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Labelling *in vivo* and chirality of griseofulvin-derived *N*-alkylated protoporphyrins

Francesco DE MATTEIS,* Anthony H. GIBBS,* Stephen R. MARTIN† and Richard L. B. MILEK*
*MRC Toxicology Unit, MRC Laboratories, Woodmansterne Road, Carshalton, Surrey, SM5 4EF, and †Division of Physical Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

1. We have compared the response to griseofulvin of rats and mice and, in mice, the effect of griseofulvin itself with that of two of its analogues. The severity of protoporphyria shows a correlation with the accumulation of both types of *N*-alkylated porphyrins previously described after treatment with this drug, namely *N*-methylproptoporphyrin and the *N*-griseofulvin protoporphyrin adduct. 2. Both *N*-alkylporphyrins are chiral, are labelled from 5-amino[4-^{14}C]laevulinate, and their liver accumulation can be inhibited by pretreatment with a suicide substrate of cytochrome *P*-450, which also prevents porphyria. 3. These findings suggest that cytochrome *P*-450 is involved in the mechanism of griseofulvin-induced protoporphyria by generating *N*-methylprotoporphyrin. The *N*-griseofulvin protoporphyrin adduct may also originate from cytochrome *P*-450, but more work is necessary to elucidate whether it acts as the precursor for *N*-methylprotoporphyrin.

INTRODUCTION

Griseofulvin induces hepatic protoporphyria in mice [1], a metabolic disorder characterized by inhibition of the last enzyme of haem biosynthesis, ferrochelatase [2,3], and consequent accumulation of very large amounts of protoporphyrin in the liver leading to development of cholestasis and biliary cirrhosis [4]. This drug-induced porphyria provides a partial experimental model for hereditary protoporphyria of man.

We have recently isolated and characterized [5] two *N*-alkylated protoporphyrins from the liver of griseofulvin-treated mice. One of them has been identified as *N*-methylprotoporphyrin, a previously described inhibitor of ferrochelatase [6,7], thus providing an explanation for the marked inhbition of ferrochelatase observed in mice after treatment with this drug. The second *N*-alkylprotoporphyrin, present in far larger amounts, bears the intact griseofulvin on its pyrrole nitrogen, and can therefore be defined as the *N*-griseofulvin protoporphyrin adduct, and its fragmentation pattern in tandem m.s. suggests that it may give rise to *N*-methylprotoporhyrin as a secondary product. The two alkylated porphyrins, *N*-methylprotoporphyrin and the additional, and more abundant, second pigment, will also be referred to below as the minor and major *N*-alkylprotoporphyrins respectively.

The purpose of this present study has been to investigate the significance of the two *N*-alkylated porphyrins and the role of cytochrome *P*-450 in griseofulvin-induced hepatic protoporphyria. We have compared the response to griseofulvin of rats and mice and, in mice, the effect of griseofulvin itself with that of two of its anaogues. We have also tried to ascertain whether both types of *N*-alkylated protoporphyrins originate from the prosthetic group of cytochrome *P*-450. The results suggest the involvement of liver cytochrome *P*-450 in the mechanism of griseofulvin-induced protoporphyria by a suicidal inactivation reaction leading ultimately to *N*-methylprotoporphyrin, the selective inhibitor of ferrochelatase. We also find that the accumulation in the liver of both types of *N*-alkylated protoporphyrins shows a correlation with the severity of protoporphyria, but it is not yet possible to conclude whether the major pigment is the precursor of *N*-methylprotoporphyrin.

MATERIALS AND METHODS

Sources of special chemicals

Griseofulvin (7-chloro-2′,4,6-trimethoxy-6′-methylgris-2′-ene-3,4′-dione) was either purchased from Sigma Chemical Co., Poole, Dorset, U.K., or was a gift from ICI Pharmaceucticals, Macclesfield, Cheshire, U.K., who also donated isogriseofulvin (7-chloro-4,4′,6-trimethoxy-6′-methylgris-3′-ene-2′,3-dione) and the 2′-(β-hydroxyethyl) thioether analogue of griseofulvin (HET-griseofulvin). 3,5-Diethoxycarbonyl-4-isopropyl-1,4-dihydro-2,6-dimethylpyridine (4-isopropyl-DDC) was prepared according to [8], starting with isobutyraldehyde. The absence of significant amounts of the corresponding pyridine was verified by measuring the ratio of the 233 to 343 nm absorption maxima [9]. 5-Amino-[4-^{14}C]laevulinic acid (55 mCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). The source of the Nucleosil 5 h.p.l.c. column and of the various organic solvents employed has already been given [5].

