Effects of Ketoconazole on Triazolam Pharmacokinetics, Pharmacodynamics and Benzodiazepine Receptor Binding in Mice¹

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ABSTRACT

We previously demonstrated that ketoconazole is a potent inhibitor of triazolam biotransformation in vitro and in vivo. Despite significant elevations in triazolam plasma levels with coadministration of ketoconazole, the pharmacodynamic enhancement was lower than predicted based on plasma levels of triazolam. The present study examines the effects of ketoconazole on benzodiazepine receptor binding in vitro as well as on open-field behavior in male CD-1 mice. Triazolam alone inhibited [3H]flunitrazepam binding with an IC₅₀ value of 0.85 nM and a Ki value of 0.50 nM. Ketoconazole alone also competitively antagonized [3H]flunitrazepam binding in a concentration-dependent manner with an IC₅₀ value of 1.56 μM and a Ki value of 1.17 μM. In the presence of 1, 3 or 9 μM ketoconazole, the IC₅₀ value of triazolam was increased to 1.11, 1.58 and 5.73 nM, respectively, whereas maximal binding was reduced by 36%, 69% and 89%. Coadministration of 50 mg/kg ketoconazole and triazolam (0.1–0.3 mg/kg) to intact animals significantly elevated plasma and brain triazolam levels. Ketoconazole could be measured in mouse brain at levels averaging 31% of those in plasma. Ketoconazole alone had minimal or no effect on open field activity, but it significantly potentiated the decreased activity seen with triazolam administration. The ability of ketoconazole to inhibit triazolam displacement of [3H]flunitrazepam binding may explain the muted pharmacodynamic effect of this benzodiazepine in the presence of ketoconazole. Based on these results, it is likely that ketoconazole acts as a neutral ligand at the benzodiazepine receptor.

Ketoconazole is an imidazole-piperazine compound that is effective against a wide range of fungal pathogens (Como and Dismukes, 1994; Kauffman and Carver, 1997). This commonly prescribed antifungal is a potent inhibitor of cytochrome P450–3A activity, a property that affords the potential for clinically relevant interactions with the many substrates of this enzyme. As expected, in addition to its primary use, ketoconazole significantly decreases the in vivo clearance of cyclosporine (Gomez et al., 1995), terfenadine (Honig et al., 1993), benzodiazepines (Olkkola et al., 1994, von Moltke et al., 1996; Wright et al., 1997) and several other compounds that are biotransformed by the P450–3A isoforms.

Triazolam, a short-acting triazolobenzodiazepine, is metabolized by oxidation to α-hydroxytriazolam and 4-hydroxy-triazolam in the liver and in the gastrointestinal tract by cytochrome P450–3A (Kronbach et al., 1989). von Moltke et al. (1996) have shown that ketoconazole is a highly potent inhibitor of both hydroxylation reactions in vitro and in vivo. In addition, Varhe et al. (1994) have shown that both ketoconazole and itraconazole increased the AUC of triazolam >20-fold and prolonged the elimination half-life 7-fold.

In a recent study, we found that ketoconazole was a highly potent inhibitor of triazolam biotransformation in vitro compared with other potential inhibitors of P450–3A isoforms (von Moltke et al., 1996). In this same study, the coadministration of ketoconazole and triazolam in human volunteers significantly potentiated triazolam-induced deficits in digit-symbol substitution and word recall test performance and increased electroencephalographic beta activity. However, examination of pharmacokinetic and pharmacodynamic data revealed that the degree of impairment produced by triazolam in the presence of ketoconazole was less than would be predicted based on the plasma levels of triazolam alone. The present study was undertaken to determine whether the

ABBREVIATIONS: Bₘₐₓ, receptor density; PEG 400, polyethylene glycol 400; Vₘₐₓ, maximum reaction velocity in vitro; HPLC, high-performance liquid chromatography; Sₘₐₓ, substrate concentration corresponding to a reaction velocity of 50% Vₘₐₓ; IC₅₀, inhibitor concentration reducing reaction velocity or receptor binding to 50% of control.

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blunted effect of triazolam in the presence of ketoconazole could be due to an interaction of ketoconazole at the γ-aminobutyric acid$_A$ receptor benzodiazepine site. To determine this, we studied in vitro benzodiazepine receptor binding as well as open-field activity in an experimental model.

