Effect of Omeprazole on Diazepam Disposition in the Rat: in vitro and in Vivo Studies

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Purpose. The inhibitory effects of omeprazole on diazepam metabolism in vitro and in vivo are compared in the rat.

Methods. 3-Hydroxylation and N-demethylation of diazepam was investigated in the presence of a range of omeprazole concentrations (2-500μM) in hepatic microsomes and hepatocytes. Zero order infusions together with matched bolus doses of omeprazole were used to achieve a range of steady state plasma concentrations (10-50mg/L) and to study the diazepam-omeprazole interaction in vivo.

Results. The 3-hydroxylation pathway was more prone to inhibition (K\textsubscript{i} = 108 ± 30 and 28 ± 11 μM in microsomes and hepatocytes, respectively) than the demethylation pathway (K\textsubscript{i} of 226 ± 76 and 59 ± 27 μM in microsomes and hepatocytes, respectively). In both in vitro systems, the mechanism of inhibition was competitive with Km/K\textsubscript{i} ratios larger than 1 for the 3HDZ pathway and smaller than 1 for the NDZ pathway. There was an omeprazole concentration dependent decrease in diazepam clearance in vivo which could be modelled using a simple inhibition equation with a K\textsubscript{i} of 57μM (19.8μg/mg/L). In contrast, there was no statistically significant change in the steady state volume of distribution for diazepam in the presence of omeprazole.

Conclusions. The in vivo K\textsubscript{i} for the omeprazole: diazepam inhibition interaction shows closer agreement with the K\textsubscript{i} values obtained in hepatocytes than with those observed in microsomes.

KEY WORDS: omeprazole; diazepam; inhibition of cytochrome P450; drug–drug interactions; in vitro—in vivo correlations.

INTRODUCTION

Several substituted imidazoles and benzimidazoles are known to inhibit cytochromes P450 (CYP) to a clinically important degree (1,2). We have investigated omeprazole, a substituted benzimidazole used widely for the treatment of gastrointestinal ulcers, as a CYP inhibitor under in vitro and in vivo conditions. In man and rat, omeprazole is rapidly distributed to extravascular sites, is more than 90% bound to plasma proteins and is rapidly and extensively metabolised by CYP to numerous compounds (3,4). The most substantial inhibitory effect of omeprazole observed to date concerns the elimination of diazepam and results in a 50% reduction in clearance (5,6).

The present study examines the use of in vitro screening systems for assessing the inhibitory potency of omeprazole in the rat. The effect of omeprazole on the kinetics of diazepam metabolism to form the primary metabolites 3-hydroxydiazepam (3HDZ) and nortriazepam (NDZ) has been investigated in both liver microsomes and freshly isolated hepatocytes. In addition, in vivo studies have been performed to determine diazepam clearance in animals receiving infusions of omeprazole, with matching loading doses, to achieve a range of steady state omeprazole concentrations. As diazepam has a high extraction ratio in rat, this drug was administered via the hepatic portal vein (IP) to avoid blood flow limitations and allow direct assessment of its intrinsic clearance (7).

MATERIALS AND METHODS

Chemicals. Omeprazole, 3HDZ (temazepam) and prazepam (internal standard) were gifts from Astra Pharmaceuticals Ltd (Mölndal, Sweden), Wyeth (Maidenhead, Berks, UK) and Warner & Co. (Pontypool, Gwent, UK) respectively. Diazepam and NDZ were purchased from Sigma (Poole, Dorset, UK). All other chemicals were obtained from either BDH (Lutterworth, Leics, UK) or Sigma (Poole, Dorset, UK).

Animals. Male Sprague-Dawley rats (250-300g), obtained from the Biological Sciences Unit, Medical School, University of Manchester, were housed and cannulated as described previously (8).

