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Effects of some hepatomegalic agents on liver DNA content: relationship to changes in liver blood flow

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Hepatic microsomal enzyme induction is the mechanism underlying a number of interactions between therapeutic agents. Phenobarbitone is the drug which is best known for this action but it has been pointed out that the increase in enzyme activity alone is not sufficient to account for all the changes in pharmacokinetics observed during the administration of this compound (Ohnhaus et al 1971). More recently it has been shown that, in the rat, phenobarbitone increases liver blood flow (Nies et al 1976; Yates et al 1978; Hiley et al 1981) as well as increasing liver size and enzyme activity and this would help to account for the changes in pharmacokinetics observed for some drugs (Wilkinson & Shand 1975). Other agents, including antipyrine and chlordiazepoxide, do not produce concurrent increases in liver blood flow and hepatic microsomal drug metabolising activity although, like phenobarbitone, they do increase liver mass (Nies et al 1976; Yates et al 1978).

These previous studies have shown that action on hepatic microsomal enzyme activity does not itself lead to changes in liver blood flow. Hence differences in other cellular changes produced by these hepatomegalic agents may be responsible for their failure to increase liver blood flow in a manner similar to phenobarbitone. The basis of the liver enlargement resulting from phenobarbitone treatment is now thought to be cellular hypertrophy, evidence having been gained from studies of both hepatic DNA content and hepatocyte size (Stäubli et al 1969; Shenoy & Peraino 1977; Sweeney et al 1978). Hepatic DNA was also used by Miner & Gaito (1979) as an index of the degrees of cellular division and growth obtained following treatment of rats with phenobarbitone and the glucocorticoid, 6-methylprednisolone. They found that both compounds increased cell size rather than cell number.

In order to attempt to correlate the change in liver blood flow and mechanism of liver enlargement produced by some hepatomegalic agents, whether hypertrophy of the cells or cellular division, we have determined the effect of 6-methylprednisolone on liver blood flow and measured hepatic DNA content in rats treated with this compound and two other liver enlarging agents, antipyrine and chlordiazepoxide.

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Materials and methods

Treatment of animals Groups of weight-matched male Wistar rats (Bantin & Kingman Ltd) were given the compounds being investigated twice daily by intraperitoneal injections for 5 days. The volume of injection was 2 ml kg^{-1} and all drug solutions were made fresh daily. Antipyrine was obtained from Sigma; 6-methylprednisolone succinate (Solu-Medrone for injection) from Upjohn Ltd and chlordiazepoxide hydrochloride (Librium for injection) from Roche Products Ltd. Dose values given in the text refer to the quantity of parent compound, not that of the derivative.

Determination of liver blood flow Hepatic blood flow and cardiac output were measured as described previously (Hiley et al 1980) using 15 ± 3 μm diameter radioactive microspheres labelled with ^{85}Sr (3M Medical Products, St Paul, MN, U.S.A.). The animals were treated with vehicle or test compound for 5 days and then starved for 16 h before experimentation.

Measurement of hepatic DNA. DNA content was determined by the diphenylamine colour reaction described by Burton (1956) as modified by Bevan et al (1976). Following treatment as above, the animals were killed by cervical dislocation and their livers rapidly removed, blotted dry and weighed. Duplicate 200–300 mg samples of livers were homogenized in 4 ml ice-cold 6% (w/v) trichloroacetic acid (Fisons A.R. grade) using a Teflon-in-glass homogenizer. The homogenate, plus a 3 ml trichloroacetic acid rinse of the homogenizing tube, was centrifuged at 18 000 g (max.)

Table 1. The effect of 5 days of 6-methylprednisolone treatment on liver blood flow in male Wistar rats.

	Saline 2 ml kg^{-1} (n = 8)	6-methylprednisolone 17 mg kg^{-1} (n = 9)	6-methylprednisolone 26 mg kg^{-1} (n = 8)
Cardiac index (ml min^{-1} per 100 g body wt)	20·7 ± 0·9	18·5 ± 0·9	24·3 ± 1·3*
Liver blood flow (ml min^{-1} per 100 g body wt)	4·80 ± 0·26	5·53 ± 0·29*	7·35 ± 0·53***
Liver blood flow (ml min^{-1} per g liver)	1·31 ± 0·09	1·59 ± 0·08*	1·80 ± 0·11**
% cardiac output received by hepato-splanchnic bed	22·3 ± 2·1	29·9 ± 0·9**	30·0 ± 1·1**
Liver weight (g per 100 g body wt)	3·46 ± 0·07	3·66 ± 0·05*	4·09 ± 0·15**
Increase in body wt (%)	11·1 ± 1·1	2·6 ± 1·2***	3·6 ± 3·2*

Values are given as means ± s.e.m. and n represents the number of animals in the group. Statistical comparison was by analysis of variance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The doses given were daily doses and the quantity refers to the parent compound, not the succinate.

