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Prevention of neonatal hyperbilirubinaemia in non-human primates by Zn-protoporphyrin

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Non-human primates were used as a model of human neonatal hyperbilirubinaemia and its chemotherapeutic suppression. High levels of haem oxygenase activity were detected in the liver and the spleen of neonatal rhesus (*Macaca mulatta*) and cynomolgus (*Macaca irus*) monkeys. When 1-day-old neonatal animals were given a single injection of Zn-protoporphyrin (40 μmol/kg, subcutaneously), serum bilirubin levels declined to nearly normal adult levels within 24h and remained suppressed throughout the postnatal period (12 days). This treatment inhibited the activities of haem oxygenase and biliverdin reductase in the liver and the spleen, without affecting that of the brain. Zn-protoporphyrin treatment did not alter the activity of brain biliverdin reductase or increase brain bilirubin levels. The biological disposition of Zn-protoporphyrin was examined by measuring the biliary and urinary excretion of the metalloporphyrin complex, as well as its uptake and deposition in blood cells and tissues. Biliary excretion of the metalloporphyrin was minimal (0.12% over a 28h period), and no evidence was detected for the urinary excretion of Zn-protoporphyrin. However, the concentration of metalloporphyrin in erythrocytes increased over the duration of the experiment (11 days) to such an extent that 46% of the administered compound was taken up by the cells. It appeared that the molecular basis for the sustained suppression of haem oxygenase activity and bilirubin production by Zn-protoporphyrin involved the release of the metalloporphyrin in the normal process of the degradation of fetal erythrocytes. The scope of the biological activity of Zn-protoporphyrin to alter haem-dependent processes appeared limited in nature, insofar as the microsomal contents of cytochrome *P*-450 and b_5, as well as the aniline hydroxylase, were similar to those of the control animals. Also, the concentration of glutathione in the liver was unchanged. These findings suggest the potential usefulness of Zn-protoporphyrin in experimental and perhaps clinical conditions in which hyperbilirubinaemia occurs.

Microsomal haem oxygenase is the rate-limiting enzyme in the haem-degradative pathway (Tenhunen *et al.*, 1968; Maines & Kappas, 1974). The product of haem oxygenase activity, biliverdin, is subsequently reduced to bilirubin, a reaction catalysed by biliverdin reductase (Kutty & Maines, 1981). Previous studies have shown that, in rats, synthetic metalloporphyrins such as Zn-(zinc), Cr-(chromium) and Sn-(tin)protoporphyrins inhibit the activity of haem oxygenase *in vivo* and that the mechanism of inhibition is competitive in nature (Maines, 1981; Drummond & Kappas, 1982; Anderson *et al.*, 1984; Maines & Veltman, 1984). Since these porphyrin complexes do not possess oxygen-binding capacity, they are not degraded by haem oxygenase. Ironically, the ability of Sn-protoporphyrin, the metal moiety of which has been shown to be the most potent inducer of haem oxygenase (Kappas & Maines, 1976), to decrease serum bilirubin levels in newborn rats has been reported (Drummond & Kappas, 1982).

Similarly, the ability of Zn-protoporphyrin to decrease serum bilirubin in newborn rats (Drummond & Kappas, 1982) and phenylhydrazine-treated adult rats (Maines & Veltman, 1984) has been reported. However, since there is no clear

Abbreviation used: uro-s, uroporphyrinogen I synthase.

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evidence that post-parturition hyperbilirubinaemia occurs in rats, the ability of Zn-protoporphyrin to prevent physiological jaundice in an appropriate animal model, such as primates, remains to be elucidated. The non-human primate is recognized as the best animal model for many specific investigations of human diseases, including bilirubin metabolism (Cornelius, 1982).

Occasionally the occurrence of hyperbilirubinaemia in the human newborn presents a clinical condition that can prove to be the cause of serious neurological consequences (Lucey, 1982). As do humans, newborn monkeys develop post-parturition hyperbilirubinaemia (Gartner *et al.*, 1977). The aim of the present study was to investigate the ability of Zn-protoporphyrin to prevent neonatal jaundice in the newborn primate and to examine its tissue distribution, excretion and the mechanism by which Zn-protoporphyrin decreases the serum bilirubin levels.

