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EFFECT IN RATS OF SUBACUTE ADMINISTRATION OF ETHOSUXIMIDE, METHSUXIMIDE AND PHENSUXIMIDE ON HEPATIC MICROSOMAL ENZYMES AND PORPHYRIN TURNOVER

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Abstract—Oral administration of the closely related antiepileptic succinimides, ethosuximide, methsuximide and phensuximide, to male rats daily for 3 days, increases the activity of the hepatic microsomal enzymes as judged *in vivo* by the reduction of hexobarbitone-induced hypnosis and *in vitro* by increased oxidation of hexobarbitone and hydroxylation of aniline. Increased liver/body weight, liver microsomal cytochrome P-450 and hepatic δ-aminolaevulinic acid synthetase and a proliferation of the hepatic smooth endoplasmic reticulum are associated with the increase in drug metabolism. Daily administration of methsuximide to rats for 3 days reduces the anticonvulsant activity of the drug presumably by increasing its metabolism. In the rat, the synthesis of porphyrins by the liver is not affected by the treatment with any of the succinimides. However, methsuximide and phensuximide, but not ethosuximide, exhibit marked porphyrogenic activity in chick embryo liver. Of the three drugs, only methsuximide interacted with rat liver microsomal cytochrome P-450 to produce a type I spectral shift. The significance of the results is discussed in relation to the clinical use of these antiepileptic drugs.

THE PHARMACOLOGICAL and clinical efficacy of the anticonvulsant succinimides, ethosuximide, (2-ethyl-2-methyl-succinimide), methsuximide (N,2-dimethyl-2-phenyl-succinimide) and phensuximide (N-methyl-2-phenylsuccinimide) has been studied extensively.[1–4] In the treatment of petit mal epilepsy, these drugs must be administered regularly for considerable lengths of time. Although the acute and chronic toxicities of these succinimides in laboratory animals have been examined[4] there are no reports on the effects of repeated administration of ethosuximide, methsuximide and phensuximide on the activity and components of the hepatic microsomal enzyme system.

It is well known that chronic administration of a wide range of drugs to rats and other species may stimulate the drug metabolising enzymes of the hepatic endoplasmic reticulum.[5,6] This phenomenon has important bearing on the therapeutic use of such drugs. The increased rate of metabolism is associated with raised levels of cytochrome P-450 and NADPH-cytochrome *c* reductase which are components of the electron transport chain for the oxidation of drugs and foreign compounds. The activity of δ-aminolaevulinic acid (ALA) synthetase is also increased during the stimulation of drug metabolism.[7] This enzyme is the rate-limiting step of the haem biosynthetic pathway which provides haem for cytochrome P-450 and other haemoproteins.[8] During drug-induced porphyria, the normally delicate control of the haem biosynthetic pathway is lost and raised hepatic ALA synthetase activity is associated with

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an overproduction of the intermediates in the pathway, the porphyrinogens, which appear in high concentration in the urine as porphyrins.[8]

It, therefore, appeared important to investigate the effect of repeated administration of ethosuximide, methsuximide and phensuximide upon hepatic microsomal enzymes in the rat and on hepatic porphyrin turnover in the rat and in the chick embryo. As induction of hepatic microsomal enzymes in the rat is maximal following 3 days of daily treatment with many drugs[5] this subacute dose regimen was employed in the present study.

MATERIALS AND METHODS

Animals. Male, CFHB Wistar rats, 110–130 g, (Carworth Europe, Huntingdon, England) were given food and water *ad lib.* but were deprived of food overnight prior to sacrifice. In all experiments, measurement of biochemical and pharmacological activity of control and treated rats was conducted 84 hr after the first dose of each drug. To parallel the human situation with respect to dose levels,[9] an effective anticonvulsant dose of ethosuximide, methsuximide and phensuximide was employed *viz.* (0·5 mmole/kg/day).[4] The drugs were administered by stomach tube for 3 days as two equally divided daily doses suspended in 1% (w/v) sodium carboxymethylcellulose solution. Control animals received sodium carboxymethylcellulose solution orally (10 ml/kg/day). Additional groups of animals received a higher daily dose of the drugs (2 mmole/kg) in the same regimen.

