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Dose-related inhibition of the drug-metabolizing enzymes of rat liver by the pyrrolizidine alkaloid, monocrotaline

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Adult, male Sprague-Dawley rats were given 0, 10, 20, 40 or 80 mg kg^{-1} of monocrotaline intraperitoneally and the following toxicity parameters determined 24 h post treatment. Compared with the control none of the doses caused significant change in either the relative liver weight or the hepatic microsomal protein concentration. Microsomal cytochrome P450 content and activities of benzphetamine *N*-demethylase and aniline hydroxylase did not differ from the control at 10 or 20 mg kg^{-1} dosage. But, there was a significant loss of cytochrome P450 at 40 and 80 mg kg^{-1} dosages and decrease in the activity of the two enzymes only at the highest dose. Similarly, the highest dose caused a marked elevation of serum sorbitol dehydrogenase and glutamic pyruvic transaminase activity suggestive of severe liver damage.

Monocrotaline is a pyrrolizidine alkaloid present in the seeds of *Crotalaria spectabilis* and also found in several other plant species growing in different parts of the world. The toxic alkaloid not only poses a serious hazard to farm animals through their direct exposure to the toxic plants, but also to humans through contaminated grains and oil seeds, and herbal tea (McLean 1970). In addition to being mutagenic and carcinogenic, monocrotaline is mainly a hepatotoxic alkaloid (Culvenor 1980; Schoental 1982; Petry et al 1984; Bruggeman & van der Hoeven 1985). It has been reported that monocrotaline is biotransformed and bioactivated in the liver (Swick 1984; Mori et al 1985) and this hepatic metabolism of the alkaloid is increased by phenobarbitone pretreatment and decreased by SKF 525-A administered to rats before monocrotaline (Lafranconi & Huxtable 1984). The alkaloid has also been found to cause increased liver copper content and disturbance in iron metabolism (Swick et al 1984).

We studied the effect of various doses of monocrotaline in rats on the hepatic microsomal enzymes and also on serum enzymes as markers of liver toxicity.

Materials and methods

Monocrotaline was obtained from Sigma Chemical Co., St Louis, MO. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide-adenine dinucleotide phosphate (NADP) were purchased from Boehringer Mannheim Corp., Indianapolis, IN and used to prepare the NADPH-generating system required for studying the activity of microsomal drug-metabolizing enzymes. All other chemicals used in these studies were of analytical or reagent grade.

Male Sprague-Dawley rats (Southern Animal Farms, Prattville, AL), 180–220 g, were housed in standard stainless steel cages placed in a temperature-controlled room (25 °C) on a 12 h light-dark cycle and allowed free access to water and Purina rat chow. Monocrotaline was dissolved in distilled water by neutralizing with hydrochloric acid, and given i.p. in single doses of 10, 20, 40, and 80 mg kg^{-1} to respective groups of 5 rats each. The control group received distilled water alone. The animals were decapitated 24 h after treatment, and blood and liver samples collected for serum enzyme assays and for the isolation of microsomes, respectively. The serum samples were used to determine the activities of sorbitol dehydrogenase (SDH), serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxalacetic transaminase (SGOT). Activities of the serum enzymes were determined spectrophotometrically as described elsewhere (Dalvi 1985).

The livers were immediately perfused and used for the isolation of microsomes according to Dalvi & Howell (1977). Activities of microsomal benzphetamine *N*-demethylase and aniline hydroxylase were assayed using benzphetamine and aniline as substrates, respectively (Dalvi & Peeples 1981). Cytochrome P450 content of the microsomes was determined using the procedure reported by Omura & Sato (1964). The microsomal protein concentration in each sample was measured by the Biuret method modified to include deoxycholate (Dalvi et al 1975). The data were analysed using Duncan's multiple-range test. Significance of mean differences was based on a P value of 0·05.

Results and discussion

It is evident from Table 1 that administration of various doses of monocrotaline (0–80 mg kg^{-1}) to rats did not cause significant changes in either the relative liver weights or the hepatic microsomal protein concentrations. The results are consistent with those of Roth et al (1981) who also found no change in relative liver weights of rats given monocrotaline in drinking water over 4 weeks. A slight increase in microsomal protein content and relative liver weight of the animals receiving the 80 mg kg^{-1} dose may have been due to liver enlargement which is frequently observed after administration of many foreign compounds (Bond & DeMatteis 1969).