Materials and methods

Porphyrin complexes were obtained from Porphyrin Products, Salt Lake City, UT, U.S.A. All other reagents were purchased from Sigma Chemical Co. St. Louis, MO, U.S.A. *Macaca irus* (cynomolgus) and *Macaca mulatta* (rhesus) monkeys were used. The newborns remained with their mothers throughout the experiments, unless otherwise indicated. The first day after birth, a blood sample was obtained, and the animals were injected subcutaneously with 40 μmol of Zn-protoporphyrin/kg. Control animals received an equal volume of saline (0.9% NaCl). During the next 11 days, blood samples (about 0.5 ml) were collected in heparinized tubes and were used for the determination of the plasma bilirubin concentration (MacDonald, 1965), erythrocyte Zn contents and the activity of uroporphyrinogen-I synthetase (uro-s) (Granick *et al.*, 1972). At 12 days after birth, cerebrospinal fluid samples were obtained, the animals were killed, and tissues were collected for enzyme assays. In other experiments, the newborn animals were killed 24 h after Zn-protoporphyrin treatment. Solutions of Zn-protoporphyrin were prepared as described previously (Maines, 1981). Tissue preparation and cellular fractionations were performed also as detailed previously (Maines, 1981). The activity of haem oxygenase was determined in microsomal preparations (Maines & Kappas, 1975), and biliverdin reductase activity was measured in the cytosolic fractions (Kutty & Maines, 1981). Tissue bilirubin concentrations were measured in the homogenate preparations (Hargreaves, 1965). The microsomal concentrations of cytochromes *P*-450 and b_5 were determined spectrophotometrically (Omura & Sato, 1964). Aniline hydroxylase activity was assessed by a colorimetric procedure (Imai *et al.*, 1966), and glutathione concentration was measured by a fluorimetric method (Cohn & Lyle, 1966).

For the measurement of the biliary excretion of Zn-protoporphyrin, juvenile primates were used. The common bile duct was cannulated, and bile was collected under subdued lighting. The animals were hydrated by the constant infusion of lactated Ringer's solution [obtained from Travenol Laboratories, Deerfield, IL, U.S.A., and containing (mequiv./litre): Na^+, 130; K^+, 4; Ca^{2+}, 3; Cl^-, 109; lactate, 28]. Anaesthesia was maintained throughout the experiment with pentobarbital sodium. The animals were maintained in an incubator (37°C) throughout the experiment.

Urine was analysed for the presence of haemoglobin, and whole cells with Biodynamics Chemstrip-8. The presence of Zn-protoporphyrin in the urine, cerebrospinal fluid, erythrocytes and the bile was determined fluorimetrically (Hart & Piomelli, 1981). Zn was measured by atomic-absorption spectrophotometry. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

All spectral studies were performed on an Aminco DW-2 spectrophotometer. Fluorimetric measurements were made with a Perkin–Elmer fluorescence spectrophotometer. Zn concentrations were determined with a Varian atomic-absorption spectorphotometer. The results are those obtained with a best-matched set of control and treated animals, and are representative of two to three determinations. A total of 12 monkeys were used for the present study.

Results

The potent and prompt action of Zn-protoporphyrin in causing a subsidence in post-parturition hyperbilirubinaemia is demonstrated in Fig. 1. A single subcutaneous injection of Zn-protoporphyrin (40 μmol/kg) to 1-day-old cynomolgus or rhesus newborns lowered plasma bilirubin to near-normal adult levels within 24 h, and the plasma bilirubin levels remained at these low values for the duration of the experiment (12 days). The effect of the Zn-protoporphyrin treatment on the activity of haem oxygenase in the newborn primates is shown in Table 1. In the newborn rhesus monkey treated with Zn-protoporphyrin the activity of haem oxygenase in the spleen and the liver was decreased, whereas that of the kidney and the brain remained unchanged when compared with that of control newborn. Moreover, the level of the bile pigment in the brain was not increased in the treated newborn primates as compared with the