Fertilized hens' eggs (C. V. Bartlett Ltd., Chepstow, England) were used to examine the porphyrogenic activity of the succinimides. The eggs were incubated at 38° and 90 per cent relative humidity for 17 days. The succinimides (8 mg/egg) were dissolved in 0·1 ml absolute alcohol. The eggshell was pierced above the air-sac and the solution injected through the chorioallantois into the fluids surrounding the embryo. Incubation was continued for 30 hr, at which time the chick embryo was killed by decapitation and the entire liver removed for the extraction of porphyrins. Ten eggs were injected for each compound. The dosage and period of incubation necessary for a well defined porphyrinogenesis had been determined in preliminary studies using methsuximide.

Experiments in vivo. The sleeping time of rats following intraperitoneal administration of hexobarbitone sodium (80 mg/kg) served as an index of the rate of drug metabolism *in vivo*. Sleeping time was considered to be the time that the righting reflex was absent following the injection of hexobarbitone.

The anticonvulsant (anti-leptazol) activity of methsuximide was determined in rats pretreated for 3 days with methsuximide (0·5 mmole/kg, daily). The rats received methsuximide (100 mg/kg, orally) 1 hr before intravenous infusion of a 1% (w/v) solution of leptazol (0·5 ml/min).[10] The volume of leptazol solution causing tonic convulsions was recorded for each animal.

Preparation of microsomal fraction. Rats were killed by decapitation between 10.00 and 11.00 hr on the day following the final dose of the pretreatment. Livers were immediately removed, washed in ice-cold saline, blotted dry and weighed. The livers of each group of rats were combined and then minced with scissors and rinsed with ice-cold 1·15% (w/v) KCl solution. The liver-mince was homogenized at 0–4° with two volumes of the KCl solution in a teflon–glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 10 min at 0–4°. The supernatant fraction was then

centrifuged for 1 hr at 100,000 *g* and 0–4° to isolate the microsomes. The microsomal pellet was resuspended in 0·2 M sodium phosphate buffer (pH 7·4). The protein concentrations of the microsomal suspensions were determined by the method of Lowry *et al.*[11] with bovine serum albumin as standard.

Assays of drug-metabolising enzyme activity. Microsomal suspensions were adjusted to 5 mg protein/ml and 1·0 ml of this suspension was incubated with NADP (2 μmoles), glucose-6-phosphate (25 μmoles), $MgSO_4$ (25 μmoles), 100,000 *g* supernatant fraction (0·5 ml), substrate and sufficient 0·2 M sodium phosphate buffer (pH 7·4) to produce a total volume of 5 ml. The incubations were carried out for 10 min at 37° in 25 ml open Erlenmeyer flasks placed in a Dubnoff metabolic shaker (100 oscillations/min). The substrates were hexobarbitone (3 μmoles/flask) a type I compound[12] and aniline (10 μmoles/flask) a type II compound. At the protein and substrate concentrations used, both reactions were found to be linear over the period of incubation. The side-chain oxidation of hexobarbitone was estimated from the disappearance of substrate as assayed by the method of Cooper and Brodie[13] with the modification of Kuntzman *et al.*[14] The *p*-hydroxylation of aniline was assayed by the method of Guarino *et al.*[15]

Assay of hepatic δ-ALA synthetase activity. The activity of ALA synthetase was determined by measuring the rate of formation of ALA in liver homogenates by the method of Marver *et al.*[16] Liver homogenates (25% w/v) were prepared in 0·9% w/v NaCl containing 0·5 M disodium EDTA and 10 mM tris–HCl buffer pH 7·2. Liver homogenate (0·5 ml) was incubated with glycine (200 μmoles), disodium EDTA (20 μmoles) and tris–HCl buffer (150 μmoles) pH 7·4. The final volume of the reaction mixture was 2·0 ml. Incubations were carried out for 30 min at 37° in open 25 ml Erlenmeyer flasks shaken in a Dubnoff incubator.

Spectral studies. Microsomes were isolated as described above from male rats (110–130 g) treated intraperitoneally with phenobarbitone sodium (80 mg/kg) daily for 3 days. A microsomal suspension (2 mg protein/ml) was divided equally between 1 cm reference and sample cuvettes. Ethosuximide, methsuximide and phensuximide (final concentrations between 1 and 5 mM) were added to the sample cuvette and the spectrum of each drug-microsome combination was recorded between 490 and 350 nm on a Pye Unicam SP 800 dual beam spectrophotometer, fitted with an external expanding recorder.

