-V-H), with no further significant change after ZnBG treatment (H-Zn-H) (Fig. 6B). When HO-1 mRNA levels were assessed in the liver, spleen, and brain samples taken at 24 h after a second heme load, no significant effects were observed in any group (Table 1).

Figure 5. Effects of heme and heme/ZnBG on HO-1 protein. HO-1 protein (mean $\pm$ SD) in the liver (A), spleen (B), and brain (C) of control (V-V-V), heme-loaded (H-V-H), and heme-loaded and ZnBG-treated (H-Zn-H, 15- μ mol ZnBG/kg BW) mice was measured 24 h after a second heme load. Data were expressed as fold change from baseline (mean $\pm$ SD), $n \geq 5$ mice for each treatment group. * $p \leq 0.05$ compared with V-V-V levels; † $p \leq 0.05$ compared with H-V-H levels.

Figure 6. Effects of heme and heme/ZnBG on HO-1 mRNA, 6 h postsecond heme load. HO-1 mRNA (mean $\pm$ SD) in the liver (A) and spleen (B) of control (V-V-V), heme-loaded (H-V-H), and heme-loaded and ZnBG-treated (H-Zn-H, 15 μ mol ZnBG/kg) mice was measured 6 h after a second heme load and normalized to 5 β -actin mRNA levels in the same tissues, $n \geq 4$ mice for each treatment group. * $p \leq 0.05$ compared with V-V-V levels, † $p \leq 0.05$ compared with H-V-H levels.

Table 1. Effects of heme and heme/ZnBG on HO-1 mRNA, 24 h after a second heme load

Mean ratios of HO-1: β -actin mRNA (mean $\pm$ SD)			
Group	V-V-V	H-V-H	H-Zn-H
Liver	1.00 $\pm$ 0.29	1.16 $\pm$ 0.05	1.46 $\pm$ 0.39
Spleen	1.00 $\pm$ 0.30	0.85 $\pm$ 0.24	1.38 $\pm$ 0.50
Brain	1.00 $\pm$ 0.25	0.86 $\pm$ 0.26	0.98 $\pm$ 0.31

HO-1 mRNA levels (mean $\pm$ SD) in livers, spleens, and brains from control (V-V-V), heme-loaded (H-V-H), and heme-loaded and ZnBG-treated (H-Zn-H) mice were measured 24 h after a second heme load and normalized to β -actin mRNA levels in the same tissues. $n = 3$ mice for each treatment group.

DISCUSSION

First, to evaluate ZnBG’s inhibitory efficacy on HO activity, we orally administered vehicle or a range of ZnBG doses to 1-wk-old mice. We observed that ZnBG is orally absorbed by neonatal mice and can rapidly inhibit liver HO activity in a dose-dependent and organ-specific manner within 3 h of oral administration, with an I_{50} of 4.0 μ mol/kg BW.

Next, to evaluate the effects of ZnBG in heme-loaded newborn mice, we orally administered a higher dose of ZnBG in between two s.c. heme loads to 1-wk-old mice. A property necessary for ZnBG to be an effective antihyperbilirubinemia drug is its ability to reduce *in vivo* bilirubin production. We observed that peak bilirubin production in 1-wk-old mice receiving two heme loads and 15 μ mol ZnBG/kg BW was 19% lower than peak production in heme-loaded mice not treated with ZnBG. Similarly, previous studies of adult mice treated with 30 μ mol SnMP/kg BW at 3 h before a 30- μ mol/kg BW heme load showed a 20% reduction of peak bilirubin production as estimated from total body CO excretion (17).

In addition to effectively reducing bilirubin production, 15 μ mol ZnBG/kg BW completely eliminated heme-induced liver HO activity to baseline levels at 24 h after a second heme load. Unlike in the liver, heme loading did not significantly increase spleen HO activity in the newborn mice. This result corroborates the findings by Braggins *et al.* (20) and Maines (21) that spleen HO-1 is already maximally up-regulated in normal conditions, because it is a primary organ for red blood cell turnover. Under conditions of hemolysis, the liver can

also act as another organ for processing excess heme, which results in the induction of both liver HO activity and transcription. When ZnBG was administered at the 15 $\mu\text{mol/kg}$ BW dose between heme loads, we found that liver and spleen HO activity was reduced to baseline and to 30% below baseline levels, respectively. Inhibition to baseline or near-baseline levels of HO activity is desirable because under- or overmodulation of any key enzyme, such as HO, may be detrimental to a developing neonate (21).