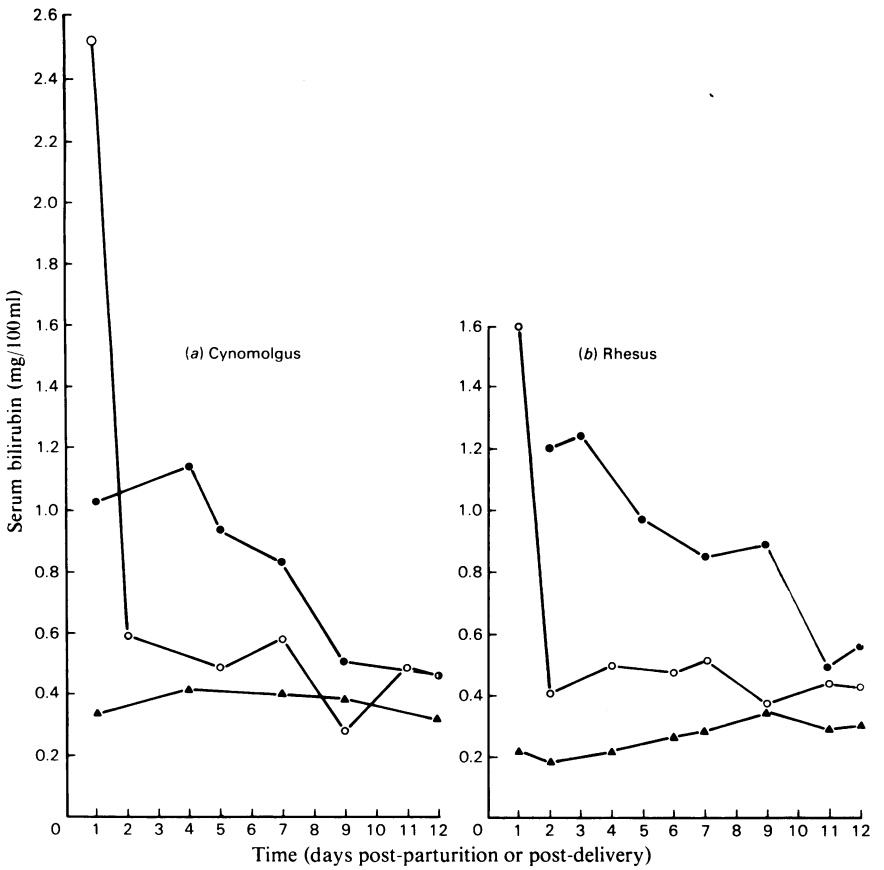

Fig. 1. *Effect of Zn-protoporphyrin treatment on plasma bilirubin concentration*
(*a*) Cynomolgus or (*b*) rhesus monkeys (1 day old) received a single subcutaneous injection of Zn-protoporphyrin (40 μmol/kg). The control newborns received an equivalent volume of saline. Plasma bilirubin was monitored during the 11 days. ●, Control newborn; ○, Zn-protoporphyrin-treated newborn; ▲, mother of Zn-protoporphyrin-treated newborn.

Table 1. *Effect in vivo of Zn-protoporphyrin treatment on liver, spleen, kidney and brain haem oxygenase and biliverdin reductase activities*
The activities of microsomal haem oxygenase and biliverdin reductase were determined 11 days after the treatment of newborn rhesus monkeys with Zn-protoporphyrin. The regimen of treatment with Zn-protoporphyrin was the same as that described in the legend of Fig. 1. The values shown are representative for two animals.

		Activity (nmol of bilirubin/h per mg of protein)	
Organ	Treatment	Haem oxygenase	Biliverdin reductase
Liver	Saline	8.44	3.28
	Zn-protoporphyrin	2.57	1.90
Spleen	Saline	47.12	45.71
	Zn-protoporphyrin	8.04	24.02
Kidney	Saline	1.90	57.84
	Zn-protoporphyrin	2.41	33.70
Brain	Saline	2.44	24.55
	Zn-protoporphyrin	1.99	26.51

controls. The average brain bilirubin concentrations in the control and the treated newborns were 0.23 and 0.22 μg/mg of tissue respectively.

The effect of the Zn-protoporphyrin treatment on the activity of biliverdin reductase, the enzyme that catalyses the conversion of biliverdin into bilirubin, is also shown in Table 1. The activity of the reductase was decreased in the liver, spleen and the kidney of the treated newborns. However, as with haem oxygenase, the activity of biliverdin reductase was not altered in the brain. Since Zn-protoporphyrin exerts a direct inhibitory action on the activities of biliverdin reductase and haem oxygenase (Maines, 1981; Kutty & Maines, 1981), the absence of an effect in the brain may reflect the inaccessibility of the metalloporphyrin to the tissue.

