Inhibitory Effect of Azole Antifungal Agents on the Glucuronidation of Lorazepam Using Rabbit Liver Microsomes in Vitro

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Azole antifungal agents (azoles) have inhibitory effects on the cytochrome P450 system. However, the effect of azoles on the drug metabolism has not been given much attention. Lorazepam (LZP), a benzodiazepine sedative agent, is known to be metabolized by uridine 5’-diphosphate (UDP)-glucuronosyltransferase. Herein we report investigation of the effect of azoles on the enzyme-kinetics of glucuronidation of lorazepam using rabbit liver microsomes in vitro. The Km and Vmax for LZP glucuronidation were determined to be 0.26±0.08 mm and 1.25±0.21 nmol/min/mg protein, respectively, when evaluated in the presence of a detergent 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) (0.8 mg/mg protein). Azoles fluconazole, miconazole, and ketoconazole competitively inhibited the glucuronidation of LZP with Ki values of 7.17±4.78 mm, 0.17±0.08 mm, and 0.092±0.026 mm, respectively. These results are comparable to the previously reported Ki values of azoles with zidovudine (AZT) glucuronidation (1.4, 0.18, and 0.08 mm for fluconazole, miconazole, and ketoconazole, respectively) [Sampol et al., Br. J. Clin. Pharmacol., 40, 83—86, 1995]. Therefore, in order to avoid possible side effects of LZP, the concomitant administration of LZP and azoles should be carefully evaluated.

Key words: drug interaction; glucuronidation; competitive inhibition; rabbit liver microsome; azole antifungal agent; lorazepam

It is well known that azole antifungal agents (azoles) exhibit inhibitory effects on the cytochrome P450 enzymes, especially CYP3A4, that are involved in phase I metabolism. Furthermore, azoles increase the plasma concentrations of concomitantly administered drugs that are metabolized by the same enzymatic systems. However, the effects of azoles on conjugative (i.e. phase II) metabolism have not been given much attention. In fact, it has been reported that plasma concentrations of zidovudine (AZT), a compound mainly metabolized by glucuronidation, are increased when an azole, fluconazole, is coadministered to patients with AIDS.1 It has also been reported, using human liver microsomes in vitro, that miconazole had an inhibitory effect on the glucuronidation of AZT.2 In clinical situations, where azoles are to be coadministered with other drugs, it is generally recommended that the other drug be to be administered on a single occasion only to phase II, and not to phase I metabolism. For example, when a compound of the benzodiazepine sedative group is to be concomitantly administered with an azole, either lorazepam or lormetazepam, both of which are chiefly metabolized by uridine 5’-diphosphate (UDP)-glucuronosyltransferase (UDPGT), is usually selected in favor of compounds such as triazolam and midazolam, which are chiefly metabolized by CYP3A4. However, if azoles inhibit the glucuronidation of either lormetazepam or lorazepam, the combination of these drugs should be clinically recognized. Therefore, we investigated the effects of the azoles fluconazole, miconazole, and ketoconazole on the enzyme-kinetics of glucuronidation of lorazepam using rabbit liver microsomes in vitro.

MATERIALS AND METHODS

Animals Male Japanese White rabbits (3.0—3.5 kg) were purchased from Nippon Bio-Supply Center (Tokyo, Japan). They were housed in cages with controlled temperature and humidity and fed ad libitum (RC4; Oriental Koubo, Tokyo).

