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Interaction of the Anticonvulsants, Denzimol and Nafimidone, with Liver Cytochrome P450 in the Rat

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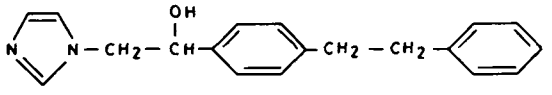
Abstract—The presence of an imidazole moiety in the chemical structure of denzimol and nafimidone suggested that these new anticonvulsants might interfere with cytochrome P450-mediated mixed function monooxygenase activities. We therefore investigated their ability to bind reversibly to rat liver cytochrome P450. Both drugs displayed a type II spectra. The K_s values of binding were 6·66 and 7·00 mM, respectively, for denzimol and nafimidone. In other in-vitro studies the IC50 of the inhibition caused by denzimol and nafimidone was determined on carbamazepine (CBZ) epoxidation and diazepam C_3-hydroxylation and N_1-dealkylation. The IC50 values for CBZ epoxidation were $4·46 \times 10^{-7}$ and $2·95 \times 10^{-7}$ M, respectively, in the presence of denzimol and nafimidone. The IC50 values for diazepam C_3-hydroxylation were $1·44 \times 10^{-6}$ and $1·00 \times 10^{-6}$ M, respectively, and those for N_1-dealkylation $6·66 \times 10^{-7}$ and $5·95 \times 10^{-7}$ M. The inhibition of CBZ metabolism was also investigated ex-vivo and in-vivo after single oral doses (15 and/or 60 mg kg^{-1}) of denzimol or nafimidone. Inhibition of CBZ-10,11-epoxidation by the two drugs was time- and dose-dependent. Further studies in-vivo showed that denzimol and nafimidone prolong pentobarbitone sleeping times indicating that both drugs bind to rat liver microsomes and are potent inhibitors in the rat of mixed function monooxygense activities both in-vitro and in-vivo.

Denzimol (I) and nafimidone (II) are two relatively new anticonvulsants undergoing clinical trials (Nardi et al 1981; Graziani et al 1983a, b; Kapetanovic & Kupferberg 1984). Clinical and pharmacological (Patsalos et al 1985; Caccia et al 1986; Kapetanovic & Kupferberg 1985) interactions have been reported between these two anticonvulsants and



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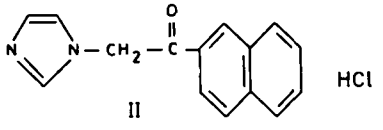

II

conventional antiepileptic agents. Recent studies in rats suggest nafimidone-mediated depression of hepatic oxidative drug metabolism as a possible mechanism underlying these interactions (Kapetanovic & Kupferberg 1984). Structurally, both nafimidone and denzimol are substituted imidazole derivatives and many such compounds have been found to bind to cytochrome P450, resulting in an inhibition of oxidatively metabolized drugs (Wilkinson et al 1972; Hajek et al 1982; Meredith et al 1985; Jensen & Gugler 1985). Accordingly, our aim has been to examine the effect of denzimol on hepatic microsomal drug metabolism and to compare its inhibitory potency with nafimidone, using the rat as a model to establish its real capacity to interfere with drug metabolism and to help establish a rational denzimol treatment protocol.

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Material and Methods

Chemicals

Denzimol hydrochloride and nafimidone hydrochloride were kindly supplied by Recordati S.p.A. Other chemicals were of the highest commercial purity.

Animals

Male CD-COBS rats (Charles River, Italy), 200–300 g, were maintained under regulated temperature with a 12 h light cycle and allowed free access to food and water.

Studies in-vitro

Microsomes were prepared according to Kato & Takayanaghi (1966). Microsomal protein concentrations were determined by the method of Lowry et al (1951). The binding spectra of denzimol and nafimidone to rat liver microsomal P450 was determined according to Schenkman et al (1967) using a double-beam double-wavelength UV 300 Shimadzu spectrophotometer. The drugs were dissolved in absolute ethanol and added to the cuvette containing 2·5 mL of microsomal suspension (1 mg mL^{-1} protein concentration) to obtain a final concentration of 50–200 μM.

The IC50 of the in-vitro epoxidation of CBZ and C_3-hydroxylation and N_1-dealkylation of diazepam were determined as follows. Two mg of microsomal protein in 50 mM phosphate buffer pH 7·4 was added to 50 μL of a methanolic solution of denzimol or nafimidone to obtain final concentrations from 10^{-4} to 10^{-10} M. Then 10 μL of a methanolic solution of CBZ or diazepam was added to obtain a final concentration of 1 mM and the microsomal reactions were started by the addition of 100 μL of a solution containing 2·73 μmol $NADP^+$, 45 μmol glucose-6-phosphate and 4·9 units of glucose-6-phosphate dehydrogenase. The reaction was carried out at 37 °C for 20 min and then

stopped by the addition of 0·6 M NaOH. The samples were extracted and chromatographed as described below.

