CYTOCHROME P450 3A4 IS THE MAJOR ENZYME INVOLVED IN THE METABOLISM OF THE SUBSTANCE P RECEPTOR ANTAGONIST APREPIPIANT

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ABSTRACT:
The contribution of human cytochrome P450 (P450) isoforms to the metabolism of aprepitant in humans was investigated using recombinant P450s and inhibition studies. In addition, aprepitant was evaluated as an inhibitor of human P450s. Metabolism of aprepitant by microsomes prepared from baculovirus-expressed human P450s was observed only when CYP1A2, CYP2C19, or CYP3A4 was present in the expression system. Incubation with CYP1A2 and CYP2C19 yielded only products of O-dealkylation, whereas CYP3A4 catalyzed both N- and O-dealkylation reactions. The metabolism of aprepitant by human liver microsomes was inhibited completely by ketoconazole or troleandomycin. No inhibition was observed with other P450 isomere-selective inhibitors. Aprepitant was evaluated also as a P450 inhibitor in human liver microsomes. No significant inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2D6, and CYP2E1 was measured in experiments with isomere-specific substrate (IC50 > 70 μM). Aprepitant was a moderate inhibitor of CYP3A4, with Ki values of ~10 μM for the 1- and 4-hydroxylation of midazolam, and the N-demethylation of diltiazem, respectively. Aprepitant was a very weak inhibitor of CYP2C9 and CYP2C19, with Ki values of 108 and 66 μM for the 7-hydroxylation of warfarin and the 4′-hydroxylation of S-mephentoin, respectively. Collectively, these results indicated that aprepitant is both a substrate and a moderate inhibitor of CYP3A4.

Aprepitant (5-[[2R,3S]-2-[[1R]-1-[3,5-bis(trifluoromethyl)phenyl]ethyl]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one; MK-0869; EMEND) is a potent and selective neurokinin 1 receptor antagonist, effective in the treatment of chemotherapy-induced nausea and vomiting (CINV) (Navari et al., 1999; Campos et al., 2001). A dosing regimen of aprepitant consists of a combination therapy with a 5-hydroxytryptamine 3 receptor antagonist, such as ondansetron, and a corticosteroid (e.g., dexamethasone, methylprednisolone) (Roila et al., 1998; Gralla et al., 1999). Thus, the use of this novel drug presents a potential for interactions with chemotherapeutic agents as well as adjuvant therapies.

In drug-drug interaction studies, it was observed that coadministration with aprepitant resulted in increases in the area under the plasma concentration versus time curve (AUC) for dexamethasone and methylprednisolone (McCrea et al., 2003). The standard dexamethasone regimen (20 mg on day 1 and 12 mg on days 2–5) was given concomitantly with aprepitant, dexamethasone AUC0–24 h increased ~2-fold on both day 1 and day 5 compared with the standard dexamethasone regimen alone. When a modified dexamethasone regimen (12 mg on day 1 and 4 mg on days 2 and 5) was given concomitantly with aprepitant, the dexamethasone AUC0–24 h also increased, making it similar to that of the standard regimen alone. When 125 mg of methylprednisolone i.v. on day 1 and 40 mg p.o. on days 2 and 3 was given with aprepitant, the AUC of methylprednisolone increased approximately 30% on day 1 (after i.v. administration) and 2-fold on day 3 (after oral administration). Aprepitant had no effect on either ondansetron (i.v.) or granisetron (p.o.) pharmacokinetics (Blum et al., 2003). Dexamethasone, methylprednisolone, and granisetron are metabolized by CYP3A4 (Glynn et al., 1986; Bloomer et al., 1994; Gentile et al., 1996; Varis et al., 1998), whereas ondansetron is metabolized by multiple P450 isoforms, including CYP1A2, CYP2B6, and CYP3A4 (Fischer et al., 1994; Dixon et al., 1995).