Treatment of animals

Male LACP rats and male MF1 mice (from these laboratories, 180–200 g body wt. and 6–9 weeks old respectively) were starved for 24 h before being given access to powdered expanded R & M no. 3 diet (Special Diet Services, Witham, Essex, U.K.) containing 2% arachis oil and, where appropriate, 1% of either griseofulvin or one of its analogues. In some experiments animals received an intraperitoneal injection of 4-isopropyl-DDC (100 mg/kg) in arachis oil (10 ml/kg) 1 h before being placed on the griseofulvin-containing diet or, alternatively, 5-amino[4-^{14}C]laevulinic acid (0.46 μCi; 8.4 nmol/mouse) 2 h before commencing drug treatment. Mice were killed by cervical dislocation, and blood was

Abbreviations used: HET-griseofulvin, 2′-(β-hydroxyethyl) thioether analogue of griseofulvin; 4-isopropyl-DDC, 3,5′-diethoxycarbonyl-4-isopropyl-1,4-dihydro-2,6-dimethylpyridine.

collected into heparinized tubes for estimation of griseofulvin content [10]. Livers were quickly removed and the left lobe taken for protoporphyrin estimation [11]; the remainder was stored frozen at −20 °C for not more than a week before being homogenized in ice-cold water for extraction of *N*-alkylporphyrins.

Isolation of hepatic green pigments

Liver homogenates were added dropwise to a 12-fold excess of 5% (v/v) H_2SO_4 in methanol and, after allowing methylation of the porphyrins and haem to proceed overnight at 4 °C, the pigments were transferred to chloroform and applied, in a 2 ml volume, to a 7 cm × 1.5 cm silica column (Merck Silica 60, 70–230 mesh) packed in hexane. After washing the column with 100 ml of hexane and 65 ml of chloroform to remove protoporphyrin the remaining chromophores were eluted with 30 ml of methanol/chloroform (1:1, v/v). The *N*-alkylporphyrins were then converted into the corresponding zinc complexes and separated from haem by chromatography on a second silica column the same size as above, but packed in methanol. After haem had been eluted with 40 ml of methanol, the *N*-alkylporphyrins were eluted with 40 ml of methanol containing 50 mM-HCl, transferred to chloroform and, after removing excess HCl, further purified on t.l.c. using Merck silica-gel 60 plates and chloroform/methanol (20:3, v/v) as a developing system. In the experiments involving labelling of porphyrins, *N*-methylprotoporphyrin was resolved into its two isomeric pairs by h.p.l.c. as described [5], and the vinyl-substituted ring isomers were taken for radioactivity measurements. The major *N*-alkylated porphyrin was estimated by its Soret absorbance, using the same ϵ^{mM} (127.8) as for *N*-methylprotoporphyrin [12]. Its specific radioactivity was verified in one experiment by copper-induced dealkylation in chloroform [5], followed by purification of the resulting copper–protoporphyrin complex and estimation of its specific radioactivity [13]: the two values of specific radioactivity agreed within 8%. Acetylation of the isogriseofulvin-derived *N*-alkylporphyrin (pigment b; see below) was carried out in pyridine containing acetic anhydride [14]. C.d. spectra were determined at a concentration of porphryins of approx. 10 μM in dichloromethane, as described in [15], using a Jasco J600 spectropolarimeter.

RESULTS AND DISCUSSION

Species differences in response to griseofulvin and effect of two analogues

In previous work [3] rats were shown to be much less responsive than mice to the porphyria-inducing effect of griseofulvin. We now find that similar differences in response between rats and mice also apply to the liver accumulation of both types of *N*-alkylated protoporphyrins. Thus, although amounts of both *N*-alkylated porphyrins were increased by griseofulvin feeding not only in mice, but also in rats, the effect was 6–10-fold greater in the mouse, where a clear protoporphyria was also seen (Table 1).