Table 2. Effect of 5 days treatment with 6-methylprednisolone on hepatic DNA and protein content in the male Wistar rat.

	Saline 2 ml kg^{-1} (n = 4)	6-Methylprednisolone 13 mg kg^{-1} (n = 4)	Saline 2 ml kg^{-1} (n = 4)	6-Methylprednisolone 26 mg kg^{-1} (n = 4)
Liver weight (g per 100 g body wt)	4·44 ± 0·06	4·74 ± 0·08*	3·44 ± 0·09	4·26 ± 0·21**
Hepatic DNA content (mg per g liver)	2·32 ± 0·07	2·18 ± 0·10	3·76 ± 0·12	3·44 ± 0·14
Hepatic DNA content (mg per 100 g body wt)	9·97 ± 0·34	9·95 ± 0·66	12·87 ± 0·25	14·55 ± 0·25**
Hepatic microsomal protein (mg per g liver)	30·1 ± 1·2	29·2 ± 1·4	32·2 ± 1·0	28·9 ± 0·4*
Protein: DNA ratio	13·1 ± 1·2	13·5 ± 0·8	8·6 ± 0·6	8·3 ± 0·9
Initial body weight (g)	177 ± 2	180 ± 4	195 ± 3	197 ± 2
Final body weight (g)	246 ± 4	222 ± 6	217 ± 2	203 ± 4
Increase in body weight (%)	39·6 ± 2·5	23·3 ± 1·8***	11·2 ± 1·6	3·2 ± 2·0*

Values are expressed as mean ± s.e.m. and n represents the number of animals in the group. Statistical comparison was carried out using Student's *t*-test between the experimental and the appropriate control: *$P < 0·05$; **$P < 0·01$. The doses given are daily doses and refer to the quantity of the parent compound, not the succinate.

for 20 min at 4 °C. The supernatant was then discarded, the pellet resuspended in 2 ml of 1 M perchloric acid (Fisons A.R.) and incubated in a water bath for 15 min at 70 °C. The suspension was then centrifuged as above, the supernatant being decanted and retained. The hot extraction and centrifugation steps were repeated on the pellet and the pooled supernatants diluted to give 0·5 M perchloric acid. 1 ml portions were used in the DNA assay.

The diphenylamine reagent was made fresh every 2 weeks using Fisons analytical grade reagents and protected from light. The reagent mixture was prepared as described by Burton (1956). Calf thymus DNA (highly polymerized, Sigma Type 1) was used as a standard; the stock solution of 4 mg ml^{-1} in 5 mM NaOH was kept refrigerated and working solutions were made every 3 weeks by the addition of an equal volume of 1 M perchloric acid, incubating at 70 °C for 15 min and diluting as required to give 0·5 M final perchloric acid concentration. The rest of the procedure followed that of Bevan et al 1976.

Microsomal protein. The remainder of the liver not required for DNA estimation was homogenized in ice-cold 1·15% KCl to give a 30% (w/v) homogenate. The microsomal fraction was obtained as described by Yates et al (1978) and the microsomal pellet, resuspended in 10 ml or 0·2 M phosphate buffer, pH 7·4, was assayed for protein content using the method of Lowry et al (1951).

Results

Table 1 shows that 6-methylprednisolone produced dose-dependent increases in liver weight and liver blood flow whether the latter is expressed relative to body weight or liver weight. With the lowest daily dose, 17 mg kg^{-1}, the increase in liver blood flow was the result of an increased distribution to the hepatosplanchnic bed whereas, at the higher daily dose of 26 mg kg^{-1}, there was also a contribution from an enhanced cardiac output. The percentage increases in hepatic blood flow relative to body weight, 15 and 53% respectively for the lower and higher daily dose, were considerably greater than the increases in liver weight relative to body weight (6 and 18%) with the result that liver perfusion per unit mass of the organ increased significantly by 21 and 37%. However, it must be noted that the greater liver weight relative to body weight in the experimental groups when compared to the controls is partly the result of the significant reduction in weight gain caused by the treatments with the glucocorticoid.

In the experiments detailed in Table 2 it may be seen that both doses of 6-methylprednisolone produced greater liver weights relative to body weight; 26 mg kg^{-1} daily produced a 24% greater liver to body weight ratio and 13 mg kg^{-1} daily an enhancement of 7%. Again, a considerable part of this apparent change was the reduced rate of weight gain observed in the 6-methylprednisolone treated animals; the mean liver weight for the group receiving 13 mg kg^{-1} 6-methylprednisolone daily was 10·5 g and that for the corresponding control was 10·9 g.

