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Porphyrinogenic activity and ferrochelatase-inhibitory activity of sydnones in chick embryo liver cells

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3-[2-(2,4,6-Trimethylphenyl)thioethyl]-4-methylsydnone was shown to be a potent porphyrinogenic agent in chick embryo liver cells. The accumulation of protoporphyrin IX was consistent with the finding that ferrochelatase activity was inhibited. 3-Benzyl-4-phenylsydnone did not inhibit ferrochelatase activity and protoporphyrin IX was found to constitute only a minor fraction of the porphyrins. These results support the idea that the porphyrinogenicity of 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone is due to its catalytic activation by cytochrome P-450 leading to heme alkylation and formation of *N*-vinylprotoporphyrin IX which inhibits ferrochelatase.

Porphyria *Ferrochelatase* N-*Alkylprotophyrin* *Sydnone* *Cytochrome P-450* *(Chick embryo liver)*

1. INTRODUCTION

The dihydropyridine, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, when administered to rodents, causes the accumulation in the liver of *N*-methylprotoporphyrin IX, a potent inhibitor of ferrochelatase (EC 4.99.1.1) activity [1–3]. The source of the *N*-methyl substituent of *N*-methylprotoporphyrin is the 4-methyl group of the dihydropyridine [4–6]. The dihydropyridine and several 4-alkyl analogues serve as suicide substrates of cytochrome P-450 [3,5]. Although there are a large number of chemicals which exhibit porphyrinogenicity, dihydropyridines which cause inhibition of ferrochelatase activity appeared to have a unique mechanism of action, particularly in chick embryo liver [7]. A report by Stejskal et al. [8] using 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (fig.1a) suggested that the mechanism of action of dihydropyridines as porphyrinogenic agents might not be unique. Thus, the sydnone (fig.1a), which was developed as a potential anti-arthritic agent, was reported to produce hepatic porphyria in dogs, rats and mice. The fact that the hepatic pigment was interpreted to be protoporphyrin IX suggested that the sydnone might be a suicide substrate for cytochrome P-450 and give rise to an *N*-alkylprotoporphyrin IX which inhibited ferrochelatase [9]. In the studies of Stejskal et al. [8], the hepatic pigment was only tentatively identified as protoporphyrin IX by its red autoflu-

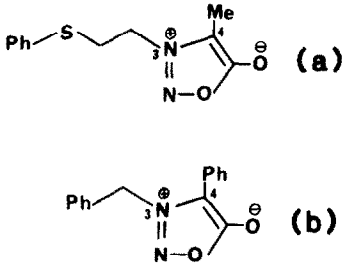

Fig.1. (a) 3-[2-(2,4,6-Trimethylphenyl)thioethyl]-4-methylsydnone, (b) 3-benzyl-4-phenylsydnone.

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orescence in ultraviolet light and by its birefringence. The objectives of this study were to confirm the structure of the pigment by biochemical methods and to determine whether the sydnone had ferrochelatase-inhibitory activity. Because of its sensitivity to porphyrinogenic agents [10], the chick embryo liver cell culture was selected for the study.

2. EXPERIMENTAL

2.1. *Determination of ferrochelatase activity*

Details of the cell culture technique have been described [11,12]. After an initial incubation period of 24 h, the medium was discarded and replaced with fresh medium. After a further 24 h incubation period, a sydnone, dissolved in 95% ethanol, was added to the dishes (maximal total volume added 20 μl). Ferrochelatase activity was assayed 6 h after addition of a sydnone as in [7,11,13] by a modification of the pyridine hemochromogen method [14] using mesoporphyrin and ferrous iron as substrates.

2.2. *Determination of total porphyrins and porphyrin precursors*

For determination of total porphyrins and porphyrin patterns, chick embryo liver cells were maintained in 6-cm diameter dishes containing 5 ml medium. Drugs were dissolved in 95% ethanol to the dishes (maximal total volume 15 μl). Total porphyrins were measured 24 h after addition of the drugs [15]. Porphyrin patterns were determined as described [7,11] by the high-performance liquid chromatographic method of Zelt et al. [16]. Results were calculated as pmol porphyrin per mg protein.