**Methods**

**Materials.** Male CD-1 mice, 6 to 8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA), maintained on a 12-h light/dark cycle and given food and water ad libitum. [3H]Flunitrazepam (specific activity, 71 Ci/mmol) and [3H]flumazenil (specific activity, 81 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Triazolam and its metabolites, ketoconazole, paroxetine, buspirone and itraconazole were generously donated by their respective pharmaceutical manufacturers. All other reagents were obtained from standard commercial sources.

**Drug administration.** Triazolam and ketoconazole were dissolved in PEG 400/saline (1:1) or PEG 400, respectively, and administered intraperitoneally. Vehicle-treated mice received either PEG 400/ saline (1:1) or PEG 400 alone depending on the experimental protocol.

**Open-field activity.** Activity for all groups, including distance traveled, rears and stereotypy, was assessed in 10-min intervals for 50 min in an Omnitech Digiscan apparatus (Columbus, OH). Ketoconazole (50 mg/kg) or vehicle was administered intraperitoneally 60 min before triazolam (0.05, 0.1 or 0.2 mg/kg) or vehicle. Behavioral testing began 10 min after the triazolam injection. Between each run, the interior of the activity chamber was cleaned with 70% ethanol and dried. All testing occurred between 9:00 a.m. and 12:00 p.m. Averages of the log parameter + 1) for the total 50-min test period were computed. Log transformation of data was used because the data were not normally distributed.

**[3H]Flunitrazepam binding.** Benzodiazepine binding in vitro was performed in mouse cortical synaptosomes (P$_2$) as previously described using [3H]flunitrazepam (Miller et al., 1988). Briefly, samples (~50 μg of protein) were incubated with [3H]flunitrazepam (0.03–30 μM) in the presence (total binding) or absence (nonspecific binding) of flumazenil (10 μM) for 60 min at 4°C. Incubations were terminated by filtration and filters were subsequently washed and counted.

**Triazolam concentrations.** Animals were injected intraperitoneally with ketoconazole (50 mg/kg) or vehicle (PEG 400) 60 min before triazolam (0.3 mg/kg). Animals were killed 30 min after the second injection. Cortical and liver tissues were weighed and homogenized in 1 ml of distilled water with a Polytron (Brinkmann, Lucerne, Switzerland) on setting 7 for 10 to 15 sec. Trunk blood samples were allowed to clot, and the serum was separated. Triazolam concentrations were determined by gas chromatography with electron capture detection as previously described (von Moltke et al., 1996).

**Ketoconazole concentrations.** Animals received 100 mg/kg ketoconazole 45 min before death (n = 12). Cortical and liver tissues were weighed and homogenized in 1 ml of distilled water with a Polytron (Brinkmann) on setting 7 for 10 to 15 sec. Trunk blood samples were allowed to clot, and the serum was separated. Ketoconazole concentrations were determined by HPLC as previously described (von Moltke et al., 1996).

**Triazolam biotransformation in vitro.** Microsomes were prepared from livers obtained from male CD-1 mice as described previously (von Moltke et al., 1994, 1993). Varying concentrations of triazolam (0–1500 μM) were incubated with microsomes, cofactors and an NADPH-regenerating system (von Moltke et al., 1996). Reactions were stopped after 20 min, and concentrations of α-OH- and 4-OH-triazolam were determined by HPLC. Rates of formation of the two metabolites were expressed in units of nmol of product formed/min/mg of protein. In a second study, a fixed concentration of triazolam (250 μM) was incubated with ketoconazole in concentrations ranging from 0 to 2.5 μM.

**Data analysis.** Data from binding studies were analyzed using the RADLIG program (version 4.0). Enzyme kinetic parameters were determined by nonlinear regression analysis of untransformed data (von Moltke et al., 1993, 1994, 1996). Comparisons among groups were performed using analysis of variance with Student-Newman-Keuls post-hoc test or Dunnett’s post-hoc test.