Table 2 also shows that only the larger of the two doses produced changes in hepatic DNA or microsomal protein. Hepatic DNA relative to liver weight was unchanged but the hepatic DNA to body weight ratio was significantly greater than in the saline-treated animals; this was again largely the result of the lesser increase in body weight in the animals given glucocorticoid. There was only a 6% increase in total hepatic DNA in these animals which was not statistically different from the amount determined in the control group.

Table 3 shows that both chlordiazepoxide and antipyrine at the single doses given produced increases in liver weight relative to body weight. There were no differences in the rate of weight gain between the experimental and the control groups in this experiment. Chlordiazepoxide at 40 mg kg^{-1} daily increased liver weight relative to body weight by 10% and the treatment with 80 mg kg^{-1} antipyrine by 8%. There were significant falls of 14% for chlordiazepoxide and 26% for antipyrine in DNA content per unit mass of liver. Antipyrine also caused a reduction in hepatic DNA content relative to body weight of 20%. Hepatic

Table 3. Effects of treatment for 5 days with chlordiazepoxide or antipyrine on hepatic DNA and protein content in the male Wistar rat.

	Saline 2 ml kg^{-1} (n = 4)	Chlordiazepoxide 40 mg kg^{-1} (n = 4)	Antipyrine 80 mg kg^{-1} (n = 4)
Liver weight (g per 100 g body wt)	3·39 ± 0·09	3·73 ± 0·07*	3·66 ± 0·06
Hepatic DNA content (mg per g liver)	3·77 ± 0·05	3·22 ± 0·05***	2·80 ± 0·04**
Hepatic DNA content (mg per 100 g body wt)	12·8 ± 0·3	12·0 ± 0·1	10·3 ± 0·2***
Hepatic microsomal protein (mg per g liver)	38·0 ± 0·8	40·8 ± 1·8	40·7 ± 1·6
Protein: DNA ratio	10·1 ± 0·3	12·7 ± 0·7*	14·5 ± 0·3***
Increase in body weight (%)	−1·9 ± 1·4	−0·5 ± 0·5	1·6 ± 0·4

Values are expressed as mean ± s.e.m. and n represents the number of animals in the group. Statistical comparison was by analysis of variance: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. The doses given are the daily dose and in the case of chlordiazepoxide quantity of it refers to the free base.

microsomal protein content was unchanged by either drug treatment but, as a result of the decreases in DNA, there were enhanced ratios of hepatic protein: DNA.

Discussion

We have shown that 6-methylprednisolone shares with phenobarbitone the property of increasing hepatic blood flow by causing a redistribution of cardiac output in favour of the organs of the hepatosplanchnic region with a consequential increase in the blood flow per unit mass of liver. The glucocorticoid also appeared to be like phenobarbitone in that liver weight relative to body weight was increased. However, the effect of 6-methylprednisolone in reducing weight gain renders difficult the interpretation of data related to body weight. This consequence of glucocorticoid treatment is well known (Winter et al 1950) and a specific effect on the liver has also been reported in that they decrease thymidine incorporation into regenerating livers (Henderson & Loeb 1970).

Miner & Gaito (1979) concluded that hepatocyte enlargement occurred following treatment with 20 mg kg^{-1} 6-methylprednisolone acetate (equivalent to 17 mg kg^{-1} of the parent compound) daily for 5 days; hepatic DNA relative to liver weight declined but was unchanged relative to body weight. In our experiments neither dose of 6-methylprednisolone (equivalent to 15 and 30 mg kg^{-1} of the acetate daily) caused significant changes in hepatic DNA content although with the largest dose the change almost reached the 5% level of significance ($0{\cdot}1 > P > 0{\cdot}05$). Consequently we are unable to confirm the conclusions of Miner & Gaito (1979) that 6-methylprednisolone produces hepatic enlargement by cellular hypertrophy. The enhancement in hepatic DNA relative to body weight observed with the larger dose in this study cannot be taken to indicate an increase in hepatocyte number since it was largely the result of decreased growth rate and there was no significant increase in total hepatic DNA.