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Chemicals Lorazepam (LZP) was a gift from Japan Wyeth (Tokyo). Fluconazole (FCZ) and ketoconazole (KCZ) were supplied by Pfizer Inc. (New York, NY, U.S.A.) and Janssen-Kyowa Co. (Tokyo). Miconazole (MCZ) was used in the form of Florid-F (Mochida, Tokyo). Uridine di-phosphate glucuronic acid (UDPGA), 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate (CHAPS), and d-saccharic acid 1,4-lactone were purchased from SIGMA Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Liver Microsome Preparation Rabbit liver microsomes were prepared by the method of Kamataki and Kitagawa.3

Liver was homogenized in ice-cold 1.15% KCl using a glass teflon homogenizer and the homogenate was centrifuged at 9000×g for 20 min at 4°C. The supernatant was again homogenized and centrifuged at 105000×g for 60 min at 4°C. The resulting precipitate was reconstituted with 1.15% KCl and again centrifuged at 105000×g for 60 min. The obtained microsomal fractions were diluted with 0.3 M sucrose in 15 mM Tris–HCl buffer (pH 7.4) and stored at −80°C. Protein concentrations were determined by the method of Lowry et al.4

LZP Metabolism in Liver Microsomes Preliminary experiments showed that, among the three detergents used (Brij, CHAPS, and Tween 20) CHAPS at a concentration of 0.8 mg/mg protein gave the most efficient and stable reaction. Thus microsomal preparations were preincubated on ice with 0.8 mg/mg protein CHAPS at 4°C for 20 min. Reactions were then initiated by incubation with various concentrations of LZP (0.05—0.4 mm), with or without addition of FCZ (0.5—5 mm), or KCZ (12.5—100 μM) or MCZ (0.125—1 mm), at 37°C for 20 min, in the presence of 10 mM MgCl2, 2 mM d-saccharic acid 1,4-lactone, 4 mM UDPGA, CHAPS 0.8 mg/mg protein and 3 mg of rabbit liver microsomal protein. The total volume of the incubation mixture was 300 μl. Reactions were stopped by addition of 100 μl of acetone and
by cooling on ice. The resulting mixtures were centrifuged at 1600×g for 5 min, and the supernatants were stored at 4°C in preparation for assay by HPLC. Glucuronidation of LZP was assessed by measuring the decrease in the concentration of unchanged LZP.

**Calculation of $K_m$, $V_{max}$, and $K_i$** Initial velocities for LZP glucuronidation were calculated for concentrations of LZP in the range of 0.05—0.4 mM. Subsequently, the enzyme kinetics parameters ($K_m$ and $V_{max}$) were determined by the method of Lineweaver–Burk plot analysis. Values for $K_i$ were determined by the inhibition of LZP glucuronidation by FCZ, MCZ, and KCZ were determined by the method of Dixon.50

**Assay for LZP Concentration** Incubation of LZP in the microsomal fractions was performed in the presence or absence of UDPGA, a cofactor of UDPGT. LZP concentrations in the incubation mixtures were determined by a modification of the HPLC-UV method of Gunawan et al.71 Ten micrograms of diazepam, as an internal standard, 100 µl of 0.01 M borate buffer (pH 10), and 5 ml of an organic solvent mixture (dichloromethane–n-hexane=1:1) were added to 100 µl of sample. After mixing by shaking for 10 min at 20°C, the mixture was centrifuged for 10 min at 1680×g, and 4 ml of the resulting organic phase was transferred to another vial and dried under N2 at 40°C. The residue was then dissolved in 100 µl of the mobile phase and subjected to HPLC.

**RESULTS AND DISCUSSION**

**Effect of UDPGA and Detergents on LZP Metabolism** The generation of glucuronide derivatives of LZP was examined in the presence and absence of UDPGA. Unchanged LZP concentrations decreased in the presence, but not absence, of UDPGA, as assessed by analysis of the rate of LZP degradation ($-0.53$ vs $-0.00013$ nmol/min/mg protein, respectively) (Fig. 1). Moreover, post-incubation treatment of the microsomal preparations with β-glucuronidase reversed the observed decrease in unchanged LZP concentration. Finally, addition of NADPH, a cytochrome P450 cofactor, did not result in a change in LZP metabolism (data not shown). Taken together, it is reasonably judged that LZP was undergoing glucuronidation via UDPGT, but not oxidative metabolism via P450. In order to release UDPGT from the microsomal membranes, a variety of detergents (Brij, CHAPS and Tween 20) were tested in a pilot experiment. The most stable glucuronidative reaction was found to occur using CHAPS 0.8 mg/mg protein with levels of glucuronidation up to 5 times that of control (without detergents). Detergents are generally regarded as suitable for this type of experiment as they affect phospholipids within microsomal membranes, but do not have a substantial effect on enzyme activity.

