Fentanyl inhibits metabolism of midazolam: competitive inhibition of CYP3A4 in vitro

Y. Oda*, K. Mizutani, I. Hase, T. Nakamoto, N. Hamaoka and A. Asada

Department of Anesthesiology and Intensive Care Medicine, Osaka City University Medical School, 1–5–7 Asahimachi, Abeno-ku, Osaka 545-8585, Japan

*Corresponding author

Fentanyl decreases clearance of midazolam administered i.v., but the mechanism remains unclear. To elucidate this mechanism, we have investigated the effect of fentanyl on metabolism of midazolam using human hepatic microsomes and recombinant cytochrome P450 isoforms (n=6). Midazolam was metabolized to 1'-hydroxymidazolam (1'-OH MDZ) by human hepatic microsomes, with a Michaelis–Menten constant (Km) of 5.0 (so 2.7) μmol litre–1. Fentanyl competitively inhibited metabolism of midazolam in human hepatic microsomes, with an inhibition constant (Ki) of 26.8 (12.4) μmol litre–1. Of the seven representative human hepatic P450 isoforms, CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4, only CYP3A4 catalysed hydroxylation of midazolam, with a Km of 3.6 (0.8) μmol litre–1. Fentanyl competitively inhibited metabolism of midazolam to 1'-OH MDZ by CYP3A4, with a Ki of 24.2 (6.8) μmol litre–1, comparable with the Ki obtained in human hepatic microsomes. These findings indicate that fentanyl competitively inhibits metabolism of midazolam by CYP3A4.

Midazolam is a widely used i.v. anaesthetic. It is metabolized by hepatic microsomal cytochrome P4503A (CYP3A) and its clearance is dependent on CYP3A activity.1–4 Some drugs, such as antimycotics, macrolide antibiotics and calcium channel blocking agents reduce the clearance of midazolam by inhibiting CYP3A activity, resulting in prolonged drowsiness and inhibition of psychomotor performance.5–7 In clinical practice, midazolam is often used in combination with opioids, and clearance of midazolam is reduced by simultaneously administered fentanyl.8 As fentanyl is also metabolized by CYP3A,9 competitive inhibition of CYP3A by fentanyl may be responsible for the reduced clearance of midazolam. Although metabolism of midazolam has been studied extensively in vitro and in vivo,1–4 no studies have examined the effect of fentanyl on the metabolism of midazolam by cytochrome P450 (P450).

In this study, we have examined the direct inhibitory effects of fentanyl on metabolism of midazolam by CYP3A using human hepatic microsomes and recombinant human hepatic P450 isoforms.

Materials and methods
The study was approved by the Institutional Human Investigational Committee. Midazolam and its metabolites, 1'-hydroxymidazolam (1'-OH MDZ), 4'-hydroxymidazolam (4'-OH MDZ) and 1',4'-hydroxymidazolam (1',4'-OH MDZ) were gifts from Hoffmann-La Roche Ltd (Nutley, USA). Fentanyl was purchased from Sankyo Pharmaceuticals Co. Ltd (Tokyo, Japan). Sulphaphenazole was a gift from Meiji Yakuhin Co. Ltd (Tokyo, Japan). Troleandomycin, quinidine and diethyldithiocarbamate were obtained from Sigma Chemical Co. (St Louis, MO, USA). Human hepatic microsomes were obtained from the International Institute for the Advancement of Medicine (Scranton, USA). These microsomes were prepared from liver samples of kidney donors by differential centrifugation, and specific contents of P450 were 0.33–0.66 nmol mg protein–1. Recombinant human P450s, expressed in human lymphoblast cells with cytochrome P450 reductase, were obtained from Gentest (Woburn, USA). These P450s were supplied as microsomes. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from the Oriental Yeast Co. (Tokyo, Japan). A C18-column (TSK gel ODS-120T, 4.6×150 mm) was obtained from the Tosoh Corp. (Tokyo, Japan). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Assay of midazolam metabolism
Metabolism of midazolam was measured using the method reported previously, with small modifications.10 Briefly,
midazolam 0.2–10.0 µmol litre\(^{-1}\) was incubated at 37°C for 2 min with hepatic microsomes (100 µg of protein) and NADPH 0.4 mmol litre\(^{-1}\) in potassium phosphate buffer 0.1 mol litre\(^{-1}\) (pH 7.4) in the presence or absence of one of the chemical inhibitors of P450 in a final volume of 500 µl (n=6). When metabolism of midazolam was measured using the P450 isoform, CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 or 3A4, 30 pmol of the P450 was used instead of human hepatic microsomes, and the incubation time was 10 min. Concentration of NADPH was saturable and the formation rates of 1'-OH MDZ were linear up to 15 and 30 min for the substrate concentrations used with microsomes and P450s, respectively. Concentrations of the P450 chemical inhibitors, sulphaphenazole, quinidine, diethylthiocarbamate and troleandomycin were 1, 10, 100 and 100 µmol litre\(^{-1}\), respectively. Concentrations of these inhibitors were sufficient to suppress more than 80% of CYP2C, 2D, 2E and 3A activities in human hepatic microsomes. In our preliminary study, troleandomycin 100 µmol litre\(^{-1}\) also inhibited formation of 1'-OH MDZ by recombinant CYP3A4 by 95%. Concentrations of fentanyl were 0, 15, 30 and 45 µmol litre\(^{-1}\). The reaction was stopped by adding 50 µl of sodium hydroxide 1 mol litre\(^{-1}\) to the incubation mixture and placing it immediately on ice. Metabolites were extracted and measured by high-performance liquid chromatography (HPLC) as reported previously. The limit of detection of 1'-OH MDZ, 4'-OH MDZ and 1',4'-OH MDZ was 20 nmol litre\(^{-1}\). Intra- and inter-assay coefficients of variation were less than 4.8% and 7.0%, respectively.