We have previously observed that phenobarbitone causes a decrease in hepatic DNA per unit mass of liver (Berman et al 1983) which may be taken as an indication that this compound produces hepatic enlargement by means of cellular hypertrophy. Similar conclusions have been reached by other workers on the basis of measurement of DNA and hepatocyte size (Stäubli et al 1969; Shenoy & Peraino 1977; Sweeney et al 1978). Phenobarbitone is only one of a number of compounds which increase activity of the enzymes of the liver microsomal mixed function oxidase system as well as liver size, but is the only one previously shown to increase liver blood flow. Accordingly, we investigated the effects of two other hepatomegalic agents, chlordiazepoxide and antipyrine, on hepatic DNA content in order to determine whether or not there was any correlation between blood flow effects and the cellular basis of the hepatic enlargement. Both compounds reduced hepatic DNA content per unit mass of liver suggesting that hypertrophy was occurring. In the case of antipyrine there was also a decrease relative to body weight compared to the control group which would appear to indicate some loss of hepatocytes despite the hepatic enlargement. Thus the increase in liver size with both of these agents is associated with a decline in hepatic DNA relative to liver weight which is also true of phenobarbitone and, perhaps, 6-methylprednisolone. However, unlike the latter two drugs neither antipyrine nor chlordiazepoxide cause a redistribution of cardiac output to the liver or the organs draining into the portal vein (Yates et al 1978). Hence it would seem that there is no relationship between the effectiveness of these therapeutic agents at redistributing cardiac output and the means by which they bring about hepatic enlargement.

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Interaction of *N*-alkylaminobenzophenones with benzodiazepine receptors

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Triazolobenzodiazepines like alprazolam and triazolam have been found to be potent anxiolytics and hypnotics in man (Fabre & McLendon 1979; Chatwin & John 1976). Correspondingly, both alprazolam and triazolam have been reported to be very potent in inhibiting the binding of [^{3}H]flunitrazepam ([^{3}H]FNZ) to rat crude rat brain membrane preparations. The result of in-vitro receptor binding assays indicates that these triazolobenzodiazepines may mediate their pharmacological activity through benzodiazepine receptors (Sethy & Harris 1982). *N*-Alkylaminobenzophenones have been reported to have anxiolytic activity in animals (Gall et al 1976). The activity of *N*-alkylaminobenzophenones on benzodiazepine receptors is not known. We have undertaken a study to determine the activity of these compounds on benzodiazepine receptors by using [^{3}H]FNZ binding to the mouse crude brain membrane preparation both in vitro and ex vivo preparations. The results of in-vitro [^{3}H]FNZ binding assays were compared with ex-vivo [^{3}H]FNZ binding assays and with in-vivo ED50's of these compounds required to protect the mice against leptazol (metrazol)- and nicotine-induced seizures.

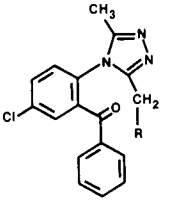
Methods

In-vitro [^{3}H]FNZ binding to crude mouse brain membrane preparation for the determination of inhibition constants (K_i) of alprazolam and *N*-alkylaminobenzophenones was carried out by the method previously described (Sethy & Harris 1982). Ex-vivo [^{3}H]FNZ binding assay was based on the guideline described by Nakajima et al (1981).

Male albino CF-1 mice bred at The Upjohn Company were used for ex-vivo [^{3}H]FNZ binding assays. Animals were kept under constant diurnal lighting and temperature conditions before use and were killed at approximately the same time of day.

Alprazolam was dissolved in 2% ethanol. Compounds I, II and III were dissolved in distilled water. All drugs were administered by the intravenous route. Control mice received equal volumes (1 ml/100 g) of the vehicle. Alprazolam was injected at a dose of 3 μmol kg^{-1}. Compounds I, II and III were administered at a dose of 10 μmol kg^{-1}. Animals were killed at 1, 3, 10, 30 and 60 min after administration of drug. Whole brain, minus cerebellum, was quickly removed and homogenized in 50 volumes of cold (4 °C) 50 mM Tris-HCl buffer, pH 7·4, using a Brinkman polytron PCU-2-110 homogenizer for 30 s at setting No. 6. [^{3}H]FNZ binding was measured by incubating 1·0 ml aliquots of the homogenate with 0·1 ml [^{3}H]FNZ (spec. act. 84·8 Ci mmol^{-1}, NEN, Boston, Massachusetts), to give a final concentration of 0·7 nM, 0·1 ml of distilled water or flurazepam (10 μm) and 0·8 ml of 50 mM Tris-HCl buffer, pH 7·4, to give a final volume of 2 ml. The mixture was incubated for 30 min at 25 °C and then filtered under vacuum through a Whatman GF/B filter.

Table 1. Effect of alprazolam and n-alkylaminobenzophenones on in-vitro [^{3}H]flunitrazepam binding to crude mouse brain membrane preparation and its correlation with in-vivo pharmacological tests.



Drug	R	Ki (nM)	ED50: μmol kg^{-1} i.p. Leptazol	Nicotine
Alprazolam		3·4	0·42	0·18
Compound I*		>100 000	3·2	0·35
Compound II*		>100 000	3·1	1·4
Compound III*		>100 000	1·3	0·5

* In-vivo test data were obtained from published report of Gall et al (1976) J. Med. Chem. 19: 1057.

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