# EFFECT OF SOME ANTINEOPLASICS ON METABOLIC HEME PATHWAY

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Abstract—1. The porphyrinogenic ability of several antineoplasics used in the therapy of the different cancers was evaluated. The action of cyclophosphamide, busulfan and 5-fluorouracil on the amount and nature of the accumulated hepatic porphyrins and on the activity of $\delta$-aminolaevulinate synthase (ALA-S), were estimated at different doses and times of drug treatment in 17-day-old chick embryos.

2. It was observed that cyclophosphamide produces a significant increase in the accumulation of hepatic porphyrins at different doses as well as in the activity of the ALA-S, at all the incubation times. Cyclophosphamide alters the pattern of porphyrins accumulated in the liver, where a coproporphyrin: protoporphyrin ratio higher than in the controls can be observed.

3. Busulfan increased the hepatic porphyrins accumulated in the liver but to a lesser degree than cyclophosphamide.

4. 5-Fluorouracil did not modify the hepatic porphyrin content when it was administered at doses up to 40 mg/embryo.

5. When the embryos were injected with busulfan or 5-fluorouracil no significant differences were observed in the activity of ALA-S up to 11 hr of incubation.

6. These results indicate that cyclophosphamide has a remarkable porphyrinogenic capacity in chick embryo while busulfan, notwithstanding the fact that it alters the haem pathway, it does so to a degree that does not impair the regulation of ALA-S activity. Fluorouracil seems to be non porphyrinogenic in this system, up to 40 mg/embryo.

## INTRODUCTION

Porphyrias are metabolic disorders characterized by an increased excretion of porphyrins and/or their precursors and by the accumulation of porphyrins in some tissues. The hepatic human porphyria cutanea tarda (PCT) is one of the most common forms of porphyria in which the disturbances of porphyrin metabolism are located mainly in liver (Meyer and Schmid, 1978).

The simultaneous appearance of PCT and tumors in internal organs has been observed (Török, 1976) and it was first pointed out by Tio *et al.* (1957). While some authors have considered the association of both diseases as a paraneoplasic event, others have regarded it only as a coincidence.

It is widely known that most PCT patients show a hepatic alteration with several degrees of severity. The high incidence of liver tumors in PCT is not merely related to hepatic cirrhosis (Solis *et al.*, 1982) and according to Rimbaud *et al.* (1972), in these patients hepatic cirrhosis incidence is five times that found in the average population. On the other hand, primary liver cancer is 100–200 times more frequent in PCT patients than in the rest of population (Kordac, 1972).

The use of antineoplasics is common in cancer therapy. However the knowledge of their effect on haem metabolic pathway is scarce and unclear (Arbus, 1981; Palma-Carlos *et al.*, 1971). In fact, at the time Arbus (1981) placed the question about what

antineoplasic could be safely used in the treatment of a PCT patient no response existed in the literature. Even today only a few reports are found: Kyle and Dameshek (1961) reported about the potential ability of busulfan, used in the treatment of a human case of chronic granulocytic leukemia, to accelerate the triggering of PCT and Palma-Carlos *et al.* (1971) reported of variable results when studying the effect of alkylating agents in rats.

Consequently, it is a matter of great interest to evaluate the porphyrinogenic capacity of different antineoplasics used to treat different types of cancer.

Therefore, the aim of the present research is to estimate the direct action of the following antineoplasics: cyclophosphamide, busulfan and 5-fluorouracil on haem metabolic pathway in chick embryos. This system is sensitive and useful to assay the porphyrinogenic capacity of numerous drugs (Racz and Marks, 1969; Rifkind *et al.*, 1973).

## MATERIALS AND METHODS

### Chemicals

Cyclophosphamide was a gift from Labinca, Argentina; 5-fluorouracil from Roche, Argentina. Busulfan was kindly provided by Dr Mac Loughlin of Wellcom, Argentina and purified from acetone.

### Animals

Seventeen-day-old chick embryos were of the White Leghorn strain.