In contrast, the alkaloid produced dose-dependent alterations in the activities of benzphetamine *N*-demethylase and aniline hydroxylase as well as the concentrations of microsomal cytochrome P450, the major component of the drug metabolizing enzyme system. Concentrations of cytochrome P450 and activi-

Table 1. Effect of varying doses of monocrotaline on the selected parameters of the drug-metabolizing enzyme system in male rats.

Dose of monocrotaline (mg kg^{-1})	Relative liver weight (% of body wt)	Microsomal		Benzphetamine *N*-demethylase activity (nmol HCHO mg^{-1} min^{-1})	Aniline hydroxylase activity (nmol *p*-aminophenol mg^{-1} min^{-1})
		Protein (mg g^{-1} liver)	Cytochrome P450 (nmol mg^{-1} protein)		
Control	3·82 ± 0·31	18·13 ± 1·72	0·769 ± 0·04[a]	4·58 ± 0·58[a]	0·340 ± 0·04[a]
10	3·84 ± 0·25	16·81 ± 5·57	0·696 ± 0·18[a]	4·41 ± 0·16[a]	0·333 ± 0·07[a]
20	3·67 ± 0·47	15·51 ± 3·26	0·677 ± 0·04[a,b]	4·55 ± 0·11[a]	0·331 ± 0·02[a]
40	3·89 ± 0·04	18·48 ± 2·59	0·466 ± 0·18[b,c]	3·75 ± 0·76[a]	0·351 ± 0·00[a]
80	4·01 ± 0·72	21·27 ± 4·44	0·439 ± 0·09[c]	2·41 ± 0·96[b]	0·219 ± 0·09[b]

Results are expressed as the mean ± s.d.m. [a,b,c] Any two means with the same superscript within the same column are not significantly different ($P < 0·05$) using the Duncan's multiple-range test.

ties of the two microsomal enzymes were depressed, but not significantly different from control at the 10 and 20 mg kg^{-1} doses of monocrotaline. However, at the 40 and 80 mg kg^{-1} doses, the amount of cytochrome P450 was significantly reduced within 24 h after monocrotaline while the activities of benzphetamine *N*-demethylase and aniline hydroxylase were depressed significantly only at the 80 mg kg^{-1} dosage level. Other workers have also found monocrotaline to decrease microsomal cytochrome P450 (White 1976) and depress the activity of aminopyrine *N*-demethylase in rats (Shull et al 1976; Eastman & Segall 1981). These results demonstrate that this pyrrolizidine alkaloid might not inhibit the drug metabolizing enzyme system at lower doses (<40 mg kg^{-1}) but will become an inhibitor at doses higher than 80 mg kg^{-1}. Furthermore, dose-dependent inhibition of the hepatic drug metabolizing enzyme system by monocrotaline should serve as a useful, sensitive indication of early liver injury (Bond & DeMatteis 1969; Urbanek-Karlowska 1980; Kloss et al 1982).

Further assessment of the dose-dependent hepatotoxicity of monocrotaline was made by determining the activities of SDH, SGPT and SGOT in the serum of animals treated with monocrotaline (Table 2). Data indicate that SDH activity increased more than 150% at the 40 mg kg^{-1} dose and almost tripled at the 80 mg kg^{-1} dose of monocrotaline. While SDH activity increased dramatically, the increase in SGPT was marked and that of SGOT was slight but significant. These dose-dependent elevations in the serum enzymes, reflecting varying degrees of liver damage, are consistent with the finding that pyrrolizidine alkaloids cause hepatotoxicity through centrilobular necrosis (Lame & Segall 1986). However, the extent of liver injury will not only depend on the dose of monocrotaline but also on the rate of its biotransformation by liver microsomes since toxicity of monocrotaline is related to its bioactivation.

Table 2. Effect of varying doses of monocrotaline on sorbitol dehydrogenase (SDH), serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxalacetic transaminase (SGOT) as parameters of liver damage in male rats.

Dose of monocrotaline (mg kg^{-1})	SDH (Sigma units mL^{-1})	SGPT (Karmen units mL^{-1})	SGOT (Karmen units mL^{-1})
Control	261 ± 47[b]	65 ± 11[c]	173 ± 25[b]
10	275 ± 36[b]	70 ± 9[b,c]	178 ± 29[b]
20	294 ± 47[b]	72 ± 4[b,c]	180 ± 9[b]
40	430 ± 233[b]	80 ± 7[b]	186 ± 16[b]
80	729 ± 226[a]	110 ± 11[b]	228 ± 36[a]

Results are expressed as the mean ± s.d.m. [a,b,c] Any two means with the same superscript within the same column are not significantly different ($P < 0·05$) using the Duncan's multiple-range test.

