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Differential interaction of porphyrins used in photoradiation therapy with ferrochelatase

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The mechanism of porphyrin accumulation by tumours is not yet established. If metabolism aids porphyrin elimination, tumours, unlike normal tissues, may not metabolize porphyrins used clinically, such as proto-, haemato-, *OO'*-diacetyl-haemato- and monohydroxyethyl-monovinyl-deutero-porphyrin. Proto-, haemato- and monohydroxyethyl-monovinyl-deutero-porphyrin are substrates for the mitochondrial enzyme ferrochelatase (EC 4.99.1.1), which can form haem analogues from exogenous porphyrins. The K_m values for proto-, haemato- and monohydroxyethyl-monovinyl-deutero-phorphyrin are 11, 22 and 23 μM respectively. However, *OO'*-diacetyl-haematoporphyrin is an effective competitive inhibitor with K_i of 11 μM. Hepatic ferrochelatase specific activity is 5.9 and 5.5 nmol of haem/h per mg of protein respectively in normal Buffalo rat and in those bearing the extrahepatic Morris 7288C hepatoma, and is only 0.13 nmol/h per mg in the hepatomas. Therefore low ferrochelatase activity in cancerous cells may provide one means whereby some porphyrins accumulate in tumours, and the ability of certain porphyrins to act as ferrochelatase inhibitors may provide another.

Photoradiation therapy, employing intravenous injection of chemical derivatives of naturally occurring porphyrins, has been used with some success to treat several different neoplasias (Dougherty *et al.*, 1978; for review see Dougherty *et al.*, 1982). The basis of the therapy lies in the photochemical cytotoxic properties of the exogenous porphyrins, which appear to be retained by both human (Gregorie *et al.*, 1968; Dougherty *et al.*, 1978; Dahlman *et al.*, 1983; Kato *et al.*, 1983) and animal cancerous cells (Gomer & Dougherty, 1979; Henderson *et al.*, 1980; Berenbaum *et al.*, 1982). Porphyrins are photoreactive and upon illumination fluoresce intensely. Consequently highly active oxygen species, which are cytotoxic, are produced intracellularly (Weishaupt *et al.*, 1976). These properties of porphyrins therefore allow both the detection and destruction of cancerous cells. We consider that the interaction of porphyrins in serum with cells involves the following processes: dissociation of the porphyrin–albumin complex (Smith & Neuschatz, 1983), cellular uptake of porphyrin, intracellular binding and elimination, which may or may not require metabolism. Since porphyrins are retained by cancerous but not by normal cells, it follows that there must be differences in one or more of these processes between tumours and normal tissue.

If metabolism aids porphyrin elimination from cells, accumulation may be caused by an inability of tumours to metabolize porphyrins. Elimination of exogenous porphyrins would be facilitated if they were substrates for the enzymes that normally form (ferrochelatase, protohaem ferro-lyase, EC 4.99.1.1) and break down (haem oxygenase, EC 1.14.99.3) haem. Since haematoporphyrin is a major component of the clinically used mixture called haematoporphyrin derivative and is known to be a substrate for ferrochelatase from *Rhodopseudomonas sphaeroides* (Jones & Jones, 1970) and duck erythrocytes (Yoneyama *et al.*, 1962), it seemed likely that several other porphyrin components of haematoporphyrin derivative might be substrates for the mammalian ferrochelatase. In contrast, other porphyrins inhibit this enzyme (Dailey & Fleming, 1983). Further, ferrochelatase activity is much lower in hepatoma tissue compared with normal liver. This differential metabolism of porphyrin components of haematoporphyrin derivative by normal and cancerous cells may provide one mechanism whereby certain porphyrins are retained by tumours but eliminated

from normal tissues, particularly liver. Also, it is postulated that a combination of lowered ferrochelatase activity from the presence of inhibitory porphyrins may lead to an accumulation of endogenous porphyrins that may become cytotoxic.