Two analogues differing from griseofulvin in porphyria-inducing activity have also been described [3]; one, isogriseofulvin, is more active than griseofulvin; the other, HET-griseofulvin, is completely inactive. A correlation between induction of porphyria (as measured by the liver concentration of protoporphyrin) and accumulation of both types of *N*-alkylated porphyrins was also found when comparing in mice the effect of griseofulvin with that of its two analogues. Concentrations of liver protoporphyrin, *N*-methylprotoporphyrin and the major *N*-alkylated protoporphyrin found after 2 days' treatment (in that order), as nmol/g of wet liver (averages for two individual observations each obtained with the pooled livers of five mice) were as follows: griseofulvin, 118 (142, 94); 0.58 (0.72, 0.44); 2.6 (3.6, 1.6); isogriseofulvin, 376 (410, 342); 1.47 (1.68, 1.26); 3.93 (4.01, 3.86); HET-griseofulvin, traces; traces; not detectable. Griseofulvin caused the appearance, in addition to *N*-methylprotoporphyrin, of a single major *N*-alkylporphyrin previously characterized as the *N*-griseofulvin protoporphyrin adduct [5]. In contrast, after isogriseofulvin feeding, the additional major *N*-alkylated porphyrin was split into two bands of approximately equal intensity: one (pigment a) had the same R_F value (0.79) on t.l.c. (chloroform/methanol, 20:3, v/v) as the major griseofulvin pigment; the other (pigment b) had a lower R_F value (0.68), but could be converted by treatment with acetic anhydride into a derivative with a higher R_F (0.79). Both fractions of the major isogriseofulvin pigment (a and b) showed a typical electronic spectrum of a *N*-monosubstituted protoporphyrin with absorption maxima (for both the neutral porphyrins and their zinc complex derivatives) indistinguishable from those reported [5] for the major griseofulvin *N*-alkylated pigment. In addition, both pigments, a and b, were found to exhibit the same c.d. spectrum as the 'major' griseofulvin pigment (see below). For these reasons, even though the structures of both these major isogriseofulvin-derived *N*-alkylated protoporphyrins still await elucidation, the most likely interpretation we can put forward at the moment is that the fast-migrating fraction (pigment a) represents the *N*-isogriseofulvin protoporphyrin adduct and the slower moving fraction (pigment b) a derivative, where one of the methoxy groups of the attached isogriseofulvin has undergone *O*-demethylation, so as to generate a free hydroxy group.

Possible involvement of cytochrome *P*-450 in griseofulvin-induced hepatic porphyria

We next turned to examine whether liver cytochrome *P*-450 was involved in the mechanism of induction of porphyria and whether its prosthetic group provided the nucleus for either or both of the *N*-alkylated protoporphyrins. Should the enzyme be involved in such a mechanism, one would expect both the minor and major pigments to be chiral [12,16] and to be labelled from 5-amino[4-^{14}C]laevulinate, a haem precursor. In addition, pretreatment with an inhibitor of cytochrome *P*-450 should result in marked inhibition of griseofulvin-induced porphyria and also in reduced liver accumulation of both types of *N*-alkylated porphyrins.

Fig. 1 shows the c.d. spectra of the two griseofulvin-derived *N*-alkylated protoporphyrins. Both minor and major pigments showed a prominent band of negative ellipticity at about 415 nm or lower wavelength; in addition, *N*-methylprotoporphyrin, but not the *N*-griseofulvin protoporphyrin adduct, showed a positive band at approx. 440 nm. Thus both types of griseofulvin pigments exhibited chiral properties, but the shapes of their c.d. spectra differed. The c.d. spectra of the *N*-alkylated porphyrins isolated after isogriseofulvin treatment were also recorded and the shape of the spectrum and $\Delta\epsilon$ values were both very similar to those shown in Fig. 1 for the corresponding griseofulvin pigments. The minor isogriseofulvin pigment gave a spectrum similar to that shown in Fig. 1 for *N*-methylprotoporphyrin, whereas both fractions of the major isogriseofulvin *N*-alkylated porphryin (pigments a and b) resembled the *N*-griseofulvin protoporphyrin adduct (results not shown).

Both griseofulvin-derived *N*-alkylated porphryins could be labelled from intraperitoneally administered 5-amino[4-^{14}C]-laevulinate. When mice, after a preliminary labelling time of 2 h, were given griseofulvin for either 5 or 24 h, the following specific radioactivities were found at the end of the experiment in purified *N*-methylprotoporphyrin (vinyl-substituted ring isomers) and the *N*-griseofulvin protoporphyrin adduct, in that

Table 1. Species difference in the response to the porphyria-inducing effect of griseofulvin and effect of pretreatment with a suicide inhibitor of cytochrome *P*-450

Animals were starved for 24 h, then given an intraperitoneal injection of arachis oil (10 ml/kg) or of 4-isopropyl-DDC (100 mg/kg) in oil. After 1 h they were fed a diet containing 1% griseofulvin or a control diet, as indicated, and killed 24 h later. Results are given as averages for two individual observations or as means ± S.E.M. for the number of observations in parentheses. *$P = 0.05$; **$P = 0.001$, when compared with corresponding values obtained without pretreatment with 4-isopropyl-DDC. † indicates that the blood concentration of griseofulvin was also measured in these animals at time of killing; values obtained (means ± S.E.M. for five observations; μg/ml of blood) were 8.5 ± 2.1 and 32.4 ± 3.9 in the absence and presence of pretreatment with 4-isopropyl DDC, respectively; $P = 0.001$; – indicates that no pigment could be detected.