**Results**

**In vitro receptor binding.** Flunitrazepam binding to mouse cortical synaptosome labeled receptors was best described by a single site with a $K_d$ value of 2.24 nM and a $B_{max}$ value of 3.51 pmol/mg protein (fig. 1). Binding of 5 nM [3H]flunitrazepam was displaced by triazolam (0.05–100 nM) in a dose-dependent manner with a mean IC$_{50}$ value of 0.85 ± 0.08 nM and a mean $K_t$ value of 0.50 ± 0.04 nM. With the addition of ketoconazole (1, 3 or 9 μM), the mean IC$_{50}$ value of triazolam was increased to 1.11, 1.58 and 5.73 nM, respectively, whereas mean [3H]flunitrazepam maximal specific binding was decreased by 42%, 69% and 89% (fig. 2a). The addition of ketoconazole shifted the $K_t$ value of triazolam slightly to the right. In a separate series of experiments, it was determined that the reduction of [3H]flunitrazepam binding by ketoconazole was most likely due to a competitive interaction of this ligand for the benzodiazepine site labeled by flunitrazepam (fig. 2b). Ketoconazole competitively displaced [3H]flunitrazepam in a concentration-dependent manner. Ketoconazole concentrations ranged from 0.01 to 500 μM with a mean IC$_{50}$ value of 1.6 ± 0.1 μM and a mean $K_t$ value of 1.17 ± 0.08 μM.

To determine whether this competitive interaction of ketoconazole and triazolam was unique, various compounds covering several psychotropic drug classes were incubated with

![Fig. 1. Representative saturation plot of [3H]flunitrazepam binding to cortical benzodiazepine receptors. Inset, Scatchard plot of the same data set. [3H]flunitrazepam (0.03–30 nM) was added to thoroughly washed cortical synaptosomes and incubated in the presence (total binding) or absence (nonspecific binding) of 10 μM flumazenil for 60 min at 4°C in 50 mM Tris buffer (pH 7.4). Each concentration was assayed in triplicate. Analysis of ligand binding was performed using the RADLIG program (version 4.0). Nonlinear curves fit to each data set were computed to determine both apparent affinity ($K_d$) and receptor density ($B_{max}$). The average $K_d$ value was 2.24 ± 0.32 nM (n = 5), which was used in later analyses to determine the $K_t$ value for both triazolam and ketoconazole (see fig. 2).](image-url)
5 nM [3H]flunitrazepam (table 1). Concentrations used (1, 3 and 9 μM) greatly exceed the clinically relevant range. Data from triazolam and ketoconazole are included for comparison. Ketoconazole significantly displaced [3H]flunitrazepam in a dose-dependent manner with a mean IC50 value of 0.85 ± 0.08 nM and a mean Ki value of 0.50 ± 0.04 nM. Ketoconazole at 1, 3 and 9 μM inhibited the displacement of [3H]flunitrazepam by triazolam and slightly shifted the Ki value of triazolam to the right.

Open-field activity. Triazolam significantly decreased (P < .05) all three open-field parameters measured (distance traveled, number of rears and number of stereotypies) in a dose-dependent manner (fig. 3). Ketoconazole alone (50 mg/kg) had no effect on the average distance traveled or the average number of stereotypies but did significantly decrease the average number of rears (P < .05). Coadministration of ketoconazole (50 mg/kg) and triazolam (0.1 mg/kg) significantly decreased all three open-field parameters measured (P < .05). The combination of drugs yielded significantly lower activity than either drug alone (P < .05).

Triazolam concentrations. Triazolam concentrations in both brain and serum were significantly increased (P < .05) in animals receiving 50 mg/kg ketoconazole (table 2). Ratios...
of brain to serum concentrations or liver to serum concentrations were unchanged with ketoconazole administration.

**Ketoconazole concentrations.** Average ketoconazole concentrations for serum, liver and brain were 80.9 μg/ml, 137.1 μg/g and 25.4 μg/g, respectively. The mean brain/serum ratio was 0.31, and the mean liver/serum ratio was 1.88.