Studies ex-vivo

Animals were given a single dose of 15 or 60 mg kg^{-1} of denzimol or nafimidone and killed at various times thereafter (from 30 min to 24 h). The livers were immediately removed and microsomes were prepared as above and frozen at −80 °C until analysis. Within two days of preparation the microsomes capacity to inhibit carbamazepine-10,11-epoxidation was measured as described for the in-vitro studies.

Studies in-vivo

CBZ and carbamazepine-10,11-epoxide (CBZ-E) disposition was studied after a single i.v. bolus dose of CBZ (15 mg kg^{-1}) administered through a chronic jugular cannula to seven control rats and seven given denzimol hydrochloride (15 mg kg^{-1} p.o., 30 min before CBZ injection). The cannula was then flushed with 0·5 mL normal saline to avoid contamination of samples drawn from the same tubing. Serial 0·2 mL blood samples (and one 0·5 mL for plasma protein binding determination) were drawn at 0·25, 0·5, 1, 2, 3, 4 6, 8 and 24 h. Blood lost due to sample collection was not replaced in either control or denzimol-treated rats. Blood samples were collected in heparinized tubes, centrifuged and the plasma stored at −20 °C until analysis.

The kinetic data were analysed by computer-assisted non-linear least squares regression with an inverse weighting for drug and metabolite concentrations. The data were most appropriately fitted to a one-compartment open model which was extended to describe the kinetic profile of the metabolite (Gibaldi & Perrier 1982; Pang & Gillette 1980). The coefficients and exponents of the equations were used to calculate the volume of distribution, total body clearance and elimination half-life of the drug and its metabolite. The areas under the plasma concentration–time curve (AUC) of CBZ and CBZ-E were calculated from standard equations (Gibaldi & Perrier 1982).

Plasma protein binding was assessed by equilibrium dialysis: 0·25 mL aliquots were dialysed overnight at 37 °C against isotonic Krebs-bicarbonate buffer (pH 7·4) in a Dianorm apparatus. Each side of each dialysis cell was then analysed for CBZ and metabolite content. Statistical analysis was done using the Mann-Whitney U-test and probabilities less than 0·05 were considered statistically significant. The influence of denzimol and nafimidone upon sleep induced by pentobarbitone in rats was measured using the loss of righting reflex as an indicator of narcosis. The drugs were administered orally to fasted rats 1 h before intravenous injection (caudal) of sodium pentobarbitone (30 mg kg^{-1}). Sleeping time was defined as the interval, in minutes, starting from the time when the rats could be placed on their back and lasting until their righting reflex was regained.

The results are reported as the mean ± s.e.m. and differences between treatments were evaluated by ANOVA and Dunnett's test. ED50 values were evaluated by quantal log dose-probit regression analysis considering in each group the number of animals with sleeping time double or more then mean sleeping time of the control group.

Chemical analysis

Concentrations of CBZ and CBZ-E in each sample were determined by high pressure liquid chromatography (HPLC) using the extraction procedure of Pachecka et al (1976). Butabarbitone and secobarbitone were added as internal standards for quantitation of the drug and its metabolites respectively. The mobile phase was 0·01 M K_2HPO_4-acetonitrile (60:40 v/v), buffered to pH 7 with phosphoric acid. All solvents were HPLC grade. Flow was 1 mL min^{-1} through a 25 cm × 4·6 mm i.d. Ultrasphere-ODS reversed phase column. Under these experimental conditions, approximate retention times were 4·2 min for CBZ-E, 5·2 min for butabarbitone, 6·4 min for CBZ and 8·2 min for secobarbitone. Eluting peaks were quantitated by absorption at 240 nm (Hewlett-Packard 1084 B chromatograph). Concentrations of diazepam metabolites in all samples were determined by gas chromatography with electron capture detection (Caccia et al 1986).

Results

Studies in-vitro

Fig. 1 reports the binding spectra to rat liver microsomes of denzimol (Panel A) and nafimidone (Panel B). These spectra gave a maximum peak at 430 nm and a minimum at 388 nm. Thus both drugs correspond to the type II classification. To calculate the K_s value (half maximal spectral change) for each compound, the effects of different substrate concentrations on the magnitude of spectral change were determined. Changes in absorbance were determined at 430 nm relative to 417 nm. Fig. 2 reports the results for denzimol (Panel A) and nafimidone (Panel B), their K_s values being, respectively, 6·66 and 7·00 mM.