The cytochrome P-450 content of microsomal suspensions was determined by the method of Omura and Sato.[17] Approximately 10 mg sodium dithionite was added to the microsomal suspension (2 mg protein/ml) which was then divided equally between two 1 cm cuvettes. Carbon monoxide was bubbled through the sample cuvette for 30 sec. The absorbance at 450 nm, minus the baseline absorbance at 490 nm, was determined for the difference spectrum obtained with the SP 800 spectrophotometer.

Determination of hepatic porphyrins. The porphyrin content of rat and chick embryo liver was estimated by the method of Racz and Marks.[18] Coproporphyrin I (Sigma) was used as standard because Granick[19] has shown that this substance constitutes more than 80 per cent of the total porphyrins present in porphyric liver cells.

Electron microscopy. Cubes (1 mm^3) of rat liver were fixed by glutaraldehyde and post fixed by Palade's buffered osmium technique.[20] After dehydration, tissues were embedded in Araldite and sectioned. The sections were stained with lead citrate[21] and then viewed under a Siemens Electron Microscope.

RESULTS

The effect of subacute (3 days) oral administration of ethosuximide, methsuximide and phensuximide on body and liver weights is shown in Table 1. At a daily dose of 0·5 mmole/kg, liver weight was not significantly affected by the administration of the succinimides. However, at the higher dose level (2 mmoles/kg) these drugs significantly

TABLE 1. EFFECT OF SUBACUTE ORAL ADMINISTRATION* OF ANTICONVULSANT SUCCINIMIDES ON BODY AND LIVER WEIGHT OF MALE RATS

Pretreatment	Daily dose (mmole/kg)	Body wt. (g)	Liver wt. (g)	Liver wt. (as % of body wt.)
Control		113 ± 2 (8)	4·48 ± 0·23	3·95 ± 0·17
Ethosuximide	0·5	116 ± 1 (4)	4·39 ± 0·24	3·78 ± 0·19
	2·0	121 ± 4 (5)	5·28 ± 0·24†	4·35 ± 0·07†
Methsuximide	0·5	112 ± 2 (4)	4·75 ± 0·45	4·22 ± 0·35
	2·0	119 ± 3 (5)	5·61 ± 0·18†	4·72 ± 0·08†
Phensuximide	0·5	105 ± 2 (4)	4·32 ± 0·12	4·10 ± 0·05
	2·0	114 ± 3 (6)	4·39 ± 0·20†	4·31 ± 0·11†

* Drugs given daily in two equally divided doses for 3 days.
† Values are significantly different ($P < 0.05$) from controls.
Results are the mean ± standard error.
Figures in parentheses indicate number of animals.

increased liver weight and the ratio of liver to body weight. The effect was most marked in the methsuximide-treated rats. Electron micrographs of the liver from rats receiving the higher dose level of these succinimides showed that there was a proliferation of the smooth endoplasmic reticulum. Liver from animals at the lower dose level were not examined by electron microscopy.

TABLE 2. EFFECT OF SUBACUTE ORAL ADMINISTRATION* OF ANTICONVULSANT SUCCINIMIDES ON HEXOBARBITONE SLEEPING TIME IN MALE RATS

Pretreatment	Daily dose (mmole/kg)	Sleeping time (min)†
Control		28·82 ± 3·52 (8)
Ethosuximide	0·5	9·98 ± 0·67 (4)
	2·0	6·53 ± 1·01 (4)
Methsuximide	0·5	5·00 ± 0·92 (4)
	2·0	7·57 ± 2·25 (4)
Phensuximide	0·5	9·03 ± 1·95 (4)
	2·0	8·22 ± 0·62 (4)
Phenobarbitone‡	0·3	4·50 ± 0·20 (4)

* Drugs given daily in two equally divided doses for 3 days.
† Time righting reflex is absent following hexobarbitone sodium 80 mg/kg, i.p.
‡ Phenobarbitone given intraperitoneally.
Results are the mean ± standard error.
Figures in parentheses indicate number of animals.
All test values were significantly ($P < 0.05$) different from controls.