Materials and methods

OO'-Diacetyl-haematoporphyrin was prepared and characterized by using the method of Bonnett *et al.* (1981), and other porphyrins were obtained commercially (Porphyrin Products, Logan, UT, U.S.A.). To check the purity of these porphyrins high-pressure liquid chromatography was carried out with a Varian C_{18} reverse-phase column (0.5cm × 25cm) with methanol/water (17:3, v/v) containing 2mM-tetrabutylammonium dihydrogen phosphate as solvent. The flow rate was 1ml/min, and the eluted porphyrins were detected by their absorbance at 405nm. Their purity was judged to be generally greater than 90–95%. The monohydroxyethyl-monovinyl-deuteroporphyrin used was a mixture of the two isomers. Stock solutions of porphyrins (1mM) were freshly made up in dimethyl sulphoxide and their concentrations determined by using published absorption coefficients (Furhop & Smith, 1976; Tipping *et al.*, 1978; Bonnett *et al.*, 1981). For the bisglycol- and disulphonic-deuteroporphyrins an estimated millimolar absorption coefficient of 13.7 for the II band in 0.1M-HCl was used in this work.

Bovine ferrochelatase purified from hepatic mitochondria by using published procedures (Dailey & Fleming, 1983) was used in this work Activity was assayed in a final reaction volume of 1ml, containing 50mM-Tris acetate buffer, pH8.1, 5mM-dithiothreitol, 0.2mM-ferrous ammonium citrate, 0.2% Triton X-100, 0.1mM-porphyrin and the enzyme preparation. For most assays with tissues and for the inhibition determinations, deuteroporphyrin was used as the porphyrin substrate. The reaction was carried out at 37°C in the dark for 30min and stopped by the addition of 0.5ml of 50mM-iodoacetamide. The product was quantified with a Cary 219 spectrophotometer as the pyridine haemochromogen by using the absorption coefficients given by Furhop & Smith (1976). For the determination of the haem of the disubstituted deuteroporphyrins an estimated $\Delta\varepsilon$ of $15.3 \times 10^3 M^{-1} cm^{-1}$, for the difference between the maximum of the α-band and the trough between the α- and β-bands, was used.

The Morris hepatoma 7288 C, kindly provided by Dr. J. Mapes (L.S.U. Medical Center), was originally developed by Dr. E. Thompson (Thompson *et al.*, 1966). Frozen cells (about 2×10^7 cells in 1.5ml) were thawed rapidly and injected intramuscularly into both hind legs of ether-anaesthetized adult male Buffalo rats (220–250g body wt.; from Harlan, Indianapolis, IN, U.S.A.) on the caudal side of the femur. After about 14 days solid tumours had grown at the injection sites, and the hepatoma tissue was removed by using aseptic techniques, avoiding the necrotic areas. The hepatoma cells were resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) containing penicillin (1000i.u./ml) and streptomycin (100μg/ml) and repassaged. After 7–10 days tumours of 7–10g wet wt. were produced from these cells and used in these experiments.

Normal Buffalo rats were matched by weight and fed and housed under the same conditions as those bearing tumours. Liver tissue was obtained from normal and tumour-bearing animals (termed experimental liver) after perfusion with ice-cold 0.9% NaCl (Smith & Morgan, 1979). Portions of these tissues were frozen immediately in liquid N_2. For ferrochelatase assays 1g tissue samples were thawed and suspended in 1ml of 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH7.5, containing 10mM-dithiothreitol. Cells were disrupted by homogenization, and large cell debris was removed by centrifugation (2000*g* for 5min). Immediately before the assay Triton X-100 was added to give 1% (w/v) and the sample sonicated for 30s. Ferrochelatase activity was then assayed as described above.

Results and discussion

The mitochondrial enzyme ferrochelatase can use a variety of porphyrins as substrates to form the corresponding haems. We have surveyed several representative porphyrins present in the porphyrin mixture administered to patients for their ability to serve as substrate for the purified ferrochelatase. These include haematoporphyrin, protoporphyrin, monohydroxyethyl-monovinyl-deuteroporphyrin and *OO'*-diacetyl-haematoporphyrin. Fig. 1 demonstrates that monohydroxyethyl-monovinyl-deuteroporphyrin is a good substrate for the enzyme with a K_m of 23μM. As expected, both haematoporphyrin and protoporphyrin, the natural substrate, are substrates for this enzyme purified from bovine liver (Table 1).

