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Diazepam–omeprazole inhibition interaction: an *in vitro* investigation using human liver microsomes

K. ZOMORODI & J. B. HOUSTON
Department of Pharmacy, University of Manchester, Manchester, UK

1 The metabolism of diazepam to its primary metabolites 3-hydroxydiazepam (3HDZ) and nordiazepam (NDZ) was evaluated in human liver microsomes. The 3HDZ pathway was the major route of metabolism representing 90% of total metabolism with a V_{max}/K_m ratio of 0.50–7.26 μl min^{-1} mg^{-1} protein.

2 Inhibition of the two metabolic pathways of diazepam by omeprazole was investigated. The NDZ pathway was not affected by omeprazole whilst a K_i of 201 ± 89 μM was obtained for the 3HDZ pathway (K_m/K_i ratio of 3.0 ± 0.9).

3 Inhibitory effects of omeprazole sulphone on the 3HDZ and NDZ pathways were also investigated. Omeprazole sulphone inhibited both pathways with similar K_is of 121 ± 45 and 188 ± 73 μM respectively (K_m/K_i ratios of 5.2 ± 2.3 and 3.3 ± 1.5 respectively).

4 These *in vitro* data provide direct evidence for cytochrome P450 inhibition as the mechanism for the well documented diazepam-omeprazole clinical interaction and indicate that omeprazole sulphone, as well as the parent drug, contribute to the inhibition effect.

Keywords omeprazole diazepam omeprazole sulphone human microsomes *in vitro* metabolism drug interactions CYP3A CYP2C19

Introduction

Omeprazole is a substituted benzimidazole which inhibits the gastric proton pump in the secretory membrane of the parietal cells. It is absorbed rapidly with an oral bioavailability of about 50% and is eliminated completely through metabolism by cytochrome P450 (CYP) [1, 2]. In man the main metabolites are the sulphone, the sulphide and hydroxyomeprazole [2]. Recent work by Andersson *et al.* [3] showed that hydroxyomeprazole production was catalysed by CYP2C19, whilst omeprazole sulphone formation was mediated by CYP3A. Furthermore, CYP2C19 was also responsible for the hydroxyomeprazole sulphone formation from omeprazole sulphone, while CYP3A was the major enzyme involved in the formation of this secondary metabolite from hydroxyomeprazole.

There have been reports of both inhibition and induction of CYP by omeprazole. Diaz *et al.* [4] provided both *in vitro* and *in vivo* evidence on induction of CYP1A1 and CYP1A2 by omeprazole. Inhibition studies with omeprazole have led to conflicting results. Metabolism of caffeine, theophylline, metoprolol, propranolol and quinidine was not affected by omeprazole co-administration [5]. The inhibitory effect of omeprazole on cyclosporin and phenytoin proved to be dose dependent [5, 6–9] and on warfarin was enantiomeric selective [5]. The most substantial effect observed however, concerns the elimination of diazepam which has been shown to be significantly affected by omeprazole in several studies [8, 10, 11]. For example, a 54% decrease in the clearance and a 130% increase in the half-life of diazepam was observed [8].

Nordiazepam (NDZ) and 3-hydroxydiazepam (3-HDZ) are the two major metabolites of diazepam formed by CYP and both can be further metabolized to 3-hydroxynordiazepam (3HNDZ) [12]. Some correlation has been found between diazepam metabolism and mephenytoin hydroxylase (CYP2C19, [13]) and it has been suggested that the omeprazole-diazepam interaction is associated with CYP2C19 polymorphism [11]. Inaba *et al.* [14] showed that diazepam and NDZ inhibited the hydroxylation of mephenytoin in human liver *in vitro*, however mephenytoin does not inhibit either NDZ or 3HDZ pathways [15]. Further studies by Forrester *et al.* [16], using a human liver panel, indicated that CYP3A4 expression was correlated with both 3HDZ and NDZ production and both pathways were also correlated with CYP2C8. Recent investigations by Yasumori *et al.* [17] using various antibodies have

Correspondence: Dr J. B. Houston, Department of Pharmacy, University of Manchester, Manchester, M13 9PL, UK

concluded that diazepam 3-hydroxylation was mainly catalysed by CYP3A while diazepam *N*-demethylation was catalysed mainly by a CYP2C isoenzyme at low substrate concentrations.

