LIVER PRODUCTION OF *N*-ALKYLATED PORPHYRINS CAUSED IN MICE BY TREATMENT WITH SUBSTITUTED DIHYDROPYRIDINES

Evidence that the alkyl group on the pyrrole nitrogen atom originates from the drug

F. DE MATTEIS, A. H. GIBBS, P. B. FARMER and J. H. LAMB
Toxicology Unit, MRC Laboratories, Carshalton, Surrey SM5 4EF, England

Received 21 May 1981

1. Introduction

The porphyrogenic drug, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine causes a marked inhibition of the enzyme protohaem ferro-lyase (EC 4.99.1.1) in the liver of rats, mice and chick embryos [1–4] an effect which is thought to be responsible for the very pronounced accumulation of protoporphyrin seen in this type of experimental porphyria. We have isolated a potent inhibitor of protohaem ferro-lyase from the liver of mice made porphyric by treatment with this drug [5,6] and have identified the inhibitor as *N*-methyl protoporphyrin [7]. Inhibition of protohaem ferro-lyase has also been obtained both in vivo and in vitro with synthetic *N*-alkylated porphyrins [7–9], and the size of the alkyl group present on the pyrrole nitrogen atom has been shown to be important for the inhibitory effect, *N*-ethylmesoporphyrin being less active than *N*-methylmesoporphyrin [8].

Isotopic experiments have suggested that the *N*-methylated protoporphyrin produced by treatment with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine originates from liver haem [5,6,10], but the source of the methyl group bound onto the pyrrole nitrogen atom has not yet been determined. The following findings have raised the possibility that the methyl group may originate from the 4-methyl substituent of the drug: under relatively mild chemical conditions certain dihydropyridines lose their 4-alkyl substituent on oxidation and that this alkyl group can be donated to suitable nucleophiles [11]. A series of dihydropyridine analogues have been compared for their ability to inhibit liver protohaem ferro-lyase activity when given to chick embryos in vivo and it was found that the presence and nature of the 4-alkyl markedly affects the inhibitory activity of the corresponding drug, the analogue lacking a 4-alkyl substituent being almost completely inactive [12]. We have now compared 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine with the corresponding 4-ethyl analogue (3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine) for their ability to produce accumulation of green pigments in the liver of mice in vivo. We find that the two drugs, henceforth referred to as the 4-methyl and 4-ethyl dihydropyridine, cause liver accumulation of *N*-methyl and *N*-ethyl protoporphyrin, respectively. By using 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine labelled with deuterium in the 4-methyl substituent, conclusive evidence has also been obtained that the alkyl group on the pyrrole nitrogen atom of the resulting *N*-alkylated protoporphyrin originates from the 4-alkyl substituent of the porphyrogenic drug.

2. Materials and methods

Male MFI mice were obtained from Olac Ltd (Blackthorn, Oxon OX6 OTP). The methods followed for treatment of animals, assay of protohaem ferro-lyase of isolated mitochondria, extraction and purification of green pigments and formation of their zinc complexes and dication derivatives have all been given [6,8,13].

The dihydropyridines were prepared and purified by a modification [14] of the procedure in [15], starting with acetaldehyde and propionaldehyde for the synthesis of the 4-methyl and 4-ethyl dihydro-

pyridine, respectively. 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine was labelled with deuterium in both the 4-H and the 4-methyl positions by using deuterated acetaldehyde [$C^2H_3C^2HO$, 99 + atom % deuterium, Sigma (London) Chemical Co. (Poole, Dorset BH17 7NH)]. The two 4-methyl dihydropyridines (whether deuterated or unlabelled) had a melting point of 128.5–129.5°C and the 4-ethyl analogue 109.5–110°C (128–129.5 and 108–110°C, respectively in [16]). The identity of the various drugs was confirmed by NMR and also by chemical ionization mass spectrometry using isobutane (VG Micromass 70/70). A weak protonated molecular ion was found at *m/e* values of 268, 272 and 282 for (a) the unlabelled 4-methyl, (b) the corresponding deuterated compound, and (c) the 4-ethyl dihydropyridine, respectively. Stronger peaks were found at *m/e* values of 252 (a), 253 (b) and 252 (c), originating in all cases from loss of the 4-alkyl group; the base peaks in the spectra had *m/e* values of 222, 226 and 236 for (a), (b) and (c), respectively, possibly originating from cleavage of an ester bond and loss of a OCH_2CH_3 grouping, but all retaining the 4-alkyl substituent.

