Inactivation of cytochrome *P*-450 and production of *N*-alkylated porphyrins caused in isolated hepatocytes by substituted dihydropyridines

Structural requirements for loss of haem and alkylation of the pyrrole nitrogen atom

F. de Matteis, C. Hollands, A.H. Gibbs, N. de Sa and M. Rizzardini*

Biochemical Pharmacology Section, Toxicology Unit, MRC Laboratories, Carshalton, Surrey SM5 4EF, England

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1. INTRODUCTION

Drugs with unsaturated side chains and certain dihydropyridines both convert liver haem into *N*-alkylated porphyrins, but the underlying mechanisms differ in the two cases. With unsaturated drugs, for example 2-allyl-2-*iso*propylacetamide (AIA), a monooxygenated derivative of the drug becomes bound [1] onto one of the pyrrole nitrogen atoms [2–4] of liver haem; whereas with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) only the 4-methyl substituent of the drug is transferred onto the pyrrole nitrogen atom [5–7] and the product, *N*-methyl protoporphyrin, is a powerful inhibitor of ferrochelatase (EC 4.99.1.1) [8,9].

There is strong evidence that the *N*-alkylated porphyrins produced by unsaturated compounds all originate from the haem of cytochrome *P*-450 [10–12], but it is less clear from which pool of hepatic haem *N*-methyl protoporphyrin originates after treatment with DDC; and the detailed mechanism of the transmethylation reaction involved has not yet been elucidated.

The aim of the experiments described in this paper has been to clarify wether *N*-methyl protoporphyrin originates from the haem of cytochrome *P*-450 and whether exogenous haem can be utilized for the formation of *N*-alkylated porphyrins. Several analogues of DDC have also been investigated to ascertain what structural characteristics the 4-substituent of a dihydropyridine must possess in order to be transferred onto the pyrrole nitrogen of haem. Most of this work has been carried out in vitro by using isolated hepatocytes, but some of the findings have been confirmed in the whole animal in vivo.

2. MATERIALS AND METHODS

2.1. *Synthesis of dihydropyridines*

DDC and its 4-desmethyl and 4-ethyl analogues were prepared as described in [13] starting with acetaldehyde, formaldehyde and propionaldehyde, respectively. The 4-propyl, 4-*iso*propyl, 4-phenyl and 4-benzyl analogues of DDC were prepared according to [14], using *n*-butyraldehyde, *iso*-butyraldehyde, benzaldehyde and phenylacetaldehyde, respectively. All drugs were recrystallized from ethanol and had melting points in good agreement with those in [14,15]. Their identity was confirmed by NMR and infrared spectroscopy and also by chemical ionization mass spectrometry.

2.2. *Treatment of animals*

Fed male mice of the MFI strain were given an intraperitioneal injection (100 mg/kg) of either DDC or of its 4-*iso*propyl or 4-benzyl analogues dissolved in arachis oil (10 ml/kg) and were killed 1 h later. Liver mitochondria and microsomes were

* Present address: Istituto di Ricerche Farmacologiche 'Mario Negri', Milano, Italy.

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obtained [16] to estimate ferrochelatase (with mesoporphyrin and Co^{2+} [17]), and microsomal cytochrome *P*-450 [18] and haem [19], respectively. The *N*-alkylated porphyrins were extracted from liver homogenates and isolated by chromatography on Sephadex LH-20 [9].

Microsomes isolated from the liver of mice were incubated at 37°C with shaking (60 cycles/min) in the presence of phosphate buffer pH 7.4 (80 mM), EDTA (1 mM), NADPH (0.6 mM), a NADPH-generating system [16], and either the 4-ethyl or 4-*iso*propyl analogue of DDC (0.5 mM). Cytochrome *P*-450 and total haem were measured before and after incubation by dual-wavelength spectrophotometry in a Perkin–Elmer, model 356 instrument.