The biological disposition of Zn-protoporphyrin was examined by measuring the biliary and urinary excretion of the metalloporphyrin complex, as well as its uptake and deposition in blood cells and tissues. The biliary excretion pattern of Zn-protoporphyrin is shown in Fig. 2. Bile samples were collected at the indicated intervals, the concentration of Zn-protoporphyrin was measured, and the volume of bile excreted was determined. As shown, 2 h after the administration of Zn-protoporphyrin, the metalloporphyrin appeared in the bile. However, during a 28 h period, only 0.12% of the total Zn-protoporphyrin administered was excreted through this route. A fluorimetric analysis did not reveal Zn-protoporphyrin in the urine and cerebrospinal fluid. The limit of detectability of this method is in the picomolar range. On the other hand, as shown in Fig. 3, the metalloporphyrin was apparently readily taken up by the erythrocytes and accumulated in the cells. The concentration of Zn-protoporphyrin, when measured as Zn in the erythrocytes, increased with time, and after 11 days, approx. 46% of the administered compound was contained within the cirulating erythrocytes. The pattern of increase in the concentration of Zn in the erythrocytes was not monophasic; rather, the gradual increase in the concentration noted during the first 3 days post-injection was followed by a marked increase during the next 2 days. This finding suggests the possibility of the dissociation of Zn-protoporphyrin from the initial tissue and organ binding sites and its entry into the circulatory system with the subsequent sequestration by the erythrocytes. The initial tissue binding sites for the metalloporphyrin most likely would include haem oxygenase and biliverdin reductase.

The accumulation of Zn-protoporphyrin in erythrocytes did not appear to affect adversely the maturation of reticulocytes. As shown in Fig. 4, the erythrocyte uro-s activity during the first 2 days of

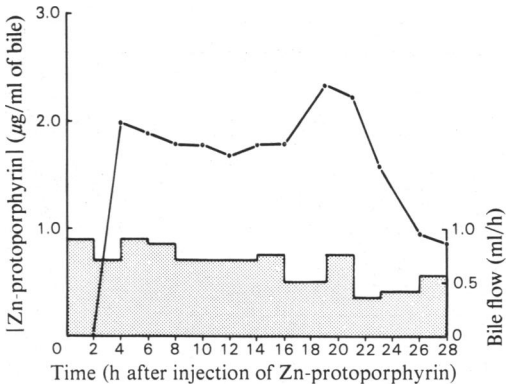

Fig. 2. *Biliary excretion of Zn-protoporphyrin in primates*
The common bile duct was cannulated and bile was collected for 6 h before the injection of Zn-protoporphyrin (40 μmol/kg, subcutaneously). Bile samples were collected at the designated intervals and used for the fluorimetric determination of Zn-protoporphyrin levels (●) (Hart & Piomelli, 1981). Biliary flow is indicated by the histogram (▨).

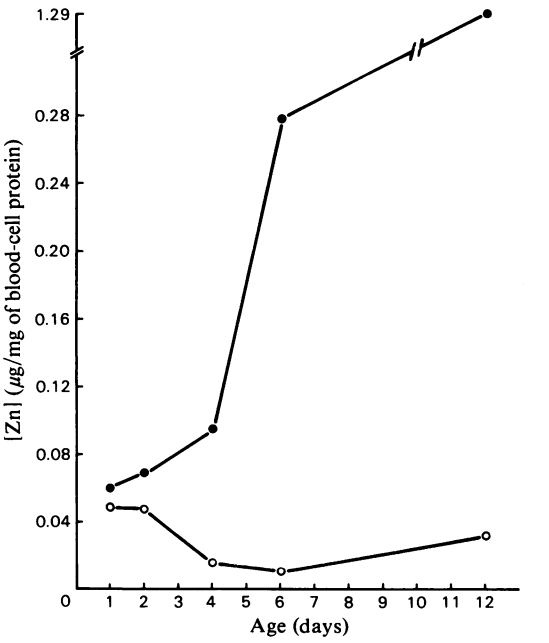

Fig. 3. *Levels of Zn in erythrocytes of newborn primates treated with Zn-protoporphyrin*
Newborn rhesus monkeys (1 day old) were treated with Zn-protoporphyrin as described in the legend to Fig. 1. Blood samples were obtained at the indicated intervals, and the Zn concentration was determined by atomic-absorption spectroscopy. ○, Control newborn; ●, Zn-protoporphyrin-treated newborn.