3. RESULTS AND DISCUSSION

Doses of 0.01, 0.1, 1.0 and 10.0 μg/ml of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine and the sydnone (fig.1a) were compared for porphyrinogenic activity in chick embryo liver cells (fig.2); the results show that the sydnone (fig.1a) is considerably more potent than the dihydropyridine as a porphyrinogenic agent. Doses of 0.1 and 1 μg/ml of the dihydropyridine and sydnone (fig.1a) were compared for ferrochelatase-inhibitory activity in chick embryo liver cells

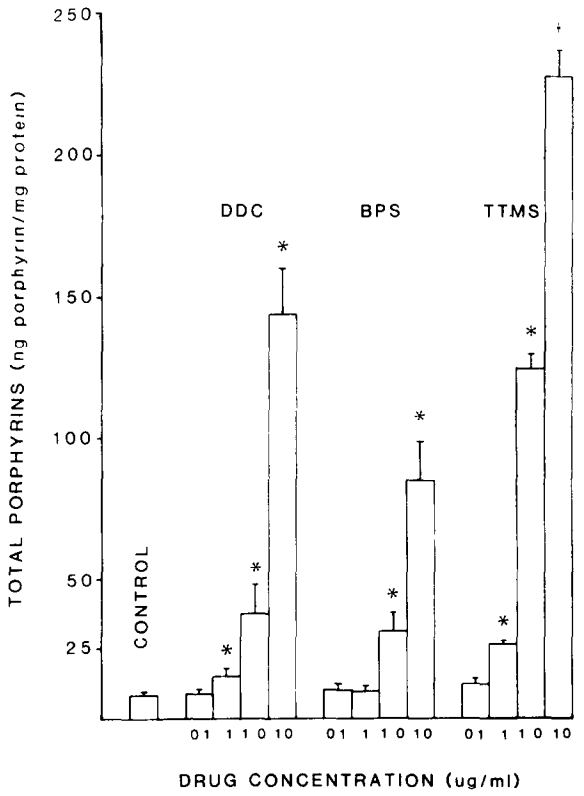

Fig.2. Porphyrin accumulation 24 h after the addition of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), 3-benzyl-4-phenylsydnone (BPS), and 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone(TTMS) to chick embryo liver cell culture. Each bar represents the mean of 4 determinations ± SD in one experiment and the results were confirmed in 2 additional experiments. * Significantly different from control by Newman-Keuls test ($p \leqq 0.05$).

Fig.3). The sydnone (fig.1a) at 0.1 and 1.0 μg/ml was shown to have ferrochelatase-inhibitory activity (fig.3). According to current ideas, the dihydropyridine owes its porphyrinogenic action to (i) ferrochelatase inhibition via *N*-alkylprotoporphyrin IX formation, and (ii) a second mechanism whereby an elevation of δ-aminolevulinic acid synthetase (EC 2.3.1.37) is induced independent of inhibition of heme biosynthesis or of stimulation of heme degradation [17]. It has been pointed out that all drugs that induce δ-aminolevulinic acid synthetase by this second mechanism have the property of lipid solubility [17]. It is likely that the sydnone similarly owes its porphyrinogenic action to the ac-