## Treatment

A small hole was made in the egg shell above the air sac of chick embryos. Two or three drops of sterile saline were dropped on the chorioallantoic membrane to make it transparent. Drugs were injected through this membrane under sterile conditions into the fluids surrounding the embryo. The opening in the shell was then covered with cellophane tape and the eggs were returned to the incubator at 38 C and maintained there for 24 hr in order to analyze porphyrin accumulation or, when indicated, to assay ALA-S activity.

At the indicated times, the embryos were killed by decapitation. The livers were removed and carefully separated from the gall bladder.

The different doses of cytostatic agent were injected in 0.1–0.4 ml of dimethylsulfoxide, except for fluorouracil, which was supplied as a saline solution. Dimethylsulfoxide alone did not affect the parameters measured.

## Hepatic porphyrin analysis

Individual livers from chick embryos (0.4–0.5 g) were separately homogenized in ethylacetate acetic acid (4:1 v/v). Porphyrins were extracted from the whole organ according to the method of Racz and Marks (1969), except that porphyrins were extracted with 5% HCl, instead of 3 N HCl, and were estimated spectrophotometrically (San Martin de Viale et al., 1977). Porphyrin methyl esters were separated by thin-layer chromatography according to the number of carboxyl groups (Doss, 1970).

## ALA-S activity

The livers were removed, immediately cooled on ice, and washed with cold saline. They were homogenized with 0.9% NaCl containing 0.5 mM EDTA and 10 mM Tris–HCl buffer (pH 7.2), in order to obtain 4 ml of homogenate/g liver. The activity of ALA-S was assayed in whole homogenates by the method of Marver et al. (1966). For the chick embryo liver assay the incubation mixture contained 0.5 ml of homogenate, 0.075 M glycine, 0.01 M EDTA, 0.06 M Tris–HCl buffer (pH 7.2) and 150 μM pyridoxal phosphate in a final volume of 2 ml. Assay mixtures were incubated at 37 C for 60 min.

## Statistical treatment of results

The nonparametric test of Mann–Whitney U test (Siegel, 1956) was used since there was not homoscedasticity and normal distribution could not be assured.

## RESULTS

### Effect of cyclophosphamide, busulfan and 5-fluorouracil on accumulation of porphyrins in liver

Figure 1 shows that cyclophosphamide significantly increases accumulation of liver porphyrins when quantities equal or higher than 10 mg/embryo are given. Considering the median values, increases of 33–60 times with respect to the controls are seen; individual values up to 25 μg porphyrins/g liver were found, which represent 400-fold enhancement as compared to controls.

As can be seen in Fig. 2, cyclophosphamide produces a great accumulation of coproporphyrin (85%) and a lower proportion of proto-(7%), pentacarboxy-(3%), hexacarboxy-(2%), heptacarboxy-(2%) and uroporphyrin (1%). Considering the nature of porphyrins accumulated in control liver embryos (56% coproporphyrin and 44% protoporphyrin) coproporphyrin increases at the expense of protoporphyrin.

Figure 3 shows that busulfan also augments accumulation of liver porphyrins as compared to controls but to a lesser degree than cyclophosphamide. Thus a 10 mg/embryo dose of busulfan produces a low increment of the median value which is not significant. Just starting with 15 and 20 mg/embryo, significant increases at 0.05 level (8 and 28 times, respectively) can be seen.

Figure 4 shows that concentrations of 5, 10, 20 and 40 mg embryo of 5-fluorouracil do not affect the liver porphyrin content and a 30 mg/embryo dose only produces a 3-fold increment. When given 60, 80 and 100 mg/embryo, low increases are found, with a
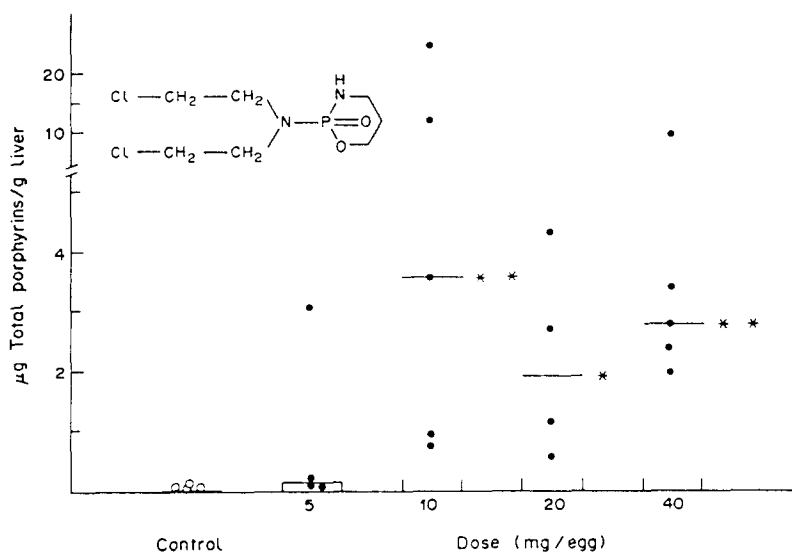