Furthermore, clinical drug interaction studies indicated that administration of aprepitant at two different dosing regimens for 5 days altered the pharmacokinetics of the CYP3A4 probe substrate midazolam, when administered on days 1 and 5 of aprepitant therapy. The pharmacokinetic changes included 2.3- and 1.5-fold increases in midazolam AUC and maximum observed plasma concentration, respectively (Majumdar et al., 2003). Collectively, these results suggested that aprepitant is a moderate inhibitor of CYP3A4.

Other clinical drug interaction studies conducted with aprepitant suggest that it is not an inhibitor of CYP2C9 or CYP2D6 [EMEND (aprepitant) capsules product information; Merck & Co. Inc., White House Station NJ, 2003]. Moreover, when a single 125-mg dose of aprepitant was administered on day 5 of a 10-day regimen of 600 mg/day ketoconazole, a strong inhibitor of CYP3A4, the AUC of aprepitant increased approximately 5-fold [EMEND (aprepitant) capsules product information; Merck & Co. Inc., White House Station NJ, 2003], suggesting that aprepitant is a substrate of CYP3A4.

ABBREVIATIONS: CINV, chemotherapy-induced nausea and vomiting; AUC, area under the plasma concentration versus time curve; HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; P450, cytochrome P450; TAO, trroleandomycin; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TFA, trifluoroacetic acid; MRM, multiple reaction monitoring.
The metabolic pathways of aprepitant have been established in vivo (Huskey et al., 2004). Briefly, two oxidative pathways have been identified, namely, N- and O-dealkylation, and incubation of aprepitant in human liver microsomes allowed the identification of the major P450 isoforms involved in the catalysis of several intermediate reactions in these pathways. The experiments presented in this paper were aimed at the identification of the major P450 isoforms involved in the metabolism of aprepitant in human liver and the evaluation of this drug as a potential P450 inhibitor.

Materials and Methods

Chemicals. All chemicals were of the highest analytical purity available. Bufuralol, 1'-hydroxybufuralol, furafylline, R,S- and S-warfarin, 7-hydroxywarfarin, S-mephenytoin, 4-hydroxy-mephenytoin, and 6-hydroxychlorzoxazone were purchased from BD Gentest (Woburn, MA). Aprepitant, M-1, M-2, M-3, and M-4 were synthesized by Merck Basic Chemistry (Rahway, NJ). [Morpholine-2-14C]aprepitant (specific activity 29.8 μCi/mg) and [4-fluorophenyl-3-1H]aprepitant (specific activity 11.62 μCi/mg) were prepared by the Merck Labeled Compound Synthesis Group (Rahway, NJ). Midazolam malate, 1'-hydroxymidazolam, 4-hydroxymidazolam, and desmethyl-diltiazem were provided by the Merck Labeled Compound Synthesis Group. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Liver Microsomes from Humans. Microsomal fractions were prepared from frozen livers from human subjects (International Institute for the Advancement of Medicine, Exton, PA) according to a published procedure (Huskey et al., 1995). Protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s recommendations. The P450 content was determined as described previously (Omura and Sato, 1964).

Incubation Conditions. All incubations contained 100 mM phosphate buffer, pH 7.4 and an NADPH-regenerating system consisting of 5 mM glucose 6-phosphate, 1 mM NADP, and 0.7 μM glucose-6-phosphate dehydrogenase. All reactions were initiated by addition of NADP. Substrates and inhibitors were prepared in methanol or acetonitrile so that each reaction contained <2% solvent.

Incubations with Recombinant P450s. The reaction mixtures (200 μL) contained 10 μM [3H]aprepitant, and microsomes prepared from baculovirus-infected SF-21 cells expressing recombinant P450 isoforms (20 or 30 pmol; CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4). The reactions proceeded for 30 min at 37°C and were terminated by the addition of 200 μL of methanol. The resulting suspensions were centrifuged and aliquots (200 μL) of the supernatants were analyzed by HPLC as described under “HPLC Methods.” Identification of reaction products was based on similarity of retention time and MS/MS fragmentation pattern to those of synthetic standards as described elsewhere (Huskey et al., 2004).