2.3. *Experiments with isolated hepatocytes*

Hepatocytes were prepared by a collagenase perfusion technique [20] from fed male rats of the Porton strain (some given phenobarbitone sodium, 0.1% in drinking water for a week before use). They were incubated in 100 ml round-bottom flasks under O_2/CO_2 (95:5, by vol) in a modified Weymouth medium (40 ml, 85–110 × 10^6 cells/flask) in presence of glutamine (200 mM) gentamycin (50 μg/ml), $NaHCO_3$ (0.19%), heparin (12.5 U/ml) and heat-inactivated horse serum (12.5%, by vol). Incubation was at 37°C and pH 7.4 with gentle rotation [21] (58 cycles/min, the direction being inverted every minute). Haematin, in some experiments prelabelled from 5-amino-[5-^{14}C]laevulinate [22], was added as methaemalbumin [23]. Viability (Trypan blue exclusion) declined from 95–87% to 85–75% after incubation. Hepatocytes were homogenized by Ultraturrax (10 s at 0°C) and mitochondrial ferrochelatase and *N*-alkylated porphyrins assayed as with intact liver, using an $\varepsilon_{mM} = 228$ for the Soret absorption of the dication derivative of the pigments. Cytochrome *P*-450 was measured as in [24] and the in vitro inhibitory activity towards ferrochelatase of green pigments extracted from hepatocytes, assayed according to [9].

3. RESULTS

3.1. *Production of* N*-alkylated porphyrins by isolated hepatocytes*

When hepatocytes were incubated with DDC the activity of their mitochondrial ferrochelatase declined (see also [25,26]), and increased amounts of a green pigment with inhibitory activity towards ferrochelatase could be isolated (fig.1). After a 30 min incubation most of the inhibitor could already be demonstrated but the mitochondrial enzyme was still normal, probably indicating that the green pigment is produced outside the mitochondrion and has to cross the mitochondrial membranes in order to inhibit the enzyme. Production of a *N*-alkylated porphyrin could also be readily demonstrated with AIA (see later).

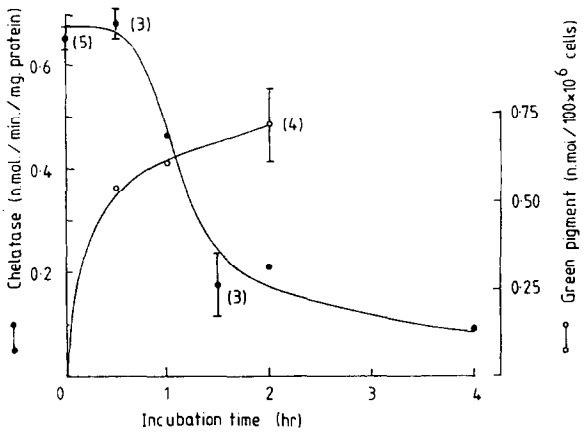


Fig.1. Decline of mitochondrial ferrochelatase and accumulation of a *N*-alkylated porphyrin (or green pigment) caused by DDC (50 μM) in hepatocytes incubated in vitro. The accumulating pigment was strongly inhibitory on the ferrochelatase activity of normal mouse mitochondria. Results are means ± SEM of the number of observations in parentheses or averages of two observations.

3.2. *Structure of the dihydropyridines required for production of* N*-alkylated porphyrins*

A decline in hepatic cytochrome *P*-450 [27] and a loss of radioactivity from prelabelled microsomal haem [28] has been reported after treatment with DDC; whereas the oxidized analogue of DDC, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine, which does not cause production of *N*-methyl protoporphyrin [8], increases (rather than decreases) the concentration of cytochrome *P*-450 in the liver [29]. These findings and recent observations ob-

Table 1

Loss of cytochrome *P*-450 and accumulation of *N*-alkylated porphyrins caused in isolated hepatocytes by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and several analogues

Dihydropyridine analogue [d] (final conc.)	Cytochrome *P*-450 (% of corresponding value in sample not incubated)	*N*-alkylated porphyrins (nmol/100 × 10^6 cells)
Control	89.5 ± 4 (6)	< 0.17
4-Desmethyl DDC (50 μM)	91.5 (99, 84)	< 0.17
4-Desmethyl DDC (235 μM)	85.0 (91, 79)	< 0.17
DDC (4-Methyl, 50 μM)	77.0 (86, 68)	0.79 ± 0.13 (4)
4-Ethyl (50 μM)	31.5 ± 3 (4)[a]	1.69 ± 0.22 (3)[b]
4-Propyl (50 μM)	27.0 ± 0.8 (4)[a]	2.54 ± 0.13 (3)[c]
4-*iso*Propyl (50 μM)	25.0 (29, 21)	< 0.17
4-Phenyl (50 μM)	74.5 (79, 70)	< 0.17
4-Benzyl (50 μM)	50.7 ± 2 (4)[a]	< 0.17