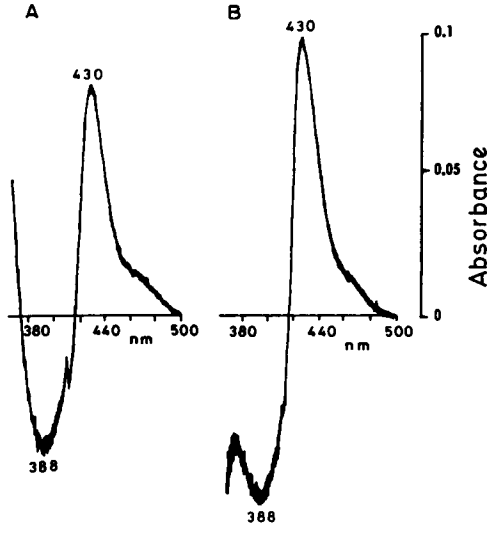

FIG. 1. Typical binding spectra to rat liver microsomes of denzimol (Panel A) and nafimidone (Panel B). Final concentration of both compounds was 62·5 μM.

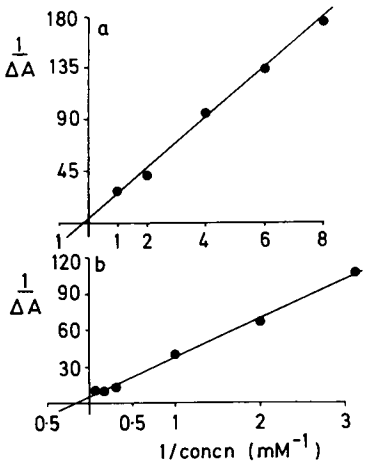

FIG. 2. Reciprocal plot of changes in absorbance (A) at 430 nm relative to 417 nm caused by consecutive additions of denzimol (Panel a) and nafimidone (Panel b) to rat liver microsomes. The K_s values were 6·6 mM for denzimol and 7·0 mM for nafimidone.

Table 1 reports the IC50 values of in-vitro CBZ-E formation and C_3-hydroxylation or N_1-dealkylation of diazepam after preincubation of rat liver microsomes with different denzimol or nafimidone concentrations. Both drugs inhibited these activities and their order of magnitude was the same.

Table 1. IC50 values of in-vitro inhibition of denzimol and nafimidone on the epoxidation of carbamazepine (CBZ) and C_3-hydroxylation or N_1-dealkylation of diazepam. The incubation mixture contained 1 mg mL^{-1} microsomal protein, substrate (1 mM) inhibitors (10^{-1}–10^{-4} M) and 0·05 M phosphate buffer, pH 7·4, in a total volume of 2·5 mL. Reactions were started by the addition of 0·273 μmol of NADPH and terminated with 0·5 mL of 0·6 M NaOH.

	IC50 (M)	
Metabolic reaction	Denzimol	Nafimidone
CBZ-10,11-epoxidation	$4{\cdot}26 \times 10^{-7}$	$2{\cdot}95 \times 10^{-7}$
Diazepam C_3-hydroxylation	$1{\cdot}44 \times 10^{-6}$	$1{\cdot}00 \times 10^{-6}$
Diazepam N_1-dealkylation	$6{\cdot}66 \times 10^{-7}$	$5{\cdot}95 \times 10^{-7}$

Studies ex-vivo

The time-courses of the ex-vivo inhibition of CBZ epoxidation by denzimol (A) and nafimidone (B) at two acute different dosages (15 and 60 mg kg^{-1}) are shown in Fig. 3. Both denzimol doses caused strong inhibition up to 2 h following treatment after which the low dose no longer caused inhibition whereas with the high dose the activity remained up to 16 h. Similarly, while the low dose of nafimidone caused significant inhibition lasting 2 h the high dose inhibition lasted to the end of the observation (i.e. 24 h).