Fig. 1. Hepatic accumulation of porphyrins induced in 17-day-old chick embryos by cyclophosphamide. Values are given for the individual animals. For each dose the median is represented by (—). Significantly different from dimethylsulfoxide controls *P < 0.05, **P < 0.01 (○) Dimethylsulfoxide controls, (●) cyclophosphamide treated embryos.
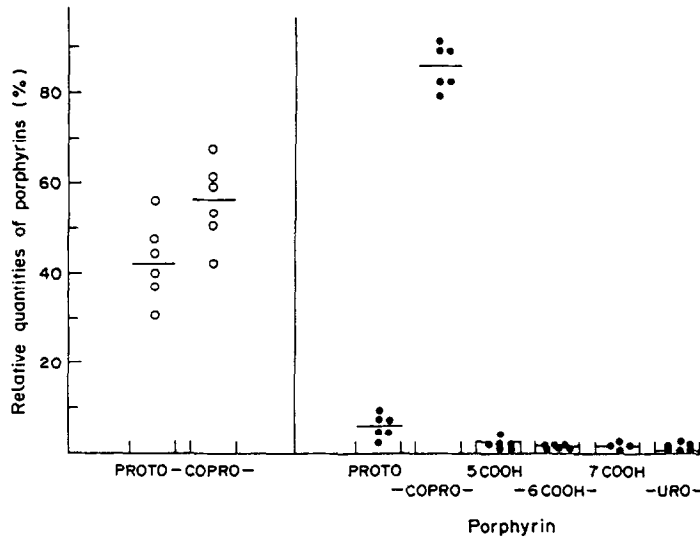
Fig. 2. Nature of porphyrins accumulated in liver of normal and cyclophosphamide treated embryos. (O) controls, (●) cyclophosphamide. Other details are given in Fig. 1.
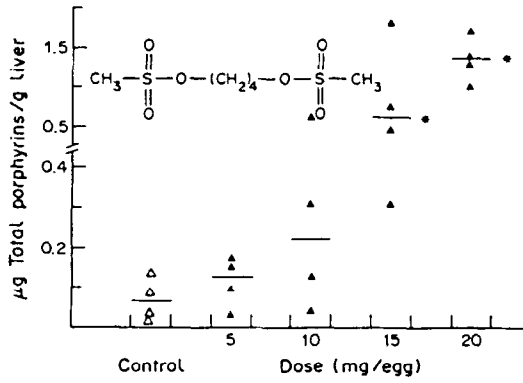


Fig. 3. Hepatic accumulation of porphyrins in 17-day-old chick embryos by busulfan. Other details are as given in Fig. 1. Significantly different from controls $*P < 0.05$. (△) Dimethylsulfoxide controls, (▲) busulfan treated embryos.
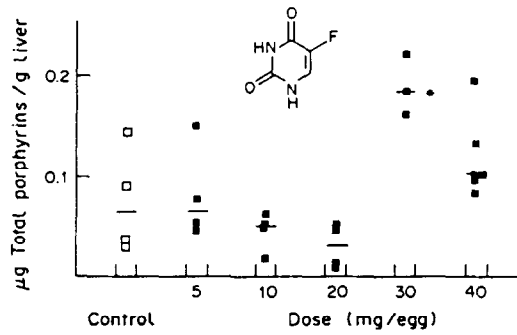


Fig. 4. Hepatic accumulation of porphyrins in 17-day-old chick embryos by 5-fluorouracil. Other details are as given in Fig. 1. Significantly different from saline controls $*P < 0.05$, (□) Saline controls, (■) 5-fluorouracil treated embryos.

maximum at 100 mg/embryo (12-times). It is interesting to point out that at these doses most embryos which accumulate porphyrins die.