TABLE 3. EFFECT OF PRETREATMENT* OF MALE RATS WITH ANTICONVULSANT SUCCINIMIDES ON THE *in vitro* ACTIVITY OF THE HEPATIC MICROSOMAL DRUG-METABOLISING ENZYMES

Pretreatment	Hexobarbitone oxidation (nmole/mg†/min)	Aniline hydroxylation (nmole/mg†/min)
Control	8·81 ± 0·29	1·85 ± 0·05
Ethosuximide	9·21 ± 0·25‡	2·90 ± 0·11‡
Methsuximide	10·06 ± 0·61‡	2·77 ± 0·04‡
Phensuximide	9·90 ± 0·85‡	2·37 ± 0·04‡

* Drugs given in two equally divided doses (0·5 mmole/kg, daily) for 3 days. The preparation of the liver microsomal fraction and the metabolism of the two substrates were performed as described in Methods.

† mg of microsomal protein.

Results are the mean ± standard error of at least four experiments.

‡ Values significantly ($P < 0·05$) different from controls.

There was a significant ($P < 0·05$) reduction (3–6 fold) in the hexobarbitone-induced sleeping time of rats pretreated with each of the succinimides at both dose levels and with phenobarbitone (Table 2). The latter drug was the most effective. For each succinimide the reduction in sleeping time produced by pretreatment at 2 mmoles/kg did not differ significantly from that produced by 0·5 mmole/kg. Consistent with the effects on hexobarbitone-induced hypnosis was the observation that the rate of metabolism (V_{max}) of both hexobarbitone and aniline by the hepatic microsomal fraction from the pretreated rats was significantly ($P < 0·05$) increased (1·1–1·5 fold) at the 0·5 mmole/kg dose level for the three succinimides (Table 3).

Rats which received methsuximide (0·5 mmole/kg) daily for 3 days showed a marked tolerance to the anticonvulsant activity of this succinimide (Table 4). Development of tolerance with ethosuximide and phensuximide was not examined.

The basal activity of hepatic ALA synthetase in control rats was 46 ± 2 nmoles ALA/g wet liver/hr. The activity of ALA synthetase was increased approximately 2-fold after 3 days treatment (0·5 mmole/kg, daily) with ethosuximide (91 ± 6 nmoles/g/hr), methsuximide (82 ± 5 nmoles/g/hr) and phensuximide (86 ± 8 nmoles/g/hr).

TABLE 4. ANTICONVULSANT ACTIVITY OF METHSUXIMIDE FOLLOWING ITS SUBACUTE ADMINISTRATION* TO MALE RATS

Pretreatment	Min convulsant dose of leptazol† (mg/kg)
Control	66·8 ± 5·2 (5)
Methsuximide	34·3 ± 5·5 (5)‡

* Methsuximide (0·5 mmole/kg/day), given in two equally divided oral doses for 3 days.

† Antileptazol activity determined 1 hr after administration of methsuximide, 100 mg/kg, orally. 1% (w/v) leptazol solution infused into a tail vein at a rate of 0·5 ml/min until tonic convulsions recorded.

Results are the mean ± standard error.

Figures in parentheses indicate number of animals.

‡ Value differs significantly ($P < 0·05$) from control.

However, the 24 hr urines of the succinimide-pretreated rats did not contain any porphyrins or porphobilinogen as estimated by the qualitative test of Rimington.[22] Normal levels of porphyrins (0·38 ± 0·05 μg coproporphyrin I/g wet wt.) were also found in the liver of these animals.

The mean level of porphyrins in the liver of untreated chick embryos was 0·188 μg coproporphyrin I/g wet liver (range 0·150–0·225). Incubation of methsuximide with chick embryo caused an approximately 50-fold increase in liver porphyrins (mean 10 μg coproporphyrin I/g liver, range 0·82–20·00). Phensuximide also raised the level of hepatic porphyrins in chick embryo (mean 0·5 μg coproporphyrin I/g liver, range 0·2–2·08) but to a smaller degree (2-fold). At the same dose and under the same incubation conditions, ethosuximide was without effect on the concentration of liver porphyrins. Absolute ethanol, the solvent used for the succinimides in this test, did not exhibit any porphyrogenic activity.