Studies in-vivo

Administration of denzimol and nafimidone to rats 60 min before barbiturate injection prolonged barbiturate sleeping times (Table 2). The ED50 values were 3 and 2·3 mg kg^{-1} in denzimol- and nafimidone-treated animals,

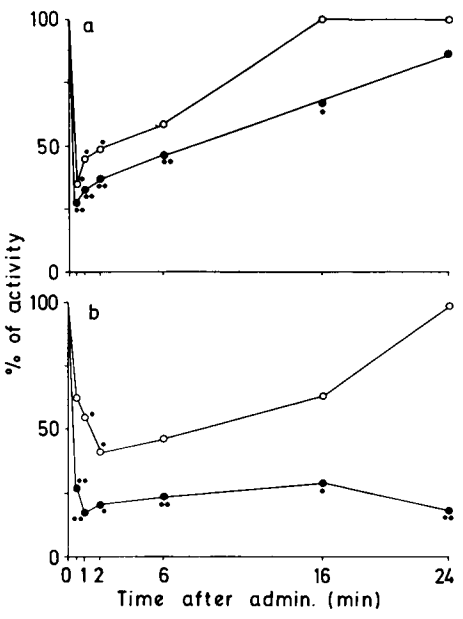

FIG. 3. Time-course of the ex-vivo inhibition caused by denzimol and nafimidone of the formation of carbamazepine-10,11-epoxide. Doses of denzimol (Panel a) and nafimidone (Panel b) were 15 (○) and 60 (●) mg kg^{-1}. $^*P < 0{\cdot}01$; $^{**}P < 0{\cdot}05$.

Table 2. Effects of oral denzimol and nafimidone on pentobarbitone sleeping time in rats. Data are the mean sleeping time (min ± s.e.m.), and [a] the number of animals with sleeping time double or more that of controls out of animals treated and the ED50 value (95% confidence limits in brackets).

Treatment (mg kg^{-1} p.o.)	Sleeping time (min)	Number of rats[a]	ED50 (mg kg^{-1} p.o)
Vehicle	61·2 ± 2·5	0/20	
Denzimol (0·94)	80·8 ± 6·4*	0/10	
Denzimol (1·87)	111·3 ± 15·4**	5/10	
Denzimol (3·75)	121·0 ± 10·5**	5/10	3·0 (2·1–4·3)
Denzimol (7·50)	148·6 ± 12·2**	8/10	
Denzimol (15·0)	206·3 ± 12·3**	10/10	
Vehicle	61·0 ± 4·2	0/20	
Nafimidone (0·94)	91·5 ± 6·5**	0/10	
Nafimidone (1·87)	125·1 ± 9·3**	4/10	2·3 (1·8–3·0)
Nafimidone (3·75)	150·9 ± 11·9**	8/9	
Nafimidone (7·5)	216·5 ± 10·1**	10/10	

$^{**}P < 0{\cdot}01$; $^*P < 0{\cdot}05$.

respectively, and were not significantly different. The in-vivo metabolism of barbiturate was also inhibited by denzimol and nafimidone, in agreement with the in-vitro finding.

The derived kinetic parameters of CBZ and its metabolite after i.v. injection (15 mg kg^{-1}) to control and denzimol pretreated rats (15 mg kg^{-1} p.o., 30 min before CBZ) are listed in Table 3. The volume of distribution of CBZ was not significantly different in the two groups. However, in the denzimol-pretreated rats total body clearance of the drug was significantly lower and the elimination half-life significantly longer than in control rats. These differences

were not due to differences in plasma protein binding, the mean CBZ free fraction being similar in both groups (Table 3).

With denzimol, the mean (± s.d.) time to maximum concentrations tended to be longer, 202 (56) vs 132 (26) min, and the mean peak plasma concentrations of the metabolite lower, 11(3) vs 14(2) nmol mL^{-1}, but the differences were not significant. Denzimol also tended to prolong the elimination half-life of CBZ-E and the difference approached significance. The plasma AUC of the metabolite was unaltered by denzimol and the ratio between the AUC of the metabolite and the AUC of the parent drug, which describes the ratio of the formation clearance of CBZ-E from CBZ relative to the elimination of the formed metabolite, was also not significantly different in control and denzimol-treated rats. As with CBZ, denzimol did not influence the binding of CBZ-E, the free fraction of the metabolite being similar in control and denzimol-treated rats (Table 3).

Table 3. Disposition of carbamazepine (CBZ) in control and denzimol-treated rats. Rats were given denzimol hydrochloride (15 mg kg^{-1} p.o.) 30 min before CBZ (15 mg kg^{-1} i.v.). Results are the mean with (s.d.) of 7 control and 7 denzimol-treated animals.

Parameters	Control	Pretreated
CBZ		
Apparent volume of distribution (L kg^{-1})	1·8 (0·3)	1·8 (0·4)
Total plasma clearance (mL min^{-1} kg^{-1})	15·3 (3·9)	9·9 (2·7)*
Elimination half-life (min)	83 (18)	134 (57)*
Free fraction in plasma (% unbound)	38·2 (4·8)	35·0 (5·5)
CBZ-E		
Area under the curve (nmol mL^{-1} × min)	6354 (1198)	7256 (1411)
Elimination half-life (min)	184 (41)	240 (57·2)
Free fraction in plasma (% unbound)	66·7 (8·4)	65·9 (10·8)

Significant differences: $*P < 0.05$.