Preparation of rat liver microsomes and hepatocytes. The animals were sacrificed by cervical dislocation. Washed microsomal pellets were prepared by standard differential centrifugation techniques and resuspended in buffer (Sucrose 250mM, Trizma base 26mM, EDTA 5.4mM, pH 7.4) and stored at −80°C. Hepatocytes were prepared as described in a previous study (9). Cell viability was assessed by the Trypan Blue exclusion test and preparations in excess of 85% viability were used.

Microsomal diazepam incubation conditions. 0.1ml of KCl (1.15%), 0.4ml of microsomal solution (2mg of protein/ml in Trizma buffer 50mM; pH 7.4), 5μl of diazepam (in DMF) and 5μl of omeprazole (in DMF) or vehicle alone were preincubated at 37°C in a shaking water bath for 5 minutes. The reaction was started by adding 0.5ml of regenerating system (0.74mg NADP⁺, 1.94mg isocitric acid, 0.5units isocitric dehydrogenase and 10μmoles magnesium sulphate in Trizma buffer 50mM) and stopped after 10 minutes by the addition of 20μl of NaOH (10M).

Hepatocyte incubation conditions. 5μl of diazepam (in DMF) and 5μl of omeprazole (in DMF) or vehicle alone were added to 2.5ml of William’s Media E (pH 7.4) and incubated at 37°C in a shaking water bath for 5 minutes before starting the reaction with 0.5ml of hepatocytes (3 x 10⁶ cells/ml). The reaction was terminated after 10 minutes by freezing the mixture in liquid nitrogen. Samples were then thawed and 0.5ml of each sample was incubated with 0.5ml of β-glucuronidase (200 units/ml in sodium acetate 60mM; pH 4.5) in a shaking water bath at 37°C for one hour.

Analysis of diazepam metabolites. HPLC was used to assay diazepam and its metabolites simultaneously, according to the method of Reilly et al (10). Aliquots (0.5ml) of incubation mixture were extracted with ethylacetate (5ml) after adding the internal standard (prazepam, 100μl, 70μM in methanol) and carbonate buffer (1ml, 100mM; pH 10) by rotary mixing for 25 minutes and centrifuging at 2000rpm for 10 minutes. The organic layer was evaporated to dryness.
under nitrogen at 50°C. The residue was reconstituted in mobile phase and 100 µl injected via a Spectra Physics SP8780XR autosampler onto the HPLC system. The system comprised of a Hichrom Spherisorb S5 ODS2 250 × 5mm column, mobile phase of 65% methanol/35% water containing 0.02% triethylamine adjusted to pH 7.0 with phosphoric acid (delivered by a Waters 6000A pump at a flow rate of 1ml/min) and UV wavelength of 236nm (measured by an Applied Biosystems spectrflow 783). Diazepam and metabolite concentrations were determined by peak height ratio with respect to the internal standard (prazepam).

In vivo experiments. Initially, the basic pharmacokinetic parameters of omeprazole were assessed in a small number of preliminary experiments (n = 6). Omeprazole was administered in a mixture of propylene-2,5-diol and polyethylene glycol 400, 1:9 V/V, at doses of 15, 30 and 45mg/kg by a short intravenous (IV) infusion over 5 min via the jugular vein. Blood samples were taken over 90 min using the carotid artery. Convolution of a simple exponential disposition function with a zero order input function describes the plasma concentration-time data for both compounds [1] and this function was used in the nonlinear regression analysis (Simphar, Simed, Créteil, France).

\[
C = R_0 \sum_{i=1}^{n} \left( \frac{1 - e^{-\lambda t_i}}{-\lambda_i} \right) C_i e^{-\lambda t_i}
\]

where C is the concentration, \(\lambda\) is the rate constant, \(C' = C/DOSE\), \(t\) is the time, \(\theta = t\) for \(t \leq \tau\) or \(\theta = \tau\) for \(t > \tau\) (R_0 and \(\tau\) are infusion rate and duration respectively) and \(i\) refers to the particular exponential function.