The omeprazole interactions reported to date suggest that this drug inhibits selectively certain isoenzymes of CYP. The aim of the present study was to substantiate the omeprazole-diazepam interaction *in vitro*, using human liver microsomes.

Methods

Chemicals

Omeprazole and omeprazole sulphone were generous gifts from Astra Pharmaceuticals Ltd (Mölndal, Sweden). Ketoconazole was a gift from Pfizer Ltd (Sandwich, Kent, UK). Diazepam and 4′-hydroxydiazepam (4′HDZ) were kindly provided by Roche Ltd (Welwyn Garden City, Herts, UK). 3HDZ (temazepam) and 3HNDZ (oxazepam) were gifts from Wyeth (Maidenhead, Berks, UK). NDZ was purchased from Sigma (Poole, Dorset, UK). Prazepam (internal standard) was obtained from Warner & Co. (Pontypool, Gwent, UK). All other chemicals were obtained from either BDH (Lutterworth, Leics, UK) or Sigma (Poole, Dorset, UK).

Preparation of human liver microsomes

Five human liver samples were supplied by the International Institute for the Advancement of Medicine, Exon, PA, USA and stored at -80° C until required. Three samples (A, B and D) were from male donors aged 22–33 years and two samples from female donors aged 54 and 62 years (the latter, C, was a smoker and the former, E, received dexamethasone therapy). Liver pieces were washed in Tris-HCl buffer (100 mM; pH 7.4), diced and homogenized in ice-cold Tris-HCl/sucrose buffer (50 mM/250 mM; pH 7.4). Washed microsomal pellets were prepared by standard differential centrifugation techniques and resuspended in phosphate buffer (100 mM; pH 7.4) and stored at -80° C. The microsomal protein contents were measured by standard methodology using Lowry *et al.* [18] method.

Microsomal incubation conditions

Microsomal solution (0.4 ml of 2 mg protein ml^{-1} in phosphate buffer 200 mM; pH 7.4), 0.1 ml of potassium chloride (1.15%) and 5 μl of diazepam (in DMF, final concentration 0.5%) were preincubated at 37° C in a shaking water bath for 5 min. The reaction was started by adding 0.5 ml of isocitrate regenerating system (0.74 mg $NADP^+$, 1.94 mg isocitric acid, 0.5 units isocitric dehydrogenase, 10 μmol magnesium sulphate in phosphate buffer 200 mM) and stopped after 10 min by the addition of 20 μl of NaOH (10 M). Linearity with respect to time and protein concentration was confirmed. For the inhibition studies, 5 μl of inhibitor (in DMF) was added to the incubation mixture prior to the addition of diazepam and vehicle only control incubations were carried out. All incubations were carried out in triplicate and mean rates reported (coefficient of variation $<5\%$).

Analysis of diazepam metabolites

H.p.l.c. was used to assay diazepam and its metabolites simultaneously, according to the method of Reilly *et al.* [19]. The incubation mixture was extracted with ethylacetate (5ml) after adding the internal standard (prazepam, 100 μl, 70 μM in methanol) and carbonate buffer (1 ml, 100 mM; pH 10) by rotary mixing for 25 min and centrifuging at 2000 rev min^{-1} for 10 min. The organic layer was evaporated to dryness under nitrogen at 50° C. The residue was reconstituted in mobile phase and 100 μl was injected via a Spectra Physics SP8780XR autosampler onto the h.p.l.c system. The system comprized of a Hichrom Spherisorb S5 ODS2 250×5 mm column, mobile phase of 65% methanol/35% water containing 0.02% triethylamine adjusted to pH 7.0 with phosphoric acid (delivered by a Waters 6000 A pump at a flow rate of 1 ml min^{-1}) and u.v. wavelength of 236 nm (measured by an Applied Biosystems spectroflow 783). Diazepam and metabolite concentrations were determined by a peak height ratio method with respect to the internal standard (prazepam). The inhibitors did not interfere with the assays. Linearity extended to at least 12 μM (NDZ) and 20 μM (3HDZ) with coefficients of variation of 5.6% (3HDZ) and 8.3% (NDZ) at 3 μM.