Kinetic analysis of midazolam 1'-hydroxylation and inhibition by fentanyl

The maximum rate of metabolism (V\(_{\text{max}}\)) and the Michaelis–Menten constant (K\(_{\text{m}}\)) for the formation of 1'-OH MDZ from midazolam were determined by linear regression from Lineweaver–Burk double-reciprocal plots. When fentanyl was added to the reaction mixture, the inhibition pattern was determined by visual inspection of the Lineweaver–Burk double-reciprocal plots. When fentanyl was added to the reaction mixture, the apparent K\(_{\text{m}}\) for each concentration of fentanyl was estimated by linear regression analysis using unweighted raw data following a simple (one enzyme) Michaelis–Menten kinetic approach. The inhibition constant (K\(_{\text{i}}\)) values were determined by unweighted linear regression analysis using the following equation:\(^4\)

\[
\text{apparent } K_{\text{m}} = K_{\text{m}} + K_{\text{i}} I K_{\text{f}}^{-1}
\]

where K\(_{\text{m}}\) = affinity constant in the absence of inhibitor, apparent K\(_{\text{m}}\) = K\(_{\text{m}}\) in the presence of inhibitor, and I = concentration of inhibitor.

Statistical analysis was performed by one-way analysis of variance for comparison of the formation rates of 1'-OH MDZ in the absence or presence of P450 inhibitors. This was followed by Fisher’s protected least significant difference tests for comparison of the formation rates of 1'-OH MDZ in the presence of P450 inhibitors and the control values without inhibitors. Stat View II (Abacus, Berkeley, CA) was used for comparisons. All results are mean (SD), with P<0.05 considered significant.

Results

Metabolism of midazolam by human hepatic microsomes

When midazolam was incubated with human hepatic microsomes, 1'-OH MDZ was the only metabolite detected on the HPLC profiles. The total amount of residual midazolam and 1'-OH MDZ formed in the incubation mixture was approximately equal to the total amount of midazolam added to the reaction, suggesting that 1'-OH MDZ was the predominant midazolam metabolite. Of the P450 inhibitors studied, only troleandomycin significantly inhibited 1'-OH MDZ (P<0.01) while other P450 inhibitors were ineffective. Fentanyl 45 µmol litre\(^{-1}\) significantly inhibited the formation of 1'-OH MDZ (P<0.05) (Fig. 1). As troleandomycin is a selective CYP3A inhibitor, these findings suggest that CYP3A is predominantly responsible for 1'-OH MDZ, and that fentanyl is a potent CYP3A inhibitor.