Discussion

Previous studies suggested that denzimol inhibited hepatic drug oxidizing reactions both in animals (Caccia et al 1986) and man (Patsalos et al 1985). This was related to the capacity of the drug's imidazole ring moiety to impair hepatic mixed function oxygenases. We examined the capacity of denzimol to bind reversibly to liver microsomal cytochrome P450 as a possible mechanism involved in these interactions. The findings confirmed that denzimol as well as its structurally and pharmacologically related compound nafimidone, interacts with rat liver microsomes in the manner of type II class compound. This binding probably reduces the substrate interactions of other drugs which have lower affinity for the cytochrome, thus slowing down their in-vivo metabolism. While this binding may rationalize the pharmacokinetics and metabolism observed, the K_s values (6·66 and 7·0 mM) are high and mM concentrations would not be realized in the liver.

CBZ is an example of a low extraction ratio drug whose clearance is directly related to drug metabolizing activity. It is almost entirely eliminated from the body by biotransformation and it is generally assumed that the metabolic processes involved in its elimination are mainly mediated by the hepatic cytochrome P450 system (Lertratanangkoon & Horning 1982; Tybring et al 1981). These two variables have been shown to be linearly related in other animal species (Wedlund et al 1982). Thus, the observed reduction in CBZ total body clearance in denzimol-treated rats is probably due to the binding of the imidazole derivative to cytochrome P450, resulting in a reduction of the amount of functionally intact cytochrome. The fact that denzimol did not apparently alter the plasma protein binding and volume of distribution of CBZ suggests that an interaction between denzimol and CBZ at the distribution level is unlikely. The reduction in CBZ metabolic clearance in denzimol-treated rats resulted in significant prolongation of the antiepileptic agent's elimination half-life.

The precise metabolic pathways of CBZ metabolism that undergo inhibition cannot be established on the basis of our studies. Its biotransformation proceeds through a complex scheme which primarily involves parallel peroxide and multiple epoxide intermediates and ultimately may result in the formation of approximately 30 metabolites (Lertratanangkoon & Horning 1982). Of the primary metabolites only the 10,11-epoxide is stable enough for quantitative determination. There was a tendency for denzimol to prolong the time taken to reach maximum concentrations and to reduce the peak plasma concentrations of this metabolite; although not significant because of broad variability in denzimol-treated animals, these effects could be interpreted as indicative of transient inhibition of epoxide formation.

Direct in-vitro evidence for denzimol-induced inhibition of 10,11-epoxidation of CBZ was obtained in the liver homogenate studies. Dose- and time-related inhibition of epoxide formation indicated its in-vitro depressive effect on a main pathway of CBZ metabolism. Similar dose-related inhibition by this imidazole derivative was found for C_3-hydroxylation and N_1-demethylation of diazepam whose has been previously shown to be impaired when it is co-administered with denzimol (Caccia et al 1986). Similar findings were observed with nafimidone; both compounds appeared extremely active (in a submicromolar concentration range) and equally effective in inhibiting hepatic microsomal mixed function oxidation. Their inhibitory potency cannot be compared with that of other imidazole-containing drugs because these were not obtained for the same systems.

The inhibitory effect appeared to be time-dependent. The ex-vivo studies show that in rats given 15 mg kg^{-1} denzimol, which is in the anticonvulsant pharmacological range (Graziani et al 1983a, b), the drug's inhibitory effect on mixed function oxygenases was powerful in the first 2 h after administration. The transient inhibitory effect of these compounds may explain, at least in part, the modest in-vivo effect of denzimol on CBZ-E disposition. High

doses of denzimol (60 mg kg^{-1}) clearly resulted in strong lasting activity towards CBZ-10,11-epoxidation. In contrast to nafimidone, complete recovery of the inhibition of CBZ-10,11-epoxidation was observed with denzimol-treated rats after 24 h, even after the relatively large dose of 60 mg kg^{-1}. Neither denzimol nor nafimidone, when given chronically for 7 days, showed any cumulative inhibitory activity (data not reported).

Overall, these in-vitro and in-vivo studies confirm previous findings suggesting impairment of hepatic drug metabolism by denzimol and indicate that its inhibitory effect, even if lower than that of nafimidone, is directly related to its capacity to bind reversibly to hepatic cytochrome P450. Thus, in possible therapeutic use both compounds should be employed with all necessary caution to avoid clinically important interactions.

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