**Triazolam biotransformation in vitro.** Both α-OH- and 4-OH-triazolam were formed by mouse liver microsomes (fig. 4). Data points consisting of reaction velocity vs. substrate concentrations were best described by a modification of the Michaelis-Menten equation in which a sigmoidal shape is incorporated by inclusion of an exponent (von Moltke et al., 1993, 1994, 1996). The parameters for α-OH-triazolam formation were $V_{max} = 5.39$ nmol/min/mg protein and $S_{0.5} = 34$ μM. The parameters for 4-OH-triazolam formation were $V_{max} = 14.1$ nmol/min/mg protein and $S_{0.5} = 154$ μM. Comparing the $V_{max}/S_{0.5}$ ratio (intrinsic clearance) for the two pathways indicates that 63% of net clearance was accounted for by α-OH-triazolam formation and 37% by 4-OH-triazolam formation. However, it should be emphasized that the $V_{max}/S_{0.5}$ ratio represents only an approximate estimate of intrinsic clearance when the kinetic profile is modified by an exponent, as is the case with many substrates of P450-3A (Schmider et al., 1996).

Ketoconazole was a potent inhibitor of the formation of both metabolites of triazolam, with IC$_{50}$ values of 0.039 and 0.038 μM, respectively (fig. 5).

**Discussion**

Previously, we found that ketoconazole competitively inhibited the biotransformation of triazolam in vitro (von Moltke et al., 1996). Enhanced pharmacodynamic effects of triazolam with ketoconazole coadministration in the same study indicate that the pharmacokinetic interaction of these two compounds is likely of clinical importance, as had been reported previously (Varhe et al., 1994). However, the pharmacodynamic enhancement demonstrated by von Moltke et al. (1996) was less than would be predicted based on the increased plasma levels alone. The relationship of plasma triazolam concentration to clinical response was consistent with a reduced sensitivity to triazolam with ketoconazole coadministration. The present data demonstrate that ketoconazole inhibits triazolam displacement of flunitrazepam binding in a concentration-dependent manner in vitro. This modulation of triazolam binding is most likely due to competition of this ligand for the benzodiazepine site. Based on its minimal benzodiazepine agonist activity, however, it is probable that ketoconazole is acting as a neutral ligand or a very minimal benzodiazepine agonist activity, however, it is probable that ketoconazole is acting as a neutral ligand or a very minimal benzodiazepine agonist activity, however, it is probable that ketoconazole is acting as a neutral ligand or a very
Dunnett’s formation in the presence of 0 to 100 of product formed/min/mg protein. Insert, enlarged view of metabolite Rates of formation of the two metabolites are expressed in units of nmol consistent with a Michaelis-Menten equation modified by an exponent. zolam were determined by HPLC. Lines represent functions of best fit of itraconazole, buspirone, dizocilpine or paroxetine did not was not concentration-dependent. The same concentrations was a close structural analog, had only a small effect that appears to be a specific property of ketoconazole, since itraconazole, a close structural analog, had only a small effect that was not concentration-dependent. The same concentrations of itraconazole, buspironine, dizocilpine or paroxetine did not result in a similar percent receptor occupancy (>90%) as ketoconazole. Open-field behavior was evaluated as a pharmacodynamic parameter due to its noninvasive nature, simplicity of mea-

TABLE 2
Effect of ketoconazole on triazolam concentrations
Ketoconazole (50 mg/kg) or vehicle (PEG 400) was administered 60 min before triazolam (0.3 mg/kg). Animals were killed 30 min after the second injection. Triazolam concentrations were determined with gas chromatography with electron capture detection. Data represent the mean ± S.E.M. (n = 7 for each treatment group).

<table>
<thead>
<tr>
<th>Triazolam concentrations</th>
<th>Triazolam + vehicle</th>
<th>Triazolam + ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (ng/g)</td>
<td>70.1 ± 8.5</td>
<td>195.3 ± 35.9b</td>
</tr>
<tr>
<td>Liver (ng/g)</td>
<td>149.7 ± 26.9a</td>
<td>352.0 ± 59.3b</td>
</tr>
<tr>
<td>Serum (ng/ml)</td>
<td>33.3 ± 4.0</td>
<td>114.9 ± 13.1b</td>
</tr>
<tr>
<td>Brain/serum ratio</td>
<td>2.1 ± 0.1</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Liver/serum ratio</td>
<td>4.5 ± 2.9a</td>
<td>2.9 ± 0.4</td>
</tr>
</tbody>
</table>

a Mean calculated after elimination of outlying data point (726.2 ng/g).
b p < .05, significant differences from vehicle as determined by ANOVA and Dunnett’s post hoc test.