TABLE 5. EFFECT OF SUBACUTE ADMINISTRATION* OF ANTICONVULSANT SUCCINIMIDES TO MALE RATS ON THE LEVEL OF HEPATIC MICROSOMAL CYTOCHROME P-450

Pretreatment	Daily dose mmole/kg	Cytochrome P-450 (% of control)†	
		Per g liver	Per mg microsomal protein
Ethosuximide	0·5	239	184
	2·0	254	
Methsuximide	0·5	219	201
	2·0	249	
Phensuximide	0·5	198	184
	2·0	206	

* Drugs given in two equally divided oral doses for 3 days.

† Average control values: 1·7 absorbance (450–490 nm)/g liver; 0·054 absorbance (450–490 nm)/mg protein.

Results are the mean of four experiments.

Blank spaces indicate that microsomal protein was not determined.

The effect of the anticonvulsant succinimides (0·5 and 2·0 mmoles/kg, daily) on the content of cytochrome P-450 of rat liver microsomes is summarised in Table 5. The amount of cytochrome P-450 (expressed per g of liver) was increased approximately 2-fold, 84 hr after treatment at both dose levels of ethosuximide, methsuximide and phensuximide.

The addition of methsuximide (final concentration 1–4 mM) to aerobic suspensions of rat liver microsomes gave rise to a type I spectral change with an absorption peak at 390 nm and a trough at 422 nm. A maximal change in absorbance at 390 nm (Δ absorbance ≡ 0·06) was observed after the addition of 3 mM methsuximide. No spectral change was observed after the addition of either ethosuximide or phensuximide (final concentrations 1–5 mM) although the subsequent addition of hexobarbitone sodium (final concentrations 0·2–0·8 mM) still gave rise to the expected type I spectral change.

DISCUSSION

The present study demonstrates that administration of the closely related anticonvulsant succinimides, ethosuximide, methsuximide and phensuximide daily for 3 days stimulates drug metabolism *in vivo* by induction of the hepatic microsomal enzymes. In the rat, induction of these enzymes by drugs such as phenobarbitone and phenylbutazone is accompanied by an increase in liver/body weight ratio[5] and a significant increase in this ratio was observed in rats receiving the succinimides at the daily dose of 2 mmoles/kg. A similar effect, however, was not observed at the lower dose level (0·5 mmole/kg) even though there was an increased rate of drug metabolism demonstrable both *in vivo* and *in vitro*. Liver enlargement has previously been found to occur in rats receiving ethosuximide (1, 2 and 4 mmoles/kg daily; Parke Davis and Co., unpublished experiments). In this case a maximal effect was obtained after 6 months of daily administration of the drug. This treatment did not cause any significant disturbance of hepatic function.

It appears that the time-course of the induction of drug metabolising enzymes is governed by the physico–chemical nature of the inducing agent. In general, compounds which stimulate the activity of the hepatic microsomal enzymes have a high lipid solubility and a fairly slow rate of metabolism.[5] On such a basis, ethosuximide with its long plasma half-life, 16 hr in rat, and moderate lipid solubility[23] would be expected to be an effective inducer. Methsuximide and phensuximide, although both highly lipid soluble have short plasma half-lives as a result of metabolism[24,25] and might have been expected to be of low activity as inducers. This was not found to be the case. However, it is possible that the metabolites of methsuximide and phensuximide (and perhaps also of ethosuximide) are themselves active inducing agents which could obscure any simple relationship between the plasma half-lives of these drugs and their induction potential. From the results of the present experiments it is not possible to compare the relative potency of the succinimides as inducing agents because, for each drug, there was no significant difference between the effects of the two dose levels employed for pretreatment, i.e. all doses were maximally effective.

It is well known that stimulation of drug metabolism *in vivo*, by induction of the hepatic microsomal enzymes, results in altered duration and intensity of pharmacological actions of administered drugs.[26] In the present study, this was exemplified by the decrease in hexobarbitone-induced sleeping time of animals pretreated with ethosuximide, methsuximide and phensuximide. In the case of rats receiving methsuximide, the decrease in anti-leptazol activity illustrated that chronic administration of this succinimide affects its own pharmacological activity presumably by increasing its rate of metabolism. If the decrease in the pharmacological activity of methsuximide, observed here, occurs in man then the therapeutic status of the drug would need careful re-evaluation. However, the relationship between increased metabolism and overall anticonvulsant activity for this drug is complicated by the fact that its major metabolite, 2-methyl-2-phenylsuccinimide,[25] possesses anticonvulsant activity.[1]