Enzyme Kinetics Studies. Varying concentrations of [3H]aprepitant (1–40 μM) were incubated with a pool of liver microsomes from three human subjects. Reactions proceeded for 20 min in the presence of 1 mg/ml microsomal protein and were quenched with 4 volumes of acetonitrile. After centrifugation, the supernatants were transferred to clean tubes and evaporated to dryness under N2. The residues were reconstituted in 200 μL of 1:1 methanol/water and aliquots of 100 μL were analyzed by HPLC as described under “HPLC Methods.” Rates of metabolism were calculated from the peak areas of aprepitant as % of total radioactivity in the radiochromatograms.

Inhibition of aprepitant metabolism. The reaction mixtures (500 μL) containing [3H]aprepitant (10 μM) and human liver microsomes (3 different preparations, 1 mg/ml) were incubated for 30 min in the presence of furafylline (25 μM), sulfaphenazole (1 μM), quinidine (1 μM), 4-methylpyrazole (100 μM), ketoconazole (1 μM) or troleandomycin (TAO, 25 μM). The reactions were terminated and processed as described above for HPLC analysis (see “HPLC Methods”). The extent of inhibition was determined from a comparison of turnover in the presence of an inhibitor to a control reaction run simultaneously with solvent vehicle only.

Determination of the IC50 for the Inhibition of P450-Specific Reactions by Aprepitant. Midazolam. The reaction mixtures (250 μL) containing midazolam (2–250 μM), aprepitant (3–48 μM), and pooled human liver microsomes (n = 10, 0.5 mg/ml) were incubated at 37°C for 5 min. The reactions were quenched by addition of 4 volumes of acetonitrile and 10 μL of a 250 mM solution of the internal standard. After centrifugation, aliquots (20 μL) of the supernatant were analyzed by LC/MS/MS (see Table 2). Concentrations of the metabolites were determined from standard curves constructed over the range of 0.1 to 10 μM for 1'-hydroxymidazolam and 0.05 to 10 μM for 4-hydroxymidazolam.

Diltiazem. Reaction mixtures (250 μL) containing diltiazem (5–200 μM), aprepitant (3–48 μM), and pooled human liver microsomes (n = 10, 1 mg/ml) were incubated at 37°C for 10 min. Quenching and addition of internal standard was done as described for midazolam. After centrifugation, aliquots (20 μL) of the supernatant were analyzed by LC/MS/MS (see Table 2). N-Desmethyldiltiazem was quantified from standard curves constructed over the range of 0.5 to 30 μM.

R,S-Warfarin. R,S-Warfarin (5–200 μM) was incubated for 30 min at 37°C in the presence of various concentrations of aprepitant (10–400 μM) and 2 mg/ml microsomal protein (pool of 10 preparations) The reactions (500 μL) were quenched by addition of 10 μL of 70% perchloric acid, and after centrifugation, aliquots (50 μL) were analyzed by fluorescence detection (Table 1). Reaction rates were calculated from metabolite concentrations extrapolated from standard curves constructed over the range of 1 to 10 pmol.

S-Mephenytoin. Reactions (200 μL) containing 10 to 200 μM S-mephenytoin, various concentrations of aprepitant (10–90 μM), and 1 mg/ml pooled human liver microsomes were incubated for 30 min at 37°C. The reactions were quenched by addition of 4 volumes of acetonitrile, and 10 μL of a 20 μg/ml solution of 5-(4'-hydroxyphenyl)-5-phenylhydantoin (HPPH) was added as internal standard. Aliquots (20 μL) were analyzed by LC/MS/MS. The concentrations of 4-hydroxymephenytoin were determined by extrapolation from a standard curve constructed over the range of 1 to 26 μM.