Data analysis

The Michaelis-Menten equation was used to determine the V_{max} and K_m parameters (equation 1) by nonlinear regression (Siphar version 3.3, Simed, Créteil, France)

$$V = \frac{V_{max} \cdot S}{K_m + S} \quad (1)$$

where V_{max} is the maximum velocity, K_m is the substrate concentration at which the reaction is half of its maximal value and S is the substrate concentration. A substrate concentration range of 30–600 μM was used. This range was constrained by the analytical sensitivity of the methods employed and the solubility properties of the substrate.

For microsomal preparation B, three diazepam concentrations (125, 400 and 600 μM) were selected to investigate the effect of various concentrations of omeprazole (10–500 μM) and omeprazole sulphone (125–500 μM). Dixon plots were initially used to get approximate estimates of the inhibitory constant (K_i). In all cases studied, the intersection of the lines was above the x-axis (competitive inhibition). Nonlinear regression (Siphar) and a model for competitive inhi-

bition (equation 2) were used to obtain a more accurate estimate of the K_i.

$$V = \frac{S \cdot V_{max}}{S + K_m\left(1 + \frac{I}{K_i}\right)} \quad (2)$$

For microsomal preparations A, C and E the effect of omeprazole (250 μM), omeprazole sulphone (250 μM) and ketoconazole (1 μM) on diazepam metabolism was studied at 400 μM. The percentage inhibition values (ICx) were converted to K_i values assuming competitive inhibition using equation 3.

$$K_i = \frac{ICx\left(\frac{100 - x}{x}\right)}{1 + \frac{S}{K_m}} \quad (3)$$

Results

Hepatic microsomal incubations of diazepam resulted in the formation of 3HDZ and NDZ and typical rate-concentration profiles obtained under linear conditions with respect to time and protein concentration are shown in Figure 1. 3HNDZ was not produced in large enough quantities to be detected within the incubation period studied, whilst the 4-hydroxylation pathway does not occur in man [15].

The Michaelis-Menten parameters V_{max}, K_m and V_{max}/K_m (intrinsic clearance, CLint) for the five human microsomes averaged (±s.d.) 2.12 ± 1.61 and 0.18 ± 0.07 nmol min^{-1} mg^{-1} protein; 557 ± 118 and 600 ± 182 μM and 3.85 ± 2.56 and 0.30 ± 0.13 μl min^{-1} mg^{-1} protein for the 3HDZ and NDZ pathways, respectively. In view of the recent demonstration [20] of unusual kinetics for 3HDZ and NDZ formation, resulting in curved Eadie-Hofstee plots, these data were also analysed by a Hill equation. Unlike Andersson *et al.* [20] we were not able to describe our data better by the Hill equation than by the Michaelis-Menten equation (the sigmoidicity factor being not significantly different from 1 in our case). Wide interindividual variability in enzymatic parameters was observed between the human samples examined. V_{max} values ranged 16 and 3 fold for the 3HDZ and NDZ pathways respectively. K_m values showed less variability between samples with two fold differences for both the 3HDZ and NDZ pathways. There was no statistical difference between the K_m values for the two pathways ($P > 0.05$ by the paired *t*-test). On average, both V_{max} and CLint values for the 3HDZ pathway were approximately 12 times larger than the respective values for the NDZ pathway and a good correlation existed between the CLint values for the 3HDZ and NDZ pathways in different microsomal preparations ($r^2 = 0.87$).

Various concentrations of omeprazole (10–500 μM) were added to the incubation mixtures of microsomal preparation B at three different diazepam concentrations (125, 400 and 600 μM). Figure 2a shows IC_{50} plots obtained for the 3HDZ and NDZ pathways. There is