[a] $P < 0.01$, when compared to corresponding control value
[b] $P < 0.05$
[c] $P < 0.01$, when compared to corresponding DDC value
[d] The dihydropyridines used in this work differ from DDC only in the nature of the 4-alkyl substituent (absent in the 4-desmethyl analogue)

Rat hepatocytes were incubated for 2 h in presence of a dihydropyridine dissolved in ethanol (0.1 ml/40 ml of incubation mixture) or with ethanol alone (control). Results are means ± SEM of the number of observations in parentheses or averages of two observations

tained in vitro with isolated microsomes [6] suggested that cytochrome *P*-450 might be the source of *N*-methyl protoporphyrin. The effect of DDC and of several of its analogues was therefore studied in isolated hepatocytes and an attempt made to relate the accumulation of *N*-alkylated porphyrins to loss of cytochrome *P*-450 (Table 1).

4-Desmethyl DDC was inactive in both respects. On increasing the size of the 4-substituent from a methyl to a propyl residue, the production of *N*-alkylated porphyrins increased and there was a correspondingly greater loss of cytochrome. When however the 4-substituent was either branched (*iso*propyl) or very bulky (benzyl), considerable losses of cytochrome were found but accumulation of a *N*-alkylated product could not be demonstrated. In experiments in mice in vivo it was confirmed that dihydropyridines with a 4-*iso*propyl or benzyl substituent caused greater losses of cytochrome *P*-450 (and microsomal haem) than DDC; but, in contrast to DDC, neither analogue caused accumulation of a *N*-alkylated porphyrin or inhibition of ferrochelatase (results not shown).

When mouse liver microsomes were incubated with a dihydropyridine in presence of NADPH and oxygen, a loss of the cytochrome was found not only with the 4-ethyl analogue (confirming data in [6]), but also with the 4-*iso*propyl analogue; this was accompanied by loss of haem in both cases (fig.2), indicating that the prosthetic group of the cytochrome was the target of the inactivation mechanism, whether a *N*-alkylated porphyrin could be demonstrated or not.

The green pigments isolated after treatment with the 4-ethyl and 4-propyl dihydropyridines were identified as *N*-ethyl and *N*-propyl protoporphyrin, respectively [30], confirming previous conclusions [5,6] that the whole 4-alkyl substituent of a dihydropyridine is transferred to the pyrrole nitrogen.

Table 2

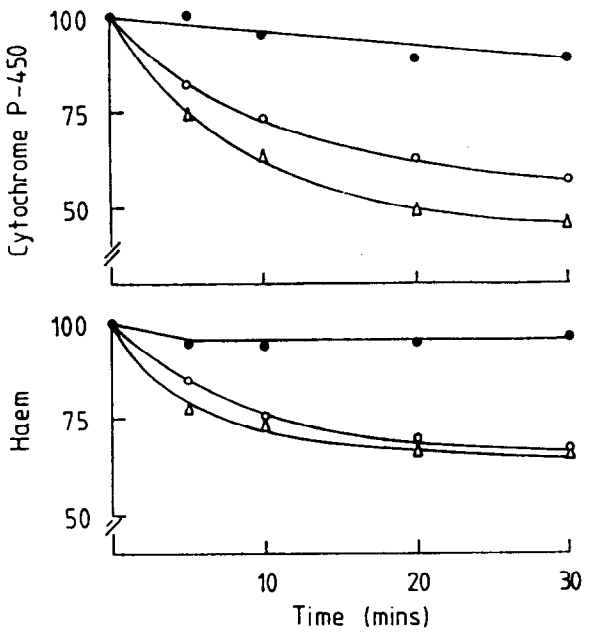
Effect of pretreatment of rats with phenobarbitone in vivo and of adding exogenous haematin in vitro on the formation of *N*-alkylated porphyrins caused in isolated hepatocytes by either AIA or the 4-ethyl dihydropyridine