Our observed inhibition of HO activity by ZnBG in the liver and spleen, but not the brain, is most likely due to the delivery route of oral administration because we have shown that *in vitro*, ZnBG inhibits HO activity to comparable degrees in tissue sonicates of liver, spleen, and brain (4). In addition, a recent *in vivo* study also showed that when ZnBG/kg BW was administered i.v. to adult mice, HO activity was inhibited in the liver, spleen, and brain within 1 h after administration (Ronald J. Wong, unpublished data). We have observed that orally administered ZnBG to adult mice results in the inhibition of HO activity in the liver, spleen, and intestine (3). In this study, we also found significant inhibition of liver and spleen HO activity after oral administration of ZnBG to 1-wk-old mice. When a drug is administered orally, it is absorbed directly by the digestive and hepatic portal system—"first pass effect"—and therefore is not available to circulate to other tissues (22), accounting for our observed lack of inhibition of brain HO activity.

At 6-h after administration of repeated heme loads (H-V-H), we found a 2.1-fold increase in spleen HO-1 mRNA. At this same time point, H-Zn-H treatment resulted in 2.3- and 2.2-fold increases in liver and spleen HO-1 mRNA, respectively, correlating with the increased HO-1 protein levels observed in these tissues 24 h later. This up-regulation of HO-1 mRNA appears to be transient, because by 24-h after administration of H-V-H or H-Zn-H, mRNA returned to baseline levels. In adult mice, we found a similar transient increase in HO-1 transcription 6-h after ZnBG administration, which returned to baseline by 24 h (3).

Even though HO-1 protein is induced 24 h after ZnBG treatment, we found that by 2 wk after treatment, liver and spleen HO-1 protein (1.0 ± 0.1 and 1.2 ± 0.2 -fold change, respectively) and HO activity ($101 \pm 4\%$ and $100 \pm 2\%$, respectively) levels have already returned to baseline levels. At 1 wk after treatment, liver HO-1 protein has already returned to baseline levels (1.1 ± 0.1 -fold), whereas spleen HO-1 protein (1.6 ± 0.1 -fold) is still increased. However, this induction was counteracted by a persistent inhibition of spleen HO activity to $77 \pm 5\%$ of baseline. Taken together, the induction of HO-1 by ZnBG most likely has no long-term effects.

Our previous work in adult mice also showed that at 24 h after two successive heme loads, liver HO-1 transcription and protein increased 7- and 2.3-fold over baseline, respectively (17). That heme loads apparently have a more dramatic and sustained effect on HO-1 transcription in adult (17) than in newborn mice may occur in part because HO is developmentally regulated in the liver, being expressed at a higher level in the neonate than the adult. This observation has been shown in the rat liver (23) and the mouse cortex (24). There may also be

suppression of neonatal HO-1 inducibility in response to stresses such as heme loading. Previous reports by Kassovska-Bratinova *et al.* (25), Di Giulio *et al.* (26), and Lavrovsky *et al.* (27) have demonstrated reduced HO-1 inducibility in the neonatal rat because of increased levels of the known HO-1 repressor Bach1 in infancy (25) or increased levels of the HO-1 inducer NF- κ B in adulthood (27). These mechanisms and their potential relevance to hemolysis merit further investigation.

It is possible that the induction of liver and spleen HO-1 in the H-Zn-H group occurs in response to not simply the exogenous heme from heme loads but also the native heme that remains unmetabolized after the inhibition of HO by ZnBG. Kappas *et al.* (28) reported that after inhibition of HO by tin protoporphyrin, the unmetabolized heme is eventually excreted into the bile, and we found that this excretion is proportional to the degree of HO inhibition (29). It is likely that after inhibition of HO by ZnBG, unmetabolized heme (including any excess from heme loading) is eventually excreted through the same route.

Our result (30) and the results of Bonkovsky and co-workers (31,32) have shown that various Mps, including SnMP, up-regulate HO-1 gene transcription by sequestering or causing downstream degradation of Bach1. However, we believe that ZnBG has much less interaction with Bach1 than CrMP or SnMP (S. Schulz, unpublished data), which suggests that ZnBG up-regulates HO-1 through an as-yet-unknown mechanism.

Although ZnBG is similar in photoreactivity to SnMP, ZnBG's high potency could make it clinically effective at much lower doses (11,15). This study showed the effectiveness of oral doses as low as 3.75 μmol ZnBG/kg BW in suppressing HO activity and also showed the effectiveness of 15 μmol ZnBG/kg BW in our hemolytic newborn mouse model. In addition, unlike SnMP, ZnBG has minimal effects on NOS and sGC (14). To further examine the clinical utility of ZnBG, ongoing work includes light exposure studies comparing the relative photosensitivities of newborn mice under treatment with SnMP, CrMP, and ZnBG.

We have demonstrated that ZnBG is orally absorbable, inhibits both basal and heme-induced HO activity in the liver and spleen, and also reduces *in vivo* bilirubin production. Our data strongly suggest that even if ZnBG does up-regulate HO-1 transcription and translation, the effect is a transient one. ZnBG is therefore an attractive compound for oral use in the treatment of neonatal jaundice caused by hemolytic disease, comparable or even preferable to SnMP.

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