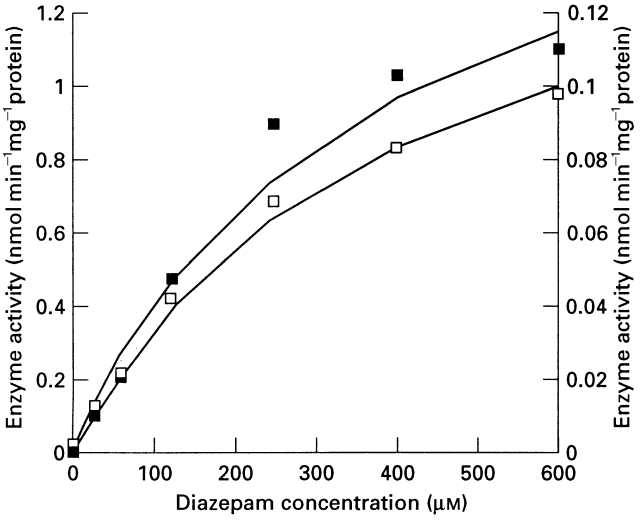

Figure 1 The relationship between rate of formation of 3-hydroxy(■, left axis) and nor(○, right axis) diazepam and substrate concentration in microsomal preparation B.

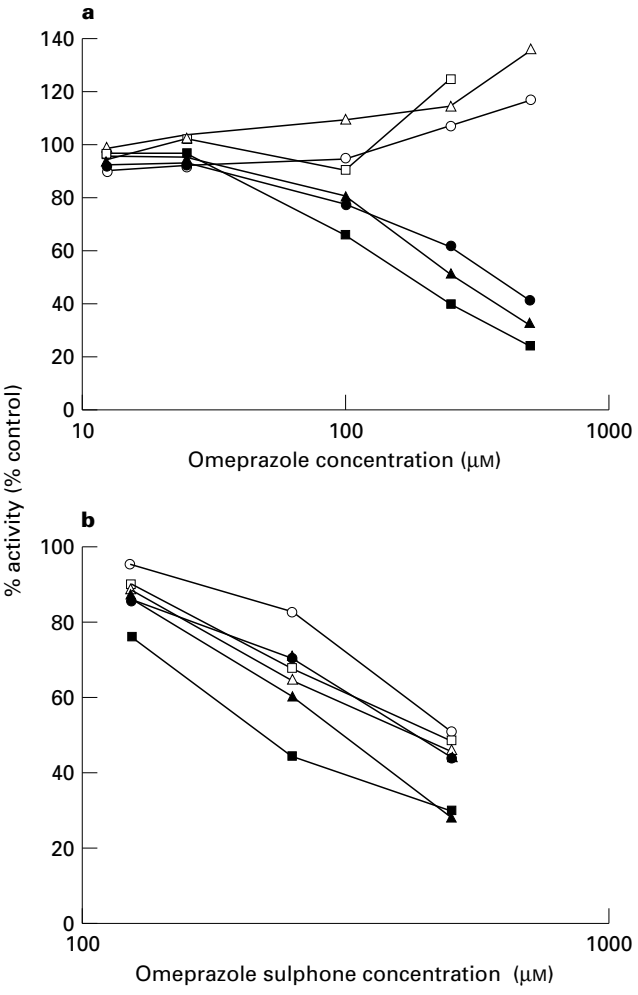

Figure 2 IC_{50} plot for a) omeprazole inhibition of the 3HDZ (closed symbols) and NDZ (open symbols) pathways in microsomal preparation B at different diazepam concentrations (125 μM □, ■; 400 μM △, ▲; 600 μM ●, ○), and b) for omeprazole sulphone inhibition of the 3HDZ and NDZ pathways in human microsomal preparation B at different diazepam concentrations (symbols as in Figure 2a).

little or no inhibition observed with the NDZ pathway, however, substantial inhibition is seen with the 3HDZ pathway at omeprazole concentrations above 100 μM. IC_{50} values for 3HDZ formation increased, 180, 260 and 400 μM, as diazepam incubation concentrations were increased, in keeping with a competitive inhibition mechanism. Inhibition data for the 3HDZ pathway at three diazepam concentrations gave an estimate of 163 μM for the K_i by nonlinear regression. This value is in reasonable agreement with that obtained by the Dixon method (115 μM). A competitive inhibition model gave the best fit to the data when compared to noncompetitive and mixed models using the Akaike and Schwarz goodness of fit criteria and residual plot analysis.