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**Fig. 4.** Triazolam biotransformation in mouse liver microsomes in vitro. Varying concentrations of triazolam (0–1500 μM) were incubated with microsomes, cofactors and an NADPH-regenerating system. Reactions were stopped after 20 min and concentrations of α-OH- and 4-OH-triazolam were determined by HPLC. Lines represent functions of best fit consistent with a Michaelis-Menten equation modified by an exponent. Rates of formation of the two metabolites are expressed as a ratio (in percent) relative to the velocity with no ketoconazole present. Ketoconazole inhibited the formation of both metabolites with IC50 values of 0.039 and 0.038 μM, respectively. Measurement and availability of data regarding acute effects of triazolam (Lopez et al., 1988). Horizontal activity, rears and stereotypy have been previously shown to be the most sensitive and reliable measures following benzodiazepine administration. All three parameters were significantly decreased in triazolam-treated animals. Coadministration of ketoconazole and triazolam further decreased all three open-field parameters measured. This potentiated pharmacodynamic effect is similar to that demonstrated in clinical studies by von Moltke et al. (1996).

Pharmacokinetic studies were consistent with pharmacodynamic data. The coadministration of ketoconazole and triazolam significantly increased triazolam concentrations in brain, liver and serum relative to the administration of triazolam alone. This is consistent with the decrease in clearance demonstrated previously in healthy human volunteers (von Moltke et al., 1996). There was no significant difference in the brain/serum and liver/serum ratios for triazolam in the two treatment groups.

**Fig. 5.** Effect of ketoconazole on triazolam metabolite formation in mouse liver microsomes in vitro. A fixed concentration of triazolam (250 μM) was incubated with ketoconazole in concentrations ranging from 0 to 2.5 μM. Reactions were stopped after 20 min, and concentrations of α-OH- and 4-OH-triazolam were determined by HPLC. Reaction velocities were expressed as a ratio (in percent) relative to the velocity with no ketoconazole present. Ketoconazole inhibited the formation of both metabolites with IC50 values of 0.039 and 0.038 μM, respectively.

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**Fig. 5.** Effect of ketoconazole on triazolam concentrations and metabolite formation in mouse liver microsomes in vitro. A fixed concentration of triazolam (250 μM) was incubated with ketoconazole in concentrations ranging from 0 to 2.5 μM. Reactions were stopped after 20 min, and concentrations of α-OH- and 4-OH-triazolam were determined by HPLC. Reaction velocities were expressed as a ratio (in percent) relative to the velocity with no ketoconazole present. Ketoconazole inhibited the formation of both metabolites with IC50 values of 0.039 and 0.038 μM, respectively.
that brain/plasma ratios as high as 0.3 would be reached in restricted, at least in part, by plasma protein binding. Be-}


dsomes. This inhibition of metabolite formation explains the increased triazolam concentrations found after coadministration with ketoconazole.

Kinetic studies of ketoconazole clearly indicated that this compound is present in brain tissue after systemic administration, with a mean brain/serum ratio of 0.31. Although ketoconazole is highly lipophilic, brain uptake is probably restricted, at least in part, by plasma protein binding. Because ketoconazole is extensively bound to plasma components in humans (Como and Dismukes, 1994), it is unlikely that brain/plasma ratios as high as 0.3 would be reached in humans. Nevertheless, the ketoconazole Kᵢ value for benzo- diazepine receptor binding averaged 1.2 μM. The usual therapeutic plasma concentration range for ketoconazole is 2 to 10 μM (Como and Dismukes, 1994). Therefore, it is possible that ketoconazole concentrations in human brain may reach sufficient levels to impair receptor binding of benzodiazepine agonists. This may explain why potentiation of benzodia- zepine agonist effects of triazolam with coadministration of ketoconazole was less than expected based on the magnitude of increase in triazolam plasma levels (von Moltke et al., 1996).

The ability of a nonbenzodiazepine compound to displace benzodiazepines from their binding sites is not novel. It has long been known that the triazolopyridazines and the β-carbo- bile-3-carboxylic acid esters, among others, are recognized by the benzodiazepine receptor (Nielson and Braestrup, 1980; Squires et al., 1979). Ketoconazole is unique, however, in that it appears to be the only compound within its class to exhibit this ability. Additional studies should further character- ize the clinical implications of benzodiazepine receptor occupancy by ketoconazole.

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References