Protoporphyrin IX dimethyl ester [Sigma (London) Chemical Co.], 3.0 mg, was heated with 1 ml of either methyl or ethyl iodide (BDH Chemicals, Poole, Dorset) at 110°C for 24 h in tubes with PTFE-lined screw caps. The resulting *N*-alkylated porphyrins were separated from the parent porphyrin and purified by repeated TLC on silica gel by using a developing system of chloroform/methanol (20:3, v/v). The identity of the product was confirmed by the characteristic bathochromic shifts of all absorption maxima and also by recording their electron impact mass spectra. The expected molecular ion at *m/e* values of 604 and 618 was found for *N*-methyl and *N*-ethyl protoporphyrin dimethyl ester, respectively, together with evidence of pyrolysis leading to partial regeneration of the parent porphyrin, protoporphyrin. The latter is in agreement with previous findings [17,18].

The methyl esters of the *N*-alkylated porphyrins were hydrolysed [19] and dissolved in dimethyl sulphoxide before testing for inhibitory activity on protohaem ferro-lyase of mouse liver mitochondria in vitro [6].

3. Results and discussion

In [12], the 4-ethyl dihydropyridine was considerably less active than the corresponding 4-methyl analogue in inhibiting protohaem ferro-lyase activity in chicken embryos in vivo, suggesting that the two drugs may stimulate to a different extent the production of the inhibitory green pigment(s) in the liver. Here, both dihydropyridines were found to cause a marked inhibition of mitochondrial protohaem ferro-lyase and accumulation of green pigment inhibitor in the liver of mice in vivo; and no great difference in activity could be demonstrated between the two drugs (table 1). However, when the inhibitory activity of the isolated *N*-alkylated porphyrin was related to its Soret absorption, a difference in specific activity was noted between the green pigments isolated after treatment with the two drugs, that obtained after 4-ethyl dihydropyridine being considerably less active. Also the wavelength of the Soret maximum exhibited by the pigments dication derivative was different according to the porphyrogenic chemical employed.

On increasing the size of the substituent of the pyrrole nitrogen atom the inhibitory activity on protohaem ferro-lyase of the resulting *N*-alkylated porphyrin decreases [8] and the electronic spectra of the porphyrin dication and zinc complex shows characteristic changes in absorption maxima [8,13]. The two green pigments were therefore purified and their specific inhibitory activity and electronic spectra compared with those of authentic *N*-alkylated protoporphyrins. It was found (tables 2,3) that in both respects the green pigments isolated after treatment with the 4-methyl and 4-ethyl dihydropyridines closely resembled authentic *N*-methyl and *N*-ethyl protoporphyrin, respectively.

The electron impact mass spectrum of the two green pigments dimethyl esters was also determined. A molecular ion at *m/e* values of 604 and 618 was found for the pigments produced by treatment with 4-methyl and 4-ethyl dihydropyridine, respectively (an additional peak being noted at *m/e* value of 590 in both cases, probably representing regenerated protoporphyrin). This is compatible with a structure of *N*-methyl protoporphyrin (see also [7,20]) and *N*-ethyl protoporphyrin for the two pigments, respectively, suggesting that in the *N*-alkylation reaction the 4-alkyl group of the dihydropyridine may be donated to the pyrrole nitrogen.