Table 2. *Effect in vivo of Zn-protoporphyrin treatment on aniline hydroxylase activity and the contents of haemoproteins and glutathione in neonatal-primate liver*

Newborn primates (rhesus monkeys) received a single injection of Zn-protoporphyrin (40 μmol/kg, subcutaneously) 1 day after birth. The control animals received saline. The treated animals were killed 11 days after receiving Zn-protoporphyrin, and the indicated parameters were measured as detailed in the Materials and methods section. The values shown are representative for two animals.

	Content			
Treatment	Cytochrome *P*-450 (nmol/mg of protein)	Cytochrome b_5 (nmol/mg of protein)	Aniline hydroxylase (nmol of *p*-aminophenol/h per mg of protein)	Reduced glutathione (μmol/g of tissue)
Control	0.45	0.23	12.44	3.01
Zn-protoporphyrin	0.47	0.25	11.35	3.05

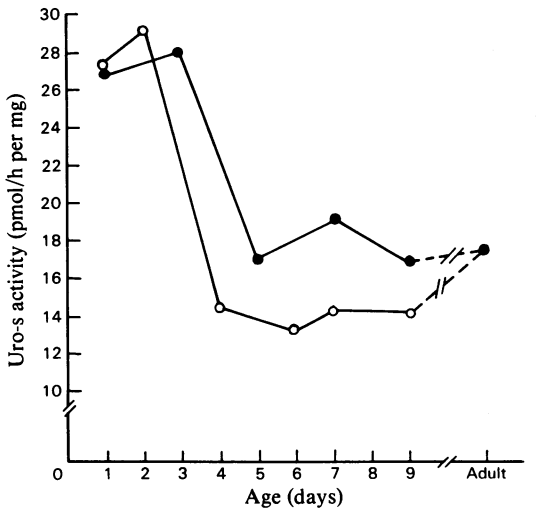

Fig. 4. *Effect of Zn-protoporphyrin treatment on the developmental pattern of uro-s activity in erythrocytes*
Enzyme activity was determined in newborn rhesus monkeys treated with Zn-protoporphyrin (40 μmol/kg, subcutaneously) or saline by a fluorimetric procedure (Granick *et al.*, 1972). ●, Control newborn; ○, Zn-protoporphyrin-treated newborn.

postnatal life was elevated when compared with adult levels. Moreover, the treatment of the newborns with Zn-protoporphyrin did not alter the developmental pattern of enzymic activity. An increase in erythrocyte uro-s activity is believed to reflect an increased frequency in the circulation of young red cells that are still active in haem biosynthesis (Sassa & Bernstein, 1977).

Previous studies with rats have suggested the selectivity of the action of Zn-protoporphyrin in inhibiting the activity of haem oxygenase (Maines, 1981). Accordingly, the response of certain haem-dependent enzymes to Zn-protoporphyrin treatment in the liver of neonatal primates was explored. As shown in Table 2, no differences between treated newborns and controls in the levels of cytochromes *P*-450 and b_5 were detected. Also, the rate of hydroxylation of aniline, a prototype substrate for the microsomal mixed-function oxidase system, did not differ in the metalloporphyrin-treated and control newborns. In addition, the concentration of glutathione in the liver was not altered in the treated newborns as compared with the controls. No abnormalities in weight gain were noted (results not shown).

Discussion

In newborn primates, increased amounts of bilirubin are produced shortly after birth, due to a post-parturition haemolysis of fetal red cells and an increase in the oxidation of haemoglobin haem. In the present study non-human neonatal primates were used to test a new approach to the prevention of hyperbilirubinaemia in the newborn. The results of the present study demonstrate that Zn-protoporphyrin reduces plasma bilirubin levels promptly and effectively (Fig. 1). The reported findings suggest that Zn-protoporphyrin prevents post-parturition hyperbilirubinaemia by inhibiting the activity of haem oxygenase (Table 1). In addition, the distinct possibility exists that the prompt decline in the plasma bilirubin levels may in part reflect the inhibition of biliverdin reductase activity (Table 1) and the circumvention of the conversion of biliverdin into the more toxic bilirubin.