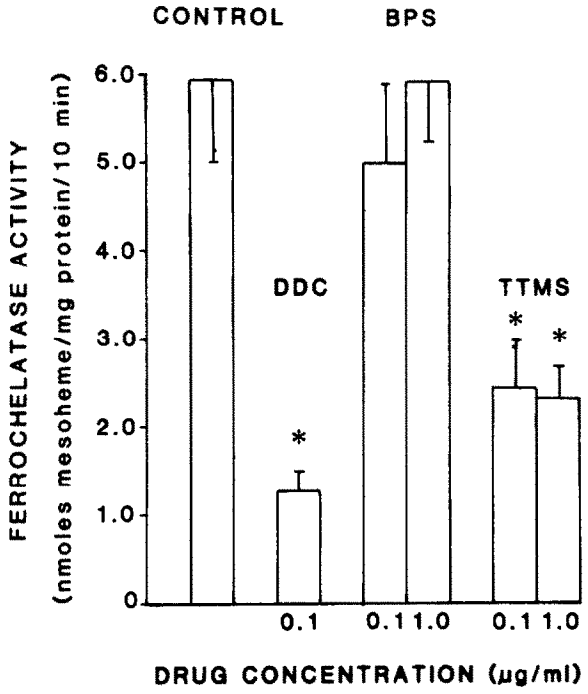

Fig.3. Ferrochelatase activity in chick embryo liver cell cultures cell cultures 6 h after addition of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), 3-benzyl-4-phenylsydnone (BPS), 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS). Each bar represents the mean of 4 determinations ± SD in one experiment and the results were confirmed in 2 additional experiments. * Significantly different from control by Newman-Keuls tests ($p \leq 0.05$).

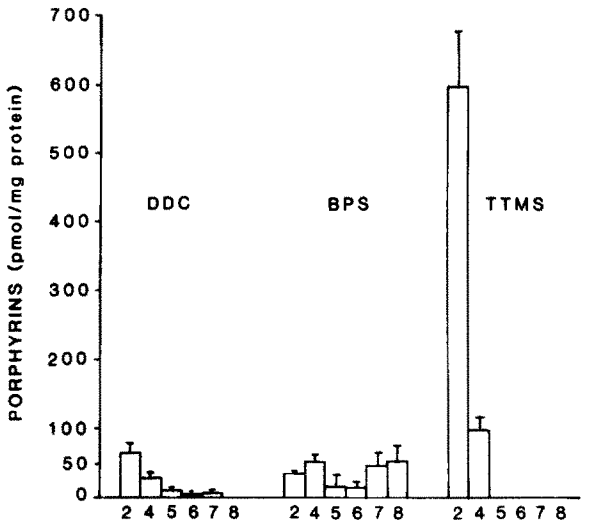

Fig.4. Porphyrin patterns obtained 24 h after the addition of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), 3-benzyl-4-phenylsydnone (BPS), 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS) (3 μg/ml medium) to chick embryo liver cell cultures. Numbers under the bars indicate the number of carboxyl groups (8, uroporphyrin; 7, heptacarboxylic porphyrin; 6, hexacarboxylic porphyrin; 5, pentacarboxylic porphyrin; 4, coproporphyrin; 2, protoporphyrin). Each bar represents the mean ± SD of 4 determinations in one experiment and the results were confirmed in 2 additional experiments.

tion described above. The higher porphyrinogenicity observed with sydnone (fig.1a) than with the dihydropyridine (fig.2) despite its lower ferrochelatase-inhibitory potency (fig.3) might therefore be attributable to higher lipophilicity. The pattern of porphyrin accumulation induced by the sydnone (fig.1a) in chick embryo liver cells was examined by high-performance liquid chromatography (fig.4); protoporphyrin IX was the major porphyrin to accumulate (approximately 85%). This pattern of porphyrin accumulation is similar to that observed with the dihydropyridine (fig.4) and is consistent with the observation that the sydnone (fig.1a) inhibits ferrochelatase. It was concluded that (i) Stejskal et al. [8] were correct in their identification of the hepatic pigment as protoporphyrin IX, and (ii) that protoporphyrin IX accumulated due to the ferrochelatase-inhibitory activity of the sydnone (fig.1a).

The sydnone (fig.1a) has been shown to cause suicidal inactivation of cytochrome P-450 when incubated with liver microsomes from phenobarbital-pretreated rats [9,18]. Following administration of the sydnone (fig.1a) to phenobarbital-pretreated rats, an *N*-alkylporphyrin was isolated from the liver and shown to be a mixture of the regio isomers of *N*-vinylprotoporphyrin IX. The lowering of ferrochelatase activity (fig.3) caused by the sydnone (fig.1a) is therefore presumably due to ferrochelatase inhibition exerted by the regioisomers of *N*-vinylprotoporphyrin IX. The formation of *N*-vinylprotoporphyrin has been suggested to occur as follows: the sydnone (fig.1a) is catalytically activated by cytochrome P-450 followed by heme alkylation of cytochrome P-450 by a fragment of the sydnone (fig.1a), viz. a vinyl group [18]. According to this scheme [18] the vinyl group is derived from the two carbon atoms in the thioethyl fragment of the 3-substituent of the syd-