Conclusive evidence that the alkyl group on the pyrrole nitrogen atom of protoporphyrin originates from the 4-alkyl group of the dihydropyridine was

Table 1
Inhibition of mitochondrial protohaem ferro-lyase activity and accumulation of a green pigment inhibitor of the enzyme caused in the liver of mice by treatment with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine or with 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine

Treatment and dose (ml or mg/kg body wt)	Mitochondrial protohaem ferro-lyase activity (nmol . min^{-1} . mg $protein^{-1}$)	Green pigment (inhibitory units/g liver wet wt)
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, 5 mg	0.60 ± 0.07	–
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, 10 mg	0.26 ± 0.05	12.5, 10.8
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine, 5 mg	0.63 ± 0.05	–
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine, 10 mg	0.39 ± 0.05	10.8, 10.4
Arachis oil, 10 ml (control)	1.11 ± 0.08	traces

Male MFI mice were given drugs dissolved in arachis oil by intraperitoneal injection and killed 1 h later. Their liver mitochondria were isolated for assay of protohaem ferro-lyase activity: results given are averages ± SEM of 4 obs, each obtained with the pooled livers of 2 animals. Green pigments were also isolated from the liver of treated animals: their inhibitory activity on protohaem ferro-lyase was determined in vitro, as in [6]: results given above refer to individual observations, each obtained with the pooled livers of 5 mice

obtained by treating mice with deuterated 4-methyl dihydropyridine (100 mg/kg) and killing them 2 h later. The resulting green pigment was purified as the methyl ester and subjected to electron impact mass spectrometry, where it produced a molecular ion at *m/e* value of 607. The value of 607 for the molecular ion was confirmed by a peak-matching technique, employing perfluorokerosene as internal standard. The increase of 3 mass units (when compared with the molecular ion of the corresponding pigment obtained by treatment with unlabelled 4-methyl dihydropyridine) is compatible with the methyl group being transferred intact, that is with its full complement of hydrogen atoms. In chemical systems [11] the 4-alkyl substituent of certain dihydropyridines is eliminated as a carbonium ion during the oxidative process leading to aromatization. A similar mechanism is compatible with our present findings and might also explain the inability of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine to promote formation of *N*-methyl

Table 2
Inhibitory activity towards protohaem ferro-lyase of *N*-methyl and *N*-ethylprotoporphyrin and of green pigments isolated from the liver after giving 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine or 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine

Porphyrin	Inhibitory activity (units/nmol)
Green pigment (with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine)	16.5 (17.5[a], 15.5[a], 16.6[b])
Green pigment (with 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine)	5.0 (4.8[a], 5.7[a], 4.6[b])
N-Methyl protoporphyrin	13.4 (14.4, 12.4)
N-Ethyl protoporphyrin	6.4 (6.3, 6.6)

Results given are averages of at least two observations, each obtained with a different bath of natural or synthetic porphyrins (results of individual batches are given in parentheses). The inhibitory activity was assayed in vitro as in [6] and is expressed as units of inhibitor/nmol porphyrin calculated from the Soret absorption by using the published ϵ-value [23] for protoporphyrin. All porphyrins were tested as the free carboxylic acids. Drugs were given at a dosage of 100 mg/kg body wt[a] or 10 mg/kg[a] and mice killed 1–2 h later. The livers of 25 mice were pooled for each batch of green pigments; these were isolated by column chromatography of a liver extract and further purified by TLC, as in [6]

Table 3

Absorption maxima (nm) of the green pigments obtained after treatment with either 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine or 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine: comparison with synthetic *N*-methyl and *N*-ethyl protoporphyrin

Porphyrin	Dication			Zinc complex			
	Soret	II	I	Soret	β	α	α
Green pigment (with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine)	412	560	604	431	547	596	633
N-Methyl protoporphyrin[a]	412	560	604	431	545.5	594.5	633
Green pigment (with 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine)	417.5	564.5	608	431.5	546	592	631.5
N-Ethyl protoporphyrin	417.4	563	606[b]	431	547	592	631.5

[a] From [7] average of values reported for 2 different chromatographic fractions