*Enzymatic activity of ALA-S*

Figure 5 shows that ALA-S activity increases with incubation time with cyclophosphamide, reaching a peak after 16 hr of incubation, with an increment of 120-times with respect to the normal value (3.3 nmol ALA/g liver/hr). Incubations for longer periods than 16 hr resulted in a dramatic decrease in activity so that after 23 hr the mean activity estimated is similar to that found for 4–6 hr incubations.

ALA-S activity was measured in embryos inoculated with busulfan or 5-fluorouracil after 2, 4, 6, 8 and 11 hr or incubation (Fig. 6). No significant differences in the activity of this enzyme were obtained with respect to the control for either of the two drugs at any time.

## DISCUSSION

Present results indicate that cyclophosphamide has a remarkable porphyrinogenic action in chick embryo, evidenced by an increase in ALA-S activity and a great accumulation of liver porphyrins (Figs 1, 2 and 5) that significantly modify its pattern as a consequence of drug treatment. Busulfan disrupts heme metabolism by significantly increasing the accumulation of porphyrins (Fig. 3) but this disturbance is not sufficient to perturb the system since ALA-S activity remains normal (Fig. 6). Fluorouracil is not porphyrinogenic in doses up to 40 mg/embryo, since its administration does not increase the liver porphyrin content or ALA-S activity. So, cyclophosphamide seems to be more porphyrinogenic than busulfan in this system. These results differ from the classification given by Rifkind (1976) in which this order is inverted, based on the studies of Palma-Carlos *et al.* (1971). These authors working with rats found that cyclophosphamide alters urinary porphyrin and precursors excretion in a variable and little defined manner; ALA-S was not assayed.

Cyclophosphamide is inactive *per se* and it requires metabolic activation by liver microsomal P-450 cytochrome, which is dependent on mixed function oxidases (Marinello *et al.*, 1984).
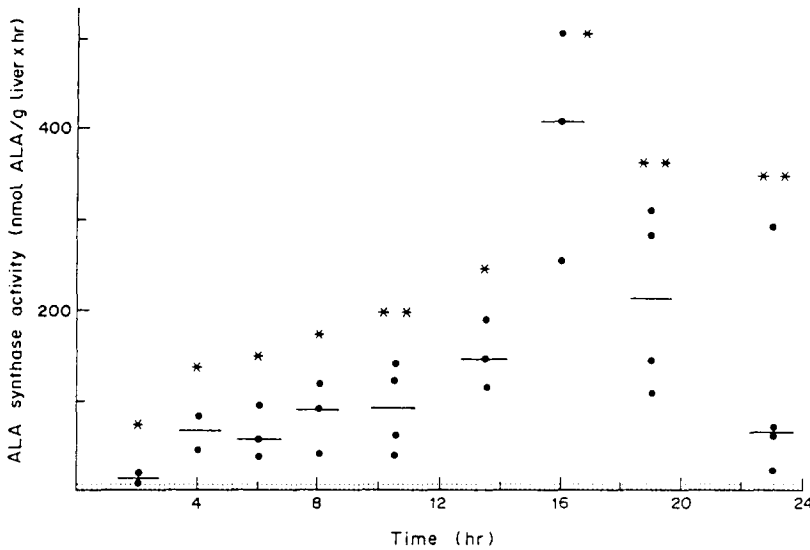