HPLC Methods. The HPLC systems used for analysis (Shimadzu Scientific Instruments Inc., Columbia, MD) consisted of two pumps (LC10A/D VP),
Microsomes. Incubation of aprepitant with a pool of human liver microsomes in the presence of the NADPH-generating system resulted in the formation of the metabolites described above (data not shown), with profiles similar to those obtained for CYP3A4 (Fig. 1, panel C). The metabolism of aprepitant (measured as parent disappearance), followed single-site Michaelis-Menten kinetics. An apparent \( K_m \) of 8.9 ± 1.3 \( \mu \)M and \( V_{\text{max}} \) of 127 ± 6 pmol/min were obtained. Based on these results, subsequent studies were performed using a 10 \( \mu \)M concentration of aprepitant.

Inhibition of the Metabolism of Aprepitant by Isoform-Selective Inhibitors. The effect of several isoform-selective inhibitors (Newton et al., 1995) on the metabolism of \([\text{H}]\)aprepitant was examined in three preparations of human liver microsomes. The metabolism of \([\text{H}]\)aprepitant was inhibited >98% by the CYP3A inhibitors ketocazone (1 \( \mu \)M) and TAO (25 \( \mu \)M), which suggested that CYP3A4 was responsible, primarily, for the metabolism of \([\text{H}]\)aprepitant in liver fractions (Fig. 3). Other selective inhibitors had no significant effect (<10% inhibition) on the metabolism of aprepitant in microsomal incubations.

Effect of aprepitant on the metabolism of specific P450 substrates by human liver microsomes. The effect of aprepitant on several metabolic conversions mediated by P450 isoforms was studied. Aprepitant showed moderate inhibition on the conversion of testosterone to its 6β-hydroxylated metabolite, but exhibited very little inhibitory effect on the activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6 and CYP2E1 (Table 3). In vitro drug-interaction studies were performed to evaluate the potential of aprepitant to interact with drugs that are commonly used as probe substrates of specific P450 isoforms. Midazolam (CYP3A4), diltiazem (CYP3A4), RS-warfarin (CYP2C9) and S-mephenytoin (CYP2C19) were used as substrates in these studies. The \( K_i \) values of aprepitant on the metabolic reactions of midazolam (hydroxylation) or diltiazem (N-demethylation), were estimated to be approximately 10 \( \mu \)M (Table 4), consistent with results obtained for testosterone 6β-hydroxylation (IC\(_{50}\) = 6.3 ± 2.8 \( \mu \)M at 10 \( \mu \)M testosterone). These results show no substrate-specific differences in inhibition (Kenworthy et al., 1999). In addition, aprepitant showed weak inhibition of (RS)-warfarin 7-hydroxylation and S-mephenytoin 4'-hydroxylation with \( K_i \) values >50 \( \mu \)M (Table 4).

**Discussion**

Aprepitant is metabolized extensively by the N- and O-dealkylation pathways (Huskey et al., 2004). Studies in animals indicated that the products derived from the initial N-dealkylation and subsequent reac-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HPLC Column</th>
<th>Flow Rate</th>
<th>Mobile Phase</th>
<th>Metabolite</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupropion</td>
<td>Zorbax 300 Extend C18, 5 ( \mu )M, 4.6 × 50 mm</td>
<td>1.5</td>
<td>20–80% B(^a) (2-min linear gradient)</td>
<td>Hydroxybupropion</td>
<td>rt-Propanolol</td>
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<td>Paclitaxel</td>
<td>BDS Hypersil C8, 5 ( \mu )M, 2 × 50 mm</td>
<td>1.5</td>
<td>20–80% B(^a) (1.9 min linear gradient)</td>
<td>4a-Hydroxypaclitaxel</td>
<td>Baccatin</td>
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<td>S-Mephenytoin</td>
<td>Zorbax RxC8, 5 ( \mu )M, 4.6 × 50 mm</td>
<td>1</td>
<td>75% B(^b) (isocratic)</td>
<td>1'-Hydroxymephenytoin</td>
<td>HPPH</td>
</tr>
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<td></td>
<td></td>
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<td>4-Hydroxymephenytoin</td>
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<td></td>
<td>342 → 293</td>
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<td>4-Hydroxymephenytoin</td>
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<td></td>
<td>342 → 297</td>
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<tr>
<td>Midazolam</td>
<td>Zorbax RxC8, 5 ( \mu )M, 4.6 × 50 mm</td>
<td>1</td>
<td>75% B(^b) (isocratic)</td>
<td>N'-Desmethyl diltiazem</td>
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<td>401 → 178</td>
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<tr>
<td>Diltiazem</td>
<td>Zorbax RxC8, 5 ( \mu )M, 4.6 × 50 mm</td>
<td></td>
<td></td>
<td>Aprepitant analog(^a)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>503 → 259</td>
<td></td>
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</tbody>
</table>