The cellular basis for the sustained decrease in plasma bilirubin levels (11 days, Fig. 1) is not fully understood. Nonetheless, the following postulated mode of action, which is compatible with the present findings, appears likely. The prolonged duration of the action of Zn-protoporphyrin may reflect the transfer of the intact metalloporphyrin from tissue binding sites into the circulation and its gradual deposition in erythrocytes. In turn, the

normal process of the degradation of erythrocytes would result in the release of Zn-protoporphyrin and the persistent bioavailability of the compound. This postulated mechanism of action is consistent with the observations that the metalloporphyrin was minimally excreted in the urine and the bile (Fig. 2), and that a large amount of Zn was detected in the erythrocytes (Fig. 3). Since Zn-protoporphyrin is not degraded by haem oxygenase, it would appear that the Zn levels of erythrocytes would rather accurately reflect the concentration of the metalloporphyrin in the cells.

The unchanged developmental pattern of uro-s activity (Fig. 4) is of physiological significance and suggests the inability of Zn-protoporphyrin to alter the natural maturation processes of the erythrocytes. Since the activity of this enzyme is much higher in the immature, as compared with the mature, erythrocytes, the high level of enzyme activity found in the neonatal-primate erythrocytes may reflect the high proportion of such cells in the peripheral circulation (Sassa & Bernstein, 1977); the decline of this enzyme activity with time is believed to reflect the maturation of these cells postnatally. The findings shown in Fig. 4 further suggest that the metalloporphyrin does not lower serum bilirubin by preventing the degradation of fetal erythrocytes.

The apparent inability of Zn-protoporphyrin to alter the degradation of fetal erythrocytes in the face of an inhibited haem oxygenase activity raises the question of the biological fate of fetal haemoglobin. It is possible that haemoglobin haem was in part disposed of via alternative pathways that might circumvent the requirement for the activity of haem oxygenase. Ostrow *et al.* (1962) have proposed the existence of alternative pathways of haem degradation. It has been reported that 20–45% of the administered ^{14}C-labelled haemoglobin haem is not converted into bilirubin (Ostrow *et al.*, 1962; Landow *et al.*, 1970). Moreover, haemoglobin haem has been shown to be a normal constituent of adult human bile (McCormack *et al.*, 1982). Similarly, in the meconium and bile of healthy human fetuses and premature newborns up to 32 weeks gestation, haem has been detected as a normal biliary constituent (Blumenthal *et al.*, 1977). Nonetheless, the possibility that haem was deposited in tissues cannot be ruled out.

A major concern when using metalloporphyrins in the treatment of hyperbilirubinaemia is the potential toxicity of the metal ion if degradation of the complex occurs. However, this does not appear to be applicable to Zn-protoporphyrin, since Zn-protoporphyrin is not degraded by haem oxygenase; therefore the possibility that Zn would be released is remote. Were this to occur, few biological consequences would be expected. Unlike other metal ions, such as tin, which accumulate in the tissues owing to the absence of an effective biological mechanism for the disposition of the toxic metal, Zn is readily excreted in the bile. Moreover, Zn is an essential mineral with a recommended daily allowance for newborns of 3 mg/day (Danford & Munro, 1980). Extrapolating from the primate data, the total amount of Zn administered as Zn-protoporphyrin to a 3.2 kg infant would be 8 mg. Moreover, the protoporphyrin moiety of the complex is a normal cellular constituent. It is noteworthy that intact Zn-protoporphyrin does not cause photosensitivity or photohaemolysis (Labbe, 1977). In addition, on the basis of the biochemical parameters measured in the present study (Table 2), Zn-protoporphyrin affects few haem-dependent parameters. Also, the finding that hepatic glutathione concentrations were unchanged in the treated primates as compared with the control animals further suggests the possible selectivity of the action of the compound. Decreases in glutathione levels are recognized as an indication of cellular toxicity (Mitchell *et al.*, 1973).

The implications of the reported findings may be of clinical significance. Neonatal jaundice is a common occurrence, and certain clinical conditions, including sepsis and low birth weight, further increase the risk of bilirubin toxicity. The treatment of hyperbilirubinaemia in low-birth-weight infants by conventional methods, such as phototherapy, has had singularly unpredictable outcomes (Lucey, 1982). However, the potential chemotherapeutic approach to neonatal jaundice reported in the present paper is different from commonly used approaches, because the formation of bilirubin by haem oxygenase is entirely prevented, and the chemotherapeutic agent appears to be relatively non-toxic.

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