Interestingly, *OO'*-diacetyl-haematoporphyrin is not a substrate, but is instead an effective competitive inhibitor, with a K_i of 11μM (see Fig. 2 and Table 1). This is not unexpected, since porphyrins with bulky substituents in the 2,4-positions, 2,4-disulphonic- and 2,4-bisglycol-deuteroporphyrin (Dailey & Fleming, 1983), and

2,4-bisacetyl-deuteroporphyrin, are competitive inhibitors with respect to deuteroporphyrin. The K_i values of these are summarized in Table 1.

Although the extent of de-differentiation of tumours varies, they commonly use glycolysis more extensively than oxidative phosphorylation, especially in rapidly growing tumours (Friedkin, 1973) and contain low activities of mitochondrial cytochrome oxidase (Weinhouse, 1960). We therefore considered that the Morris hepatoma 7288C cells would be deficient in the ferrochelatase. In fact, although normal hepatic ferrochelatase activity is 5.9 ± 0.3 nmol of deuterohaem formed/h per mg of protein, the activity is less than 2% of these

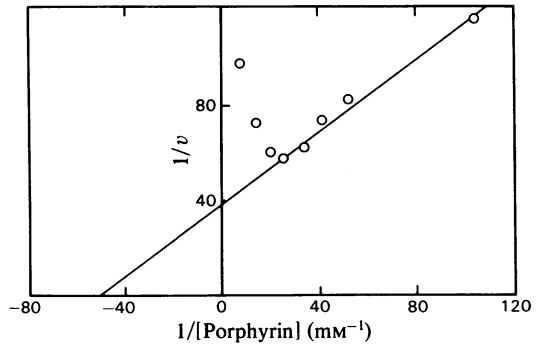


Fig. 1. *Determination of the K_m of ferrochelatase for monohydroxyethyl-monovinyl-deuteroporphyrin*

Purified bovine ferrochelatase was assayed as described in the Materials and methods section with various amounts of porphyrin. The results are plotted in a Lineweaver–Burk format, where v is nmol of deuterohaem formed/min per nmol of ferrochelatase. The upward curve seen at high substrate concentration is similar to that seen with protoporphyrin, and may result from porphyrin aggregation at higher porphyrin concentrations.

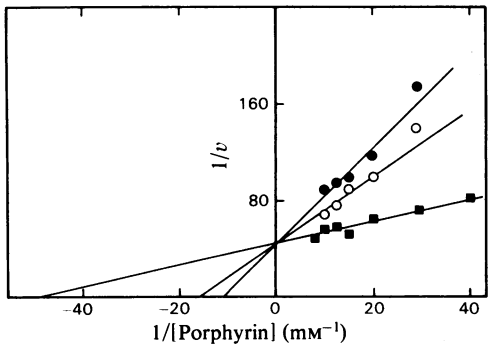
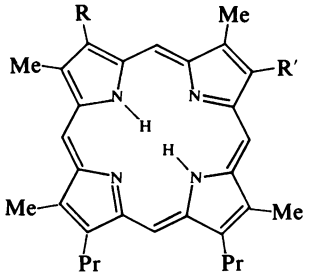


Fig. 2. *Inhibitory properties of OO′-diacetyl-haematoporphyrin on ferrochelatase*

Purified ferrochelatase was assayed as described in the text, with haematoporphyrin as the substrate. The lower line (■) is obtained for haematoporphyrin alone in the absence of *OO′*-diacetyl-haematoporphyrin. The upper two lines are obtained with 32 μM- (○) and 56 μM- (●) *OO′*-diacetyl-haematoporphyrin.

Table 1. *Kinetic parameters of bovine ferrochelatase*



Ac = O–C(=O)–CH_3; Me = CH_3 and Pr = $[CH_2]_2CO_2H$.