[b] This absorption maximum was not clearly defined

The neutral spectrum of all porphyrins listed here is of an aetio type with very similar absorption maxima to those reported for the griseofulvin-induced pigment or for synthetic *N*-methyl protoporphyrin [7,13]. The dication and zinc complexes of the various porphyrins were prepared [13] and their spectra determined in $CHCl_3$, using the porphyrin methyl ester

protoporphyrin in vivo [5], as this drug already possesses the aromatic structure and, on account of this, the 4-methyl substituent will not be readily eliminated. A monooxygenated intermediate such as formaldehyde is less likely, as this would entail loss of one of the 3 deuteriums present in the original methyl group. Similar conclusions have been reached for the methylating species generated metabolically either from physiological methyl donors, such as methionine [21] or from methylating carcinogens, such as *N*-nitrosodimethylamine [22], where too the methyl groups appear to be transferred intact.

We conclude from this work that the alkyl group present on the nitrogen of the modified protoporphyrin inhibitor of protohaem ferro-lyase originates from the 4-alkyl substituent of the porphyrogenic drug. This explains the inability of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine to inhibit the enzyme [12], as this drug does not possess a 4-alkyl group which can be donated.

References

[1] Onisawa, J. and Labbe, R. F. (1963) J. Biol. Chem. 238, 724–727.

[2] Tephly, T. R., Hasegawa, E. and Baron, J. (1971) Metab. Clin. Exp. 20, 200–214.

[3] Rifkind, A. B. (1979) J. Biol. Chem. 254, 4636–4644.

[4] Cole, S. P. C., Vavasour, E. J. and Marks, G. S. (1979) Biochem. Pharmacol. 28, 3533–3538.

[5] Tephly, T. R., Gibbs, A. H. and De Matteis, F. (1979) Biochem. J. 180, 241–244.

[6] De Matteis, F., Gibbs, A. H. and Tephly, T. R. (1980) Biochem. J. 188, 145–152.

[7] De Matteis, F., Gibbs, A. H., Jackson, A. H. and Weerasinghe, S. (1980) FEBS Lett. 119, 109–112.

[8] De Matteis, F., Gibbs, A. H. and Smith, A. G. (1980) Biochem. J. 189, 645–648.

[9] Ortiz de Montellano, P. R., Kunze, K. L., Cole, S. P. C. and Marks, G. S. (1980) Biochem. Biophys. Res. Commun. 97, 1436–1442.

[10] Tephly, T. R., Gibbs, A. H., Ingall, G. and De Matteis, F. (1980) Int. J. Biochem. 12, 993–998.

[11] Loev, B. and Snader, K. M. (1965) J. Org. Chem. 30, 1914–1916.

[12] Cole, S. P. C. and Marks, G. S. (1980) Int. J. Biochem. 12, 989–992.

[13] De Matteis, F. and Gibbs, A. H. (1980) Biochem. J. 187, 285–288.

[14] De Matteis, F. and Prior, B. E. (1962) Biochem. J. 83, 1–8.

[15] Hantzsch, A. (1982) Ann. Chem. 215, 1–82.

[16] Marks, G. S., Hunter, E. G., Terner, U. K. and Schneck, D. (1965) Biochem. Pharmacol. 14. 1077–1084.

[17] Jackson, A. H. and Dearden, G. R. (1973) Ann. NY Acad. Sci. 206, 151–174.

[18] Smith, A. G. and Farmer, P. (1980) unpublished work.

[19] Falk, J. E. (1964) BBA Libr. 2, 126.

[20] Ortiz de Montellano, P. R., Beilan, H. S. and Kunze, K. L. (1981) Proc. Natl. Acad. Sci. USA 78, 1490–1494.

[21] Du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R. and Simmonds, S. (1941) J. Biol. Chem. 140, 625–641.

[22] Lijinsky, W., Loo, J. and Ross, A. E. (1968) Nature 218, 1174–1175.

[23] Smith, K. M. (1975) in: Porphyrins and Metalloporphyrins (Smith, K. M. ed) p. 189, Elsevier/North-Holland, Amsterdam, New York.