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Diazepam and desmethyldiazepam differ in their affinities and efficacies at 'central' and 'peripheral' benzodiazepine receptors

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The in-vitro binding characteristics of three different ligands ([^{3}H]Ro 15-1788, [^{3}H]Ro 5-4864 and [^{3}H]flunitrazepam) and the structural requirements for binding to 'central' and 'peripheral' benzodiazepine receptors have been evaluated in rat cerebral cortex, cerebellum and adrenal glands. [^{3}H]Ro 15-1788 binding was detectable only in the brain. Clonazepam was the most potent inhibitor followed by diazepam and desmethyldiazepam, which showed the same affinity, and by premazepam; Ro 5-4864 did not show appreciable affinity. The same pattern was seen for [^{3}H] flunitrazepam binding in brain areas while in adrenal gland the inhibition pattern was exactly superimposable on that with [^{3}H]Ro 5-4864 in all the areas considered (Ro 5-4864 > diazepam > desmethyldiazepam > clonazepam > premazepam). These data confirm and extend previous reports. A methyl group in position 1 enhances the affinity for peripheral benzodiazepine binding sites which are labelled in the adrenal gland by [^{3}H]Ro 5-4864 and [^{3}H]flunitrazepam; in brain areas, [^{3}H]flunitrazepam, like [^{3}H]Ro 15-1788, selectively labels central binding sites. Methylation in position 1 did not change the affinity for these sites. Desmethyldiazepam is less active than diazepam as an anticonvulsant and in other tests. In-vivo experiments were therefore carried out to assess the 'intrinsic activity' of desmethyldiazepam: it appeared that this compound acts as a partial agonist at central benzodiazepine receptors.

Benzodiazepine (BDZ) binding to central and peripheral binding sites has been studied in detail (Richards et al 1982; Sieghart & Schuster 1984; Wang et al 1984). The two receptors have different structural and steric requirements. However, different ligands and receptor sources were used for those studies, making direct comparison of the two receptor sites difficult. Moreover, most of the experiments were made with [^{3}H]diazepam or [^{3}H]flunitrazepam, two ligands that, because of the presence of an alkyl group in position 1, lack selectivity between the two sites.

The study now reported aimed to verify the structural requirements for binding to these two receptors, and has considered the binding of three different ligands: [^{3}H]Ro 15-1788, reported as a selective ligand of central binding sites (Richards et al 1982; Gee & Yamamura 1983; Bonetti et al 1982); [^{3}H]Ro 5-4864 which has a methyl group in position 1 and a chloro in position 4 (Wang et al 1984) and is therefore considered a selective ligand for peripheral binding sites (Marangos et al 1982; Schoemaker et al 1983), and [^{3}H]flunitrazepam which, with a methyl group in position 1 can label both sites (Richards et al 1982). The in-vitro affinities of diazepam (methyl group in position 1), desmethyldiazepam, clonazepam (both without substituents in position 1), Ro 5-4864, and a pyrrolodiazepine, premazepam (no substituent), have been determined in the cortex and cerebellum and adrenal glands of the rat.

This characterization of the binding of various BDZ compounds to central and peripheral BDZ receptors also helped towards explaining why desmethyldiazepam is less active than diazepam as an anticonvulsant in rats (Caccia & Garattini 1984; Garattini et al 1981) and mice (Frey & Loscher 1982) and also in reducing motor activity in rats and in the anticonflict test (Babbini et al 1979).

As previously described (Mennini & Garattini 1984; Mennini et al 1985) a useful experimental approach to test this hypothesis is to measure [^{3}H]flunitrazepam binding in the hippocampus after in-vivo injection of a tracer dose of the ligand in control rats and in rats treated with equiactive doses (against metrazol convulsions) of different drugs.

Materials and methods

In-vitro binding. Male CD-COBS rats (Charles River, Italy), ca 200 g, were decapitated and their cerebella, cortex and adrenal glands dissected and stored at −80 °C until use. Binding assays were carried out on crude membrane preparations obtained as follows: the

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