none (fig.1a). To test this scheme, 3-benzyl-4-phenylsydnone (fig.1b), which lacked a thioethyl fragment in the 3-position, and therefore could not be activated to form *N*-vinylprotoporphyrin IX was investigated. 3-Benzyl-4-phenylsydnone did not inhibit ferrochelatase (fig.3) and protoporphyrin IX was found to constitute only a minor fraction (16%) of the porphyrins which accumulated (fig.4). These results support the scheme, suggested for formation of *N*-vinylprotoporphyrin IX from the sydnone (fig.1a). Since 3-benzyl-4- phenylsydnone does not lower ferrochelatase activity (fig.3) its porphyrinogenicity (figs 2 and 4) presumably resides in its property of lipophilicity.

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REFERENCES

[1] Tephly, T.R., Gibbs, A.H. and De Matteis, F. (1979) Biochem J. 180, 241–244.
[2] De Matteis, F., Gibbs, A.H. and Tephly, T.R. (1980) Biochem. J. 188, 145–152.
[3] Ortiz de Montellano, P.R., Beilan, H.S. and Kunze, K.L. (1981) Proc. Natl. Acad. Sci. USA 78, 1490–1494.
[4] De Matteis, F., Gibbs, A.H., Farmer, P.B. and Lamb, J.H. (1981) FEBS Lett. 129, 328–331.
[5] Ortiz de Montellano, P.R., Beilan, H.S. and Kunze, K.L. (1981) J. Biol. Chem. 256, 6708–6713.
[6] Tephly, T.R., Coffman, B.L., Ingall, G., Abou Zeit-Har, M.S., Goff, H.M., Tabba, H.D. and Smith, K.M. (1981) Arch Biochem. Biophys. 212, 120–126.
[7] Cole, S.P.C., Whitney, R.A. and Marks, G.S. (1981) Mol. Pharmacol. 20, 395–403.
[8] Stejskal, R., Itabashi, M., Stanck, J. and Hrnbam, Z. (1975) Virchows Arch. B18, 83.
[9] Grab, L.A., Ortiz de Montellano, P.R., Sutherland, E.P. and Marks, G.S. (1985) Fed. Proc. 44, 1610.
[10] Marks, G.S. (1978) in: Handbook of Experimental Pharmacology (De Matteis, F. and Aldridge, W.N. eds) vol. 44, pp. 210–237, Springer, Berlin.
[11] Marks, G.S., Allen, D.T., Johnston, C.T., Sutherland, E.P., Nakatsu, K. and Whitney, R.A. (1985) Mol. Pharmacol. 27, 459–465.
[12] Morgan, R.O., Fischer, P.W.F., Stephens, J.K. and Marks, G.S. (1976) Biochem. Pharmacol. 25, 2609–2612.
[13] Cole, S.P.C., Vavasour, E.J. and Marks, G.S. (1979) Biochem. Pharmacol. 28, 3533–3538.
[14] Porra, R.J., Vitols, K.S., Labbe, R.F. and Newton, N.A. (1967) Biochem. J. 104, 321–327.
[15] Granick, G. (1966) J. Biol. Chem. 241, 1359–1375.
[16] Zelt, D.T., Owen, J.A. and Marks, G.S. (1980) J. Chromatogr. 189, 209–216.
[17] De Matteis, F. (1978) in: Handbook of Experimental Pharmacology (De Matteis F. and Aldridge, W.N. eds) vol. 44, pp. 129–155, Springer, Berlin.
[18] Ortiz de Montellano, P.R., Costa, A.K., Grab, L.A., Sutherland, E.P. and Marks, G.S. (1985) in: Proceedings of International Conference on Porphyrins and Porphyrias, Paris, France (Nordman, Y. ed.) Karger, in press.