			Recovery of liver pigments		
Species	Pretreatment	Treatment	Protoporphyrin (nmol/g)	*N*-Methylprotoporphyrin (pmol/g)	*N*-Griseofulvin protoporphyrin (pmol/g)
Rat	Arachis oil	Control diet	0.25 ± 0.01 (4)	65 (68, 63)	–
		Griseofulvin diet	0.51 ± 0.03 (4)	92 (110, 73)	339 (379, 298)
Mouse	Arachis oil	Control diet	0.42 ± 0.04 (4)	173 (178, 168)	–
		Griseofulvin diet†	40.9 ± 5.6 (5)	330 ± 22.7 (3)	3468 ± 152 (3)
	4-Isopropyl-DDC	Control diet	0.38 ± 0.04 (4)	123 (130, 115)	–
		Griseofulvin diet†	2.18 ± 0.98 (5)*	116.2 ± 17.7 (3)*	679 ± 195 (3)**

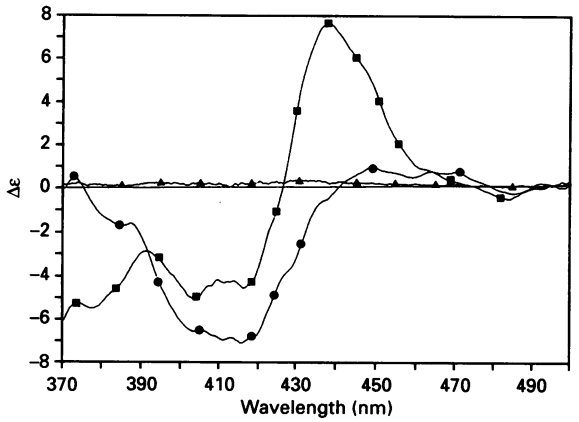

Fig. 1. C.d. spectra of the two alkylated porphyrins isolated from the livers of griseofulvin-treated mice and of synthetic racemic *N*-methylprotoporphyrin

Spectra were recorded at a concentration of the porphyrin dimethyl esters of approx. 10 μM: ■, minor griseofulvin pigment (*N*-methylprotoporphyrin); ●, major griseofulvin pigment (*N*-griseofulvin protoporphyrin); ▲, synthetic *N*-methylprotoporphyrin. Note that both biological pigments are chiral, unlike the synthetic, racemic porphyrin.

order (d.p.m./nmol; average for two observations each obtained with pooled livers of five mice): 5 h: 84 (92, 76); 535 (544, 525); 24 h: 79 (92, 66); 66 (74, 58). Previous work in rats [17] has shown that the incorporation of exogenous 5-aminolaevulinate into liver haem is essentially complete 30 min after intraperitoneal injection of the label. Assuming that the same also applies to mice, then the labelling results we have described can be taken as evidence that both griseofulvin *N*-alkylated porphyrins originate from prelabelled liver haem. The major griseofulvin pigment appears to reach high specific radioactivity at the earlier time and to decay more rapidly than *N*-methylprotoporphyrin.

In our last experiment we tested the effect of pretreatment of mice with a suicide substrate of liver cytochrome *P*-450 before feeding griseofulvin. We made use of 4-isopropyl-DDC for this purpose, since we have previously found [18] that this compound is a very effective inactivator of liver cytochrome *P*-450 in mice, but, in contrast with other 4-alkyldihydropyridines, does not cause accumulation of *N*-alkylated porphyrins or inhibition of ferrochelatase and porphyria. Pretreatment with the inhibitor almost completely prevented the appearance of porphyria and also markedly inhibited the liver accumulation of both *N*-alkylated protoporphyrins (Table 1), while significantly increasing the blood concentration of griseofulvin, an indication that the inhibitor slowed down markedly the liver metabolism of the drug without interfering with its intestinal absorption.

In conclusion, the results we have obtained suggest that cytochrome *P*-450 is involved in the mechanism of production of protoporphyria by providing its prosthetic haem group for drug-induced formation of both the *N*-alkylated protoporphyrins we have isolated, including *N*-methylprotoporphyrin, the selective inhibitor of ferrochelatase. A correlation between degree of drug-induced protoporphyria and liver concentration of both types of *N*-alkylated protoporphyrin has also been shown, but it is not yet possible to decide whether the major pigment is a precursor of *N*-methylprotoporphryin. An alternative explanation may be that the two alkylated porphyrins are formed by independent mechanisms, but that their formation is tightly coupled.

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