When midazolam was incubated with human hepatic microsomes, single-phase straight lines were obtained from the Lineweaver–Burk plots for 1'-hydroxylation (Fig. 2A). K\(_{\text{m}}\) was 5.0 (SD 2.7) µmol litre\(^{-1}\) and V\(_{\text{max}}\) was 6.3 (2.1) mmol min\(^{-1}\) mg protein\(^{-1}\) (n=6). These values are comparable with those reported previously.\(^1\) When fentanyl was added to the reaction mixture, metabolism of midazolam was inhibited in a competitive manner, with a K\(_{\text{i}}\) of 26.8 (12.4) µmol litre\(^{-1}\) (n=6) (Fig. 2A). These

![Fig 1 Effect of sulphaphenazole (Sul.), quinidine (Qui.), diethylthiocarbamate (DDC), troleandomycin (TAO), and fentanyl 15, 30 and 45 µmol litre\(^{-1}\) (F 15, 30 and 45) on midazolam 1'-hydroxylation (1'-OH MDZ) by human hepatic microsomes. Control value is without inhibitors. Midazolam 10.0 µmol litre\(^{-1}\) was incubated at 37°C for 2 min with human hepatic microsomes (100 µg of protein) and reduced nicotinamide adenine dinucleotide phosphate 0.4 mmol litre\(^{-1}\) in potassium phosphate buffer 0.1 mol litre\(^{-1}\) (pH 7.4) in a final volume of 500 µl. Formation rate of 1'-hydroxymidazolam without inhibitors was mean 2.73 (SD 0.96) mmol min\(^{-1}\) mg protein\(^{-1}\) (n=6 experiments). *P<0.05, **P<0.01 vs control values without P450 inhibitors.](Image 321x607 to 542x738)
Midazolam 0.5–10.0 µmol litre⁻¹ was incubated at 37°C for 2 min with human hepatic microsomes (100 µg of protein) (A) or for 10 min with recombinant CYP3A4 30 pmol (B) and reduced nicotinamide adenine dinucleotide phosphate 0.4 mmol litre⁻¹ in potassium phosphate buffer 0.1 mol litre⁻¹ (pH 7.4) in the presence of fentanyl in a final volume of 500 µl. Concentrations of fentanyl were 0, 15 and 30 µmol litre⁻¹. Each plot is the average of six experiments. Inhibition constants were mean 26.8 (SD 12.4) and 24.2 (6.8) µmol litre⁻¹ (n=6 experiments) for human hepatic microsomes and recombinant CYP3A4, respectively.

findings suggest that a single P450 isoform in human hepatic microsomes was involved in the formation of 1'-OH MDZ, and that the metabolic activity of the P450 isoform was inhibited by fentanyl.

Metabolism of midazolam by recombinant human P450

Only CYP3A4 catalysed hydroxylation of midazolam to 1'-OH MDZ, suggesting that CYP3A4 is selectively involved in the metabolism in human hepatic microsomes. Formation rates of 1'-OH MDZ by CYP3A4 were 0.10 (0.01) and 2.57 (0.13) nmol min⁻¹ nmol P450⁻¹ with midazolam 0.2 and 10.0 µmol litre⁻¹, respectively (n=6). Inhibition of midazolam metabolism was therefore studied using recombinant CYP3A4.

Kₘ and Vmax for midazolam 1'-hydroxylation by recombinant CYP3A4 were 3.6 (0.8) µmol litre⁻¹ and 4.7 (0.9) nmol min⁻¹ nmol P450⁻¹, respectively (n=6). Kₘ value with CYP3A4 was comparable with that obtained with human hepatic microsomes. Formation of 1'-OH MDZ by CYP3A4 was competitively inhibited by fentanyl, with a Kᵢ of 24.2 (6.8) µmol litre⁻¹ (n=6) (Fig. 2A). This Kᵢ value was also comparable with that obtained in human hepatic microsomes, suggesting that CYP3A4 in human hepatic microsomes was competitively inhibited by fentanyl, and that this inhibition was therefore responsible for the decreased clearance of midazolam in vivo.

Discussion

There are a large number of studies on the metabolism of midazolam. However, only few have elucidated the midazolam metabolic activity of each P450 isoform. Our study, using human hepatic microsomes and recombinant P450 isoforms, showed that only CYP3A4 metabolized midazolam, and that other representative P450s in human hepatic microsomes did not.