Porphyrin	R	R′	K_m (μM)	K_i (μM)
Protoporphyrin*	$CH{=}CH_2$	$CH{=}CH_2$	11	—
Mesoporphyrin	CH_2CH_3	CH_2CH_3	34	—
Deuteroporphyrin	H	H	47	—
Haematoporphyrin	$CH(OH)CH_3$	$CH(OH)CH_3$	22	—
Monohydroxyethyl-monovinyl-deuteroporphyrin†	$CH{=}CH_2$	$CH(OH)CH_3$	23	—
OO′-Diacetyl-haematoporphyrin	$CH(OAc)CH_3$	$CH(OAc)CH_3$	—	11
2,4-Bis-acetal-deuteroporphyrin	$CH_2CH(OCH_3)_2$	$CH_2CH(OCH_3)_2$	—	13
2,4-Bis-glycol-deuteroporphyrin	$CH(OH)CH_2OH$	$CH(OH)CH_2OH$	—	67
2,4-Disulphonic-deuteroporphyrin	SO_3^-	SO_3^-	—	70

* Natural substrate for this enzyme.
† A mixture of isomers.

values, i.e. 0.13 ± 0.05 nmol/h per mg, in the hepatoma tissue.

Hepatic microsomal haem oxygenase activity is increased in the liver of animals bearing the extrahepatic Murphy–Sturm lymphosarcoma (Schacter & Kurz, 1982) or the Walker 256 carcinosarcoma (Beck *et al.*, 1982), which do not metastasize to the liver. In contrast, hepatic ferrochelatase activity in the liver of Buffalo rats 10 days after transplantation of the Morris hepatoma is the same as in normal animals (Table 2).

In conclusion, certain exogenous porphyrins used in phototherapy are substrates for purified ferrochelatase, and this enzyme is present in low amounts in Morris hepatoma 7288C tissue. Protoporphyrin and deuteroporphyrin are transported into intact mitochondria and iron is incorporated there (Romslo & Husby, 1980; Koller & Romslo, 1980). Also, exogenous porphyrins are transported into Morris 7288 hepatoma cells *in vitro* (Smith & Neuschatz, 1983). We therefore propose that, in normal liver, clearance of certain exogenous porphyrins, including protoporphyrin, haematoporphyrin and monohydroxyethyl-monovinyl-deuteroporphyrin, is facilitated by their metabolism to their corresponding haems. Further metabolism by haem oxygenase is likely, since the haem analogues formed will be similar in structure to those like haematohaem known to be substrates for this enzyme *in vitro* (Frydman *et al.*, 1981). Conversely, tissues with low ferrochelatase activity, including certain tumours such as the Morris hepatoma, would retain exogenous porphyrins.

Porphyrins that are inhibitors and not substrates for ferrochelatase would not be expected to cause major problems in normal hepatic tissues, owing to the relatively high ferrochelatase activity. However, in cancerous cells, where ferrochelatase activity is low, small amounts of these competitive inhibitors may profoundly effect the cell's haem metabolism. This inhibition may cause cellular haem pools to decrease, thereby de-repressing the initial rate-limiting step in the haem-biosynthetic pathway, 5-aminolaevulinate synthase (Granick & Beale, 1978). The consequent accumulation of endogenous porphyrins may then contribute to the observed photosensitivity of these cells. Accumulation of endogenous porphyrins might also contribute to the cutaneous photosensitivity observed in some patients (Zalar *et al.*, 1977; Dougherty, 1981) and to the sensitivity of certain normal tissues (Douglas *et al.*, 1983). In addition, ferrochelatase may provide one porphyrin-binding site in mitochondria, where exogenous porphyrins have been shown to localize (Cozzani *et al.*, 1981; Berns *et al.*, 1982).

Table 2. *Comparison of hepatic ferrochelatase activity with that in Morris hepatoma 7288C tissue*

Ferrochelatase activity was assayed as described in the text. Activity is expressed as nmol of deuterohaem formed/h per mg. The results are given as means ± S.D. for three individual animals assayed.

Tissue	Activity
Normal liver	5.9 ± 0.28
Experimental liver	5.47 ± 0.87
Morris hepatoma 7288C	0.13 ± 0.05

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