Pretreatment of the rat in vivo	Exongenous haematin	Incubation time	*N*-alkylated porphyrins (nmol/100 × 10^6 cells)	
			Dihydropyridine	AIA
None	Absent	2 h	1.69 ± 0.22 (3)	2.13 (2.2, 2.06)
Phenobarbitone	Absent	2 h	4.4 (4.45, 4.36)	11.8 (11.9, 11.7)
Phenobarbitone	Absent	4 h	4.92 ± 0.31 (8)	13.14 ± 0.8 (3)
Phenobarbitone	Present	2 h	5.79 (5.55, 6.03)	15.97 ± 1.6 (3)
Phenobarbitone	Present	4 h	9.49 ± 0.43 (6)[b]	27.4 ± 2.9 (3)[a]

[a] $P < 0.01$

[b] $P < 0.001$, when compared with corresponding value obtained in absence of haematin

Rat hepatocytes were incubated with AIA or 4-ethyl dihydropyridine (3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine) in presence or absence of exogenous haematin. The latter was added at the start of the incubation to a final concentration of 6.25 μM. AIA (16 mg) and the dihydropyridine (0.6 mg) were added both at the beginning and after 2 h incubation, each time in 0.1 ml ethanol



3.3. *Effect of pretreatment with phenobarbitone and of addition of haem*

Hepatocytes from phenobarbitone pretreated rats produced *N*-alkylated porphyrins on incubation with either AIA or 4-ethyl dihydropyridine in significantly greater amounts than hepatocytes from control rats. The production of both types of pigments was significantly increased by exogenous haem, particularly after the first 2 h of incubation, by which time production of green pigments from endogenous haem was virtually complete (table 2).

Fig.2. Effect of incubating mouse liver microsomes with either the 4-ethyl (○) or the 4-*iso*propyl (△) analogue of DDC (dissolved in ethanol) or with ethanol alone (●) on the concentration of cytochrome *P*-450 and total haem. Results are expressed as a percentage of the values of samples not incubated.

More direct evidence for conversion of exogenous haem into green pigment of both classes was obtained by using [^{14}C]haematin and demonstrating that the pigments, purified by TLC as the free carboxylic acid [9], were radioactive [30].

4. DISCUSSION

This work has shown conclusively that the *N*-alkylated porphyrins produced by treatment with substituted dihydropyridines originate (like the pigments obtained with unsaturated drugs [10,31]) from pre-existing haem. Both endogenous liver haem and exogenous haem can be utilized for conversion to *N*-alkylated porphyrins. The following findings strongly suggest that the endogenous precursor pool is cytochrome *P*-450: (1) a correlation was found between loss of the cytochrome and production of *N*-alkylated porphyrins, when the effects of DDC, its 4-ethyl and 4-propyl analogues were compared; (2) the yield of *N*-alkylated porphyrins could be increased by phenobarbitone, an inducer of cytochrome *P*-450, whereas the inhibitor SKF 525-A has been shown to decrease the production of *N*-methyl protoporphyrin after DDC [32]; and (3) finally, when DDC was given to mice at different times after labelling their liver haem with [5-^{14}C]aminolaevulinate, the specific radioactivity of the isolated *N*-methyl protoporphyrin declined with an apparent half-life of approx. 7 h [30], the $t_{½}$ value given in [33] for the rapidly turning-over component of cytochrome *P*-450.

We conclude that a dihydropyridine can transfer (by an oxidative mechanism leading to aromatization [5,6,14]) its 4-alkyl substituent to nucleophilic sites in the molecule of haem, among these one of its pyrrole nitrogens. The present results suggest that the process of transalkylation may be more efficient with 4-alkyl groups which give rise to stable carbonium ions, in agreement with the oxidative dealkylation of dihydropyridines in chemical systems [14]; however, for a *N*-alkylated porphyrin to accumulate, the 4-alkyl must possess special characteristics of size and shape, possibly because bulky carbonium ions will be sterically hindered in their access to the pyrrole nitrogen atom and will attack instead more accessible sites in the molecule of haem.

In [25], analogues of DDC in which the 3- and 5-ethoxycarbonyl groups were replaced by cyano or acetyl groups were far less inhibitory towards ferrochelatase than DDC itself. This finding is also compatible with the above mechanism of oxidative transfer of the 4-methyl group of DDC to a pyrrole nitrogen of haem (leading to *N*-methyl protoporphyrin, the inhibitor of ferrochelatase), as it is known from chemical systems [14] that the oxidative elimination of the 4-alkyl group of a dihydropyridine is facilitated by the presence of bulky substituents in the 3- and 5-positions.

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