CYP3A4 is the most abundant P450 isoform in human hepatic microsomes and is involved in the metabolism of a large number of agents, including fentanyl. As one drug may inhibit the metabolism of other drugs when they are metabolized by the same P450, metabolism of midazolam may be inhibited by drugs metabolized by CYP3A4. However, there have been no reports demonstrating direct inhibitory effects of fentanyl on metabolism of midazolam by CYP3A4. In this study, fentanyl inhibited metabolism of midazolam by CYP3A4, and the pattern of inhibition was competitive (Fig. 2), suggesting that fentanyl binds to the substrate binding site of CYP3A4. Alfentanil is also metabolized selectively by CYP3A4 and produces hypnotic and anaesthetic effects synergistically with midazolam. These anaesthetic interactions may also have resulted from decreased clearance of midazolam induced by inhibition of CYP3A4 activity by alfentanil, although pharmacokinetic interactions between midazolam and alfentanil have not been reported.

Multiple P450 isoforms have been detected in the CYP3A family in the liver. CYP3A5, 3A7 and CYP3A4 catalyse midazolam hydroxylation, although CYP3A5 and 3A7 were not tested in our study. CYP3A5 is found in only 20–30% of adult human livers, and CYP3A7 is found only in fetal liver, suggesting that CYP3A4 is predominantly responsible for metabolism of midazolam in human hepatic microsomes.

In addition to 1'-OH MDZ, 4'-OH MDZ and 1',4'-OH MDZ are formed by P450 from midazolam. However, 4'-OH MDZ and 1',4'-OH MDZ were not detected in our experiments, consistent with findings reported previously. This probably resulted from the higher Kₘ and lower Vmax values for 4'-hydroxylation than for 1'-hydroxylation of midazolam. In particular, the Kₘ for midazolam 4'-hydroxylation is more than 50 µmol litre⁻¹, approximately 10-fold higher than the Kₘ for 1'-hydroxylation. At the
concentrations of midazolam tested in this study, the formation rates of 4'-OH MDZ and 1'-4'-OH MDZ would have been too low to be detected. As CYP3A4 is involved in midazolam 4'-hydroxylation and 1'-hydroxylation, formation of 4'-OH MDZ should also be inhibited by fentanyl.

In this study, the \( K_i \) value for inhibition of midazolam 1'-hydroxylation by CYP3A4 was 24.2 (6.8) \( \mu \)mol litre\(^{-1}\). In contrast, plasma concentrations of fentanyl in clinical practice are usually lower than 1.0 \( \mu \)mol litre\(^{-1}\). Fentanyl is highly lipophilic, becomes highly concentrated in vascular rich tissues and concentrations of fentanyl in the liver are several fold higher than those in plasma.\(^{18} \)\(^{19} \) Therefore, the higher \( K_i \) values obtained in this study compared with concentrations of fentanyl in plasma do not rule out the occurrence of drug–drug interactions \textit{in vivo}.

In summary, we have demonstrated competitive inhibition of metabolism of midazolam by fentanyl \textit{in vitro}. Although reduction of hepatic blood flow induced by various conditions such as hypotension and mechanical ventilation may reduce clearance of midazolam in clinical practice, clearance is dependent predominantly on CYP3A4 activity in the liver,\(^4 \) and inhibition of CYP3A4 by fentanyl may be responsible for decreased clearance of midazolam when co-administered.\(^8 \) Interactions between midazolam and fentanyl produce prolonged hypoxaemia and apnoea.\(^20 \) An \textit{in vitro} study of interactions of anaesthetics using human hepatic microsomes would be useful for predicting possible interactions of anaesthetics in clinical practice.

Acknowledgements

Supported in part by the Fund for Medical Research from Osaka City University Medical Research Foundation and Grant-in-Aid for Research from the Ministry of Education, Science and Culture of Japan, No. 09771183.

References

5 Olkkola KT, Aho nen J, Neuvonen PJ. The effect of the systemic anticoagiants, itraconazole and fluconazole, on the pharmacokinetics and pharmacodynamics of intravenous and oral midazolam. Anesth Analg 1996; 82: 511–16
20 Bailey PL, Pace NL, Ashburn MA, Moll JWB, East KA, Stanley TH. Frequent hypoxemia and apnea after sedation with midazolam and fentanyl. Anesthesiology 1990; 73: 826–30