Similarly, omeprazole sulphone (125–500 μM) and diazepam (125–600 μM) were coincubated at various concentrations to investigate the inhibitory effects of this metabolite towards diazepam metabolism in microsomal preparation B. Figure 2b demonstrates that IC_{50} values of 220, 320 and 430 μM and 430, 470 and 500 μM were estimated for the inhibition of the 3HDZ and NDZ pathways, respectively, for diazepam concentrations of 125, 400 and 600 μM. Omeprazole sulphone therefore has similar inhibitory potency as its parent drug towards the 3HDZ pathway. However, this metabolite shows much higher affinity towards NDZ inhibition than omeprazole. Once more the mechanism appeared to be competitive based on the trend in IC_{50} values with increasing substrate concentration and the Dixon plot. K_i values by nonlinear regression analysis were 183 and 225 μM for 3HDZ and NDZ formation, respectively, for microsomal preparation B.

Further inhibition studies were carried out in microsomal samples A, C and E using omeprazole and diazepam concentrations which yielded around 50% inhibition in sample B. Table 1 summarizes the results obtained together with the inhibitory effects of omeprazole sulphone and ketoconazole. It is evident that the 3HDZ pathway was more prone to inhibition than the NDZ pathway regardless of the inhibitor used ($P<0.05$ by paired t-test). Furthermore, omeprazole sulphone produced a greater inhibitory effect than the parent compound on the NDZ pathway ($P<0.01$ by paired t-test). However, ketoconazole was the most potent inhibitor of both pathways (Table 1). For both omeprazole and omeprazole sulphone, the use of a higher substrate concentration (600 μM) in these livers resulted in a reduction in the percent inhibition observed, supporting a competitive inhibition mechanism.

Table 2 shows that the K_i parameters for omeprazole sulphone and omeprazole inhibition for the 3HDZ pathway were comparable in the four livers studied, as were the K_m/K_i ratios in these samples. However, although K_i values could not be determined for omeprazole, omeprazole sulphone inhibited the NDZ pathway with K_i values consistently lower than those for the 3HDZ pathway (23–117%). When expressed as K_m/K_i ratios, both sets of data are similar (average of 3.3 and 5.2 for the NDZ and 3HDZ pathways respectively) and approximately the same rank order is obtained for the four livers studied. The corresponding ratio for omeprazole inhibition of the 3HDZ pathway is 3.0.

Table 1 Inhibition of diazepam metabolism by omeprazole, omeprazole sulphone and ketoconazole in human liver microsomal preparation A,B,C,E

		Activity (% control)[a]		
Code	*Pathway*	*Omeprazole (250 μM)*	*Omeprazole sulphone (250 μM)*	*Ketoconazole (1 μM)*
A	3HDZ	67.9	44.6	9.7
	NDZ	91.2	59.0	33.2
B	3HDZ	52.0	60.3	15.2
	NDZ	113.8	63.9	40.1
C	3HDZ	53.5	36.2	18.3
	NDZ	73.6	46.1	32.1
E	3HDZ	48.3	38.5	10.3
	NDZ	93.3	46.4	27.9

[a] Diazepam concentration 400 μM.

Discussion

A 15 fold and 3 fold range in CLint for the 3HDZ and NDZ pathways seen in the present study was mainly a consequence of differences in V_{max} values since the K_m

Table 2 K_i and K_m/K_i values for omeprazole and omeprazole sulphone inhibition of the 3HDZ and NDZ pathways in human liver microsomes

	Omeprazole		*Omeprazole sulphone*			
	3HDZ pathway		*3HDZ pathway*		*NDZ pathway*	
Code	K_i *(μM)*	K_m/K_i	K_i *(μM)*	K_m/K_i	K_i *(μM)*	K_m/K_i
A	331	2.0	126	5.3	273	4.6
B	163	2.3	183	2.0	225	1.8
C	178	3.7	88	7.4	135	5.1
E	132	3.9	88	5.9	119	4.1
Mean	201	3.0	121	5.2	188	3.9
95%CI	60–340	1.6–4.4	50–192	1.5–8.9	115–304	1.5–6.3

values were relatively consistent. The parameter values are within the range reported by Inaba and co-workers [15, 21] in their studies of diazepam metabolism in 15 human livers, using a similar Michaelis-Menten model as we employed. Other workers have used the Hill equation to model data to the sigmoidal trend frequently apparent in the 3HDZ rate-concentration profile [17,20] obtaining concentration values for 50% maximal velocity comparable with the K_m values of the above studies.