Mean values of clearance (CL) and volume of distribution at steady state (Vss), calculated (8) from the preliminary experiments, were used to design loading doses (in the form of a short 5 minute infusion) and infusion rates (over 90 min) for omeprazole in order to achieve various steady state concentrations (Css, Eqs [2] and [3]).

\[
Loading\ Dose = Vss.Css
\]

\[
Infusion\ Rate = CL.Css
\]

A Sage infusion pump model 355 (A.H. Horwell Ltd., London, England) was used to deliver the infusion into the jugular vein at a flow rate of 0.15-1.1 ml/hour. Diazepam was administered in a mixture of propylene-2,5-diol, ethanol and 0.9% sodium chloride solution, 4:1:5 V/V/V, by a short infusion over 5 min at a dose of 5mg/kg into the hepatic portal vein via the lineal vein (IP) immediately after the omeprazole bolus and the start of the infusion. A range of omeprazole concentrations were achieved (10-50mg/L) and the clearance of diazepam assessed at each omeprazole concentration. Each experimental group also included a control infusion of the vehicle without omeprazole. Blood samples were collected over 90 minutes into Eppendorf tubes containing one or two drops of heparinised saline (5000 units/ml) from the carotid artery and plasma (200µl) separated for analysis as described above. Eq [1] was fitted to the data using the Simphar nonlinear regression programme.

Data analysis. Dixon plots were initially used to obtain initial estimates of the inhibitory constant (K_i). In all cases studied, the intersection of the lines lay above the x-axis (competitive inhibition). Furthermore, using nonlinear regression analysis, a model for competitive inhibition [4] was found to fit the data better than noncompetitive or uncompetitive models.

\[
V = \frac{S.V_{max}}{S + \frac{K_m}{1 + \frac{I}{K_i}}}
\]

where V is the observed rate of metabolite production (nmoles/min per mg of microsomal protein or per million cells), Vmax is the maximum velocity, Km is the substrate concentration at which the reaction is half of its maximal value, S is the substrate concentration and I is the inhibitor concentration.

Microsomal studies were carried out in two stages. First, detailed studies involving a range of substrate and of inhibitor concentrations were performed in one set of microsomes. Then, in subsequent experiments (with an additional 3 microsomal preparations) a single substrate concentration was used and Eq [5] employed to convert the ICx values (inhibitor concentration causing x% inhibition) to the corresponding K_i values.

\[
K_i = \frac{IC_x \left( \frac{100 - x}{x} \right)}{1 + \frac{S}{K_m}}
\]

For hepatocyte studies a range of substrate and inhibitor concentrations were used in 3 different rat preparations. In vivo data were analysed to estimate K_i by nonlinear regression (Simphar) using a simple inhibition model [6], described previously (8):

\[
CL = \frac{CL_0}{1 + \frac{I_o}{K_i}}
\]

where CL_0 is the clearance of the drug in the absence of the inhibitor and I_o is the steady state plasma concentration of the inhibitor.

RESULTS

In vitro studies. Omeprazole inhibition of 3HDZ formation in microsomes was concentration dependent (Figure 1). Using an inhibitor concentration range of 6-500µM at three different concentrations of diazepam (8, 16 and 31µM), IC50s were found to increase as diazepam concentrations were raised. Qualitatively similar behaviour was observed with the NDZ pathway. K_i values of 163 and 303µM were calculated by nonlinear regression from the 3HDZ and NDZ inhibition data respectively. Also, the Km/K_i ratio for the 3HDZ pathway inhibition was larger than 1 whilst this ratio was smaller than 1 for the NDZ pathway inhibition (Table 1).

In the hepatocyte studies, the effect of omeprazole on the formation of 3HDZ and NDZ was also concentration dependent, as illustrated in Figure 2 for the 3HDZ pathway in a typical preparation. Using an omeprazole concentration range of 2-100µM at three different diazepam concentrations