Fig. 5. Effect of cyclophosphamide on the activity of ALA-S in 17-day-old chick embryo liver. Dose assayed (10 mg/egg). The embryos were incubated at 38 C and killed at the times indicated above. The shaded area represents the range of normal values. Other details are given in Fig. 1. Significantly different from control values *$P < 0.05$, **$P < 0.01$.
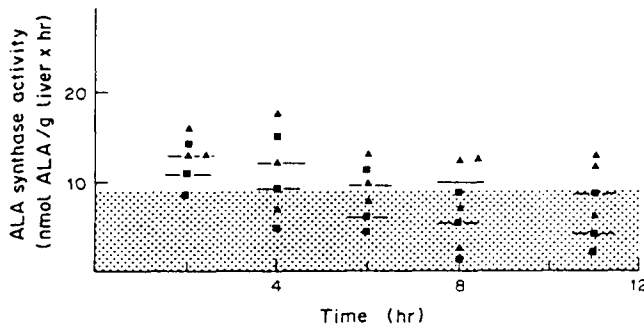


Fig. 6. Effect of busulfan (▲) and fluorouracil (■) on the activity of ALA-S in 17-day-old chick embryo liver. Dose assayed (15 mg/egg). Other details are given in Fig. 5.

Cyclophosphamide metabolism produces acrolein which binds to cytochrome P-450. Which in turn is capable of further metabolism to a reactive metabolite presumably an alkene oxide, which can alkylate proteins in microsomes. The destruction of this cytochrome takes place owing to alkylation of critical sulfhydryl groups located at or near the active site of cytochrome P-450 (Marinello *et al.*, 1984). The destruction of liver microsomal P-450 cytochrome could be the primary mechanism of induction for ALA-S as it happens with other porphyrinogenic drugs (Lim *et al.*, 1980). Cyclophosphamide would exert its porphyrinogenic action through acrolein, which by destroying cytochrome would induce its new synthesis and therefore a higher heme production rate. This would in turn increase the activity of ALA-S, the enzyme regulating heme biosynthetic pathway, thus leading to a rise in the liver porphyrin content. Thereafter the mechanism of action of cyclophosphamide to induce ALA-S would be similar to that of allylisopropylacetamide, a drug of well known porphyrinogenic capacity. The latter produces a rapid loss of cytochrome P-450 content (De Matteis, 1971) acting as a suicidal substrate that inactivates cytochrome P-450 by alkylation (Ortiz de Montellano *et al.*, 1979; Ortiz de Montellano and Mico, 1981).

Busulfan, an alkyl alkane sulfonate reacts with thiol groups of amino acids and proteins more extensively than do the nitrogen mustards. It was suggested that the cytotoxic action of busulfan could be exerted through such thiols rather than through interactions with DNA (Roberts and Warwick, 1957, 1959). Therefore busulfan could react with enzyme proteins decreasing their activity. As a consequence an increase of ALA-S activity would not be expected (Fig. 6). Accumulation of porphyrins could be explained as resulting from a block of some of the enzymes which act between uroporphyrinogen and protoporphyrin in heme pathway. It is interesting to point out that 1-(2-chloroethyl)-3-cyclohexyl-1 nitrosourea, also an antineoplasic alkylating agent, produces a decrease of hepatic ALA-S activity in rats (El-Azhary and Ahmed, 1984) perhaps as a consequence of the nitrogen cytidylate and guanylate DNA alkylation that results in DNA–DNA and DNA–protein crosslinks (Colvin, 1982).

5-Fluorouracil belongs to the group of fluorinated pyrimidines. It acts through inhibition of thymidylate synthetase. 5-Fluorouracil strongly binds to this enzyme in thymidylate synthesis and prevents formation of deoxythymidine triphosphate, one of the nucleotides required for DNA synthesis (Chabner, 1982). This produces a decreased formation of RNA and protein. Therefore, synthesis of enzyme protein, ALA-S and other enzymes of heme pathway would be consequently very depressed. This would justify the lack of increase of both ALA-S activity and porphyrin production when inoculating this antineoplasic in chick embryo (Figs 4 and 6).

It has been reported that porphyrinogenic drugs are able to precipitate attacks of porphyria in susceptible individuals even when given in low doses, normal organisms being disturbed at higher ones (Rifkind, 1976; Moore, 1980).

Hence the present findings enlarge the scarce knowledge on the porphyrinogenic ability of antineoplasics and might be potentially useful when selecting the safe drug to be employed in the treatment of cancer patients who suffer from or who are carriers of porphyria; specially in the case of cyclosphosphamide which, as is here demonstrated, strongly induces the regulatory enzyme of this metabolic pathway.

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