The alteration of drug metabolising activity caused by drug interaction is of particular importance in the treatment of epilepsy where drugs are administered chronically and where combinations of antiepileptic drugs as well as combinations of antiepileptic and other drugs are common. Recently, the serum levels of phenytoin and phenobarbitone, a combination of drugs widely employed in grand mal epilepsy, have been shown to be significantly lower in epileptic patients receiving both drugs than in

patients receiving either drug alone.[27,28] Sotaniemi *et al.*[28] tried to assess the clinical significance of this observation but were unable to relate the incidence of side effects and recurrence of seizures to the decrease in concentrations of the drugs in serum caused by accelerated biotransformation. Glazko *et al.*[24] studied the levels of drug in the serum of patients on the first day of treatment with phensuximide and again following 8 days of continued daily administration. The results indicated that the serum levels for each subject were similar on the first and eighth days of treatment although there were variations in the levels between subjects. These workers also determined the levels of drug in the serum of epileptic patients given phensuximide daily for periods of 6–13 months. In these patients the drug concentrations in serum were found to be similar to those determined in subjects receiving the drug for the first time. Recently, Haerer, Buchanan and Wiygul[29] determined the levels of ethosuximide in the serum of children receiving the drug for the chronic control of petit mal or other minor seizures. Only 3 out of 21 patients studied were successfully maintained on ethosuximide alone and in these cases the serum levels did not vary greatly from the levels of ethosuximide in patients receiving other anticonvulsants, mainly phenobarbitone and phenytoin, in combination with this succinimide. The average concentration of ethosuximide in the serum of epileptic patients receiving the drug was similar to the maximum levels obtained in serum after administration of a single dose to subjects receiving this imide alone.[30]

Thus there is no evidence to suggest that phensuximide and ethosuximide cause induction of drug metabolising enzymes in man. The reason for the difference in inducing activity of these succinimides between the rat and man is not obvious. In the present study, although the dose level of 2 mmoles/kg for these succinimides may be regarded as relatively large, the dose level of 0·5 mmole/kg is of the same order as that of the human dose on a body weight basis. Thus in children a dose of 0·14 mmole ethosuximide/kg provides good protection against petit mal seizures[29] while the recommended daily dose of methsuximide and phensuximide is about 0·2 mmole/kg for an adult.[31] Although the hepatic microsomal enzymes are generally believed to catalyse a wide range of oxidations and reductions it is possible that the main metabolic pathway for phensuximide and ethosuximide does not involve these enzymes. However, *N*-demethylation, the principal route of metabolism of methsuximide[25] is most certainly confined to the hepatic microsomes.[32] Interestingly, the spectral studies indicated an interaction between hepatic microsomes and methsuximide only, as evidenced by a type I spectrum.

The increase of *in vitro* activity of the hepatic microsomal enzymes (approximately 1·1–1·5-fold) following treatment of rats with the anticonvulsant succinimides (0·5 mmole/kg/day) was lower than the rise in hepatic microsomal cytochrome P-450 levels (approximately 2-fold). On the other hand, hepatic ALA synthetase was increased two-fold after administration of the succinimides to rats. This increase was not coupled with a higher level of hepatic porphyrins or with increased excretion of porphobilinogen and porphyrins into urine but it was probably related to the increase in hepatic microsomal cytochrome P-450. The porphyrinogenic properties of methsuximide and phensuximide were, however, demonstrated by increased levels of porphyrins in chick embryo liver. This supports the recent finding of Racz and Marks[18] with these two drugs in chick embryo. No porphyrinogenic activity was demonstrated with ethosuximide. This is supported by a clinical report where etho-

suximide did not precipitate porphyric attacks in a patient with hereditary coproporphyria.[33] However, methsuximide was implicated as a porphyrinogenic agent in this patient.[33] There are no clinical reports linking phensuximide with precipitation of symptoms of porphyria and the results with the chick embryo suggest that it is some 25 times less potent that methsuximide in stimulating porphyrinogenesis. However, Moore *et al.*[34] have suggested that all compounds which raise ALA synthetase are contraindicated in patients genetically disposed to acute intermittent porphyria.

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