\(^a\) Mobile phase consisted of A = 9:1 water/methanol with 0.05% formic acid and B = 1:9 water/acetonitrile with 0.05% formic acid.

\(^b\) Mobile phase consisted of 75:25 acetonitrile/water (v/v) with 10 mM ammonium acetate and 0.1% TFA.

\(^c\) Internal standard described by Constanzer et al. (2004).
Results presented here showed that the O-dealkylation of aprepitant was catalyzed by recombinant CYP1A2, CYP2C19, and CYP3A4, whereas the N-dealkylation pathway resulted primarily from CYP3A4 activity. However, our results indicated that CYP3A4 represents the major contributor to the metabolism of aprepitant in human liver. This
was evidenced by the ability of CYP3A4 to catalyze both metabolic pathways of aprepitant and the complete inhibition observed in human liver microsomes when ketoconazole or TAO was used as inhibitor (Fig. 3). These results are likely a reflection of the low levels of CYP1A2 and CYP2C19 in human liver relative to CYP3A4 (Rodrigues, 1999).

Aprepitant undergoes monophasic kinetics in human liver microsomes, and its $K_m$ approaches 10 $\mu$M. Furthermore, aprepitant was a moderate inhibitor of CYP3A4, with a $K_i$ of approximately 10 $\mu$M for two CYP3A4 probes, midazolam and diltiazem ($IC_{50}$ for 10 $\mu$M testosterone $= 6$ $\mu$M).

At the dosing regimen used for aprepitant administration to CINV patients (i.e., 120 mg on day 1 and 80 mg on days 2–5), maximal concentrations of aprepitant in plasma are approximately 1.5 $\mu$M (Constanzer et al., 2004). Under this regimen, clinical drug interaction studies showed a moderate inhibitory effect on CYP3A4 (Majumdar et al., 2003; McCrea et al., 2003) but not on CYP2D6, CYP2C9, or CYP2C19 substrates [EMEND (aprepitant) capsules product information; Merck & Co. Inc., White House Station NJ, 2003]. Thus, the in vitro phenotyping and inhibition studies appropriately assessed the moderate drug-drug interaction potential of the compound and helped to understand the clinical observations.

In conclusion, the data presented here support the results obtained from clinical studies, in which aprepitant, administered at the dosing regimens recommended for the treatment of CINV in conjunction with other drugs that are CYP3A4 substrates, led to increased plasma levels of those drugs. Thus, aprepitant should be used with caution when given concomitantly with drugs, including chemotherapy agents, that are primarily metabolized by CYP3A4. Increases in plasma concentrations of drugs that are metabolized by CYP3A4 may occur when coadministering these agents with aprepitant.

Interaction of aprepitant with drugs that are substrates of P450 isoforms other than CYP3A4 is unlikely to involve inhibition of their metabolism. For example, a modest, transient inductive effect on tolbutamide metabolism occurred when this drug was administered on days 4 or 8 after a 3-day aprepitant CINV therapy, but disappeared by day 15 (Shadle et al., 2004). A similar effect was observed in the same study when patients were administered intravenous midazolam after aprepitant treatment. The modest inductive effect on CYPC29 is not likely to be clinically relevant for most CYPC29 substrates, but it could be important for drugs with a low therapeutic index, such as warfarin. On the other hand, given that the inductive effect on midazolam was weak, it is unlikely that it would significantly affect the pharmacokinetics of chemotherapeutic agents administered within 12 days after completion of the aprepitant regimen for prevention of CINV.

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References


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