**Enzyme-Kinetics of LZP** The $K_m$ and $V_{max}$ for LZP glucuronidation, determined as described in the Materials and Methods section, were found to be 0.26±0.08 mM and 1.25±0.21 nmol/min/mg protein, respectively (Fig. 2). $K_m$ values of 93.3 (human) and 130 µM (rabbit), and $V_{max}$ values of 6.2 (human) and 7.6 nmol/min/mg protein (rabbit) have been reported for berupipam glucuronidation using liver microsomes. In addition, $K_m$ and $V_{max}$ values of 45.7±32.6 µM, 2.71±1.6 nmol/min/mg protein, respectively, have been reported for odapipam glucuronidation, using rabbit liver microsomes. Thus, the $V_{max}$ of LZP glucuronidation reported is similar to that of other agents that are also substrates of UDPGT.

**Inhibitory Effect of Azoles on LZP Glucuronidation** Inhibition experiments using FCZ, MCZ, and KCZ (Fig. 3) indicate that these azoles competitively inhibit LZP glucuronidation. The $K_i$ values for FCZ, MCZ, and KCZ were 7.17±4.78, 0.17±0.08, and 0.092±0.026 mm, respectively. Therefore, Such in vitro experiments indicate the distinctiveness of a drug interaction between LZP and these azoles via inhibition of glucuronidation. It is known that azoles are not metabolized by glucuronidation but instead are exclusively metabolized by CYP3A4. However, since azoles appear to inhibit the glucuronidation of AZT and LZP in a competitive fashion, it has been suggested that azoles are non-metabolizable ligands for UDPGT.

The present study was conducted on rabbit liver microsomes in vitro; nevertheless, our current findings have clinical relevance for the following reasons. The order of the inhibitory effect ($K_i$ in mm) determined in this study was KCZ (0.092)>MCZ (0.17)>FCZ (7.17), consistent with the $K_i$ values of these azoles for AZT glucuronidation (i.e., KCZ 0.08 mm, MCZ 0.18 mm, and FCZ 1.4 mm), reported by Sampol et al.9 Since FCZ has been reported to increase plasma concentration of AZT in AIDS patients and $K_i$ values determined in this study for KCZ/MCZ inhibition of LZP glu-
curonidation were lower than those for FCZ inhibition of AZT glucuronidation, it seems likely that an in vivo drug interaction would occur between LZP and KCZ/MCZ, via glucuronidative metabolism. However, there has been no study to compare sequence homology or substrate specificity of UGT2B7, which is responsible for glucuronidative metabolism of LZP, between rabbits and human. Moreover, an isoform of UDPGT to be inhibited by azoles remains to be identified, and should be clarified in the near future. Furthermore, an in vitro-to-in vivo extrapolation of metabolic inhibition requires an accurate estimation of unbound inhibitor concentration close to microsomes in the liver, which is difficult to measure.

Adverse effects of LZP administration include drowsiness, reduced blood pressure, dizziness, and respiratory depression. It has been reported that lorazepam produces a significant impairment in the tasks of driving a car and in fact automobile accidents have happened because of the drug's administration. LZP is metabolized and excreted as LZP glucuronide in human to an extent of 70% of the dose. It would be possible for drug interactions between LZP and azoles to happen in human. To avoid potentially dangerous side effects of LZP due to increase in its concentration in plasma, attention should be given to the concomitant use of LZP and azoles.

In conclusion, we have demonstrated for the first time the competitive inhibition by azoles of LZP glucuronidation under an in vitro condition. An in vivo drug-interaction study using LZP and FCZ is now being undertaken in our laboratory using rabbits, to examine in vivo vs. in vitro relationship of the drug interaction in the same animal species.

REFERENCES