Also in agreement with other investigations [15, 20, 21] we found the 3HDZ pathway to be dominant based on higher V_{max} and CLint values for this pathway in all samples studied. The correlation between the CLint values for the formation of the two diazepam metabolites is consistent with the participation of the same CYP isoform(s) (CYP3A family) in both pathways [16, 17]. However as this analysis has an intercept significantly larger than zero it does not preclude the involvement of other isoforms (i.e. CYP2C19) in the NDZ pathway [17]. The observation (Table 1) that 3HDZ is more susceptible than NDZ to inhibition at low ketoconazole concentrations (at which it acts as a selective inhibitor of CYP3A [22]) is consistent with these conclusions on specific isoform involvement on particular diazepam pathways.

Both omeprazole and omeprazole sulphone inhibit the metabolism of diazepam *in vitro*. Whereas only the sulphone reduces NDZ formation, both benzimidazoles affect 3HDZ formation. All three inhibition effects show the characteristics of a competitive inhibition mechanism and K_i values have been calculated on this basis. Average K_i values are in the range of 120–200 μM and K_m/K_i ratios, an estimate of the relative affinities of diazepam and the benzimidazoles, ranged from 3–5.9 in favour of the benzimidazoles. Although the inhibitory action of omeprazole sulphone has not been reported previously, there is one report on the inhibition of DZ metabolism by omeprazole in human microsomes [20]. These workers were able to inhibit NDZ formation substantially with omeprazole concentrations more than 10-fold excess over substrate but could not show any consistent effect on 3HDZ formation [20]. Furthermore we have demonstrated selective inhibition of 3HDZ formation by omeprazole in rat liver microsomes and isolated hepatocytes [23].

As in most *in vitro* inhibition studies, the concentrations of diazepam and inhibitors used were in excess of that encountered under therapeutic conditions. However a competitive inhibition model is applicable to these particular interactions and the K_i parameters obtained are concentration independent and hence relevant to the therapeutic concentration range. Indeed the extent of the diazepam-omeprazole interaction documented *in vivo* [8, 10, 11] is consistent with the parameters reported here *in vitro* .

One apparent anomaly between the *in vitro* observations reported here and the *in vivo* clinical interactions concerns the selectivity seen in the inhibition of the two diazepam pathways. We observed little inhibition *in vitro* by omeprazole towards the NDZ pathway in human microsomes, suggesting that the decrease observed in diazepam metabolism in the presence of omeprazole in the clinical studies must mainly be due to the inhibition of the 3HDZ pathway. However, Gugler & Jensen [8] observed that the plasma concentrations of NDZ were 34% lower with omeprazole. They concluded that the hepatic microsomal NDZ pathway was inhibited in the presence of omeprazole. Similar decreased plasma concentrations of NDZ were also observed by Andersson *et al.* [10]. In both these studies however, samples were analysed only up to 120 h, therefore, changes in the NDZ half-life and area under the curve (AUC) from time zero to infinity could not be accurately determined. NDZ plasma concentrations show a very broad peak (20–100 h) and a terminal half-life of approximately 90 h, necessitating a study of at least 14 days to adequately define the kinetics of this metabolite after oral diazepam administration. Thus the effect on NDZ elimination is unknown. As the metabolite kinetics of NDZ are elimination rate limited, changes in the NDZ plasma concentration accrual will result from changes in the parent drug elimination rate constant [24]. The latter is influenced by both the NDZ or 3HDZ pathways. This analysis together with the nonselectivity of the sulphone metabolite, which persists longer in the plasma than the parent drug [2], would indicate that the present *in vitro* data are consistent with the *in vivo* observations.

In conclusion, we have demonstrated that omeprazole and omeprazole sulphone inhibit DZ metabolism; the NDZ pathway being less susceptible to inhibition by omeprazole than the 3HDZ pathway. However, omeprazole sulphone was an inhibitor of both pathways with comparable effects to omeprazole on the 3HDZ formation. It is likely that the inhibition observed *in vivo* results from the actions of omeprazole sulphone, as well as the parent drug itself.

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