with aprepitant, the dexamethasone AUC0–24 h also increased, making (12 mg on day 1 and 4 mg on days 2 and 5) was given concomitantly with a modified dexamethasone regimen (20 mg on day 1 and 12 mg on days 2–5) was given 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 troлейдомycin. No inhibition was observed with other P450 isoform-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 observed in experiments with isofor-specific substrates (IC50 > 70 M). Aprepitant was a moderate inhibitor of CYP3A4, with KI values of ~10 M 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 M 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.

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 troлейдомycin. No inhibition was observed with other P450 isoform-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 observed in experiments with isofor-specific substrates (IC50 > 70 M). Aprepitant was a moderate inhibitor of CYP3A4, with KI values of ~10 M 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 M 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-[[2(R),3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-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) (Rola 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). 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, CYP2D6, 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, troлейдомycin; 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 aprepi

tant 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 aprepi

tant 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'-hydroxybufural, furafylline, R,S- and S-warfarin, 7-hydroxy-

warfarin, 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]aprepi

tant (specific activity 29.8 μCi/mg) and [4-fluoro-

phenyl-3-14C]aprepi

tant (specific activity 11.62 μCi/mg) were prepared by the Merck Labeled Compound Synthesis Group (Rahway, NJ). Midazolam mal-

eate, 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 BCA method (Fierce Chemical, Rockford, IL) according to the manufacture'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 dehy-

drogenase. 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]aprepi

tant, 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 (20 μl) of the supernatants were analyzed by HPLC as described below. 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]aprepi

tant (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 micro

somal protein and were quenched with 4 volumes of acetonitrile. After cen-

trifugation, 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 aprepi

tant as % of total radioactivity in the radiocromatograms.

Inhibition of aprepi

tant metabolism. The reaction mixtures (500 μl) containing [3H]aprepi

tant, (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 compar
tion of turnover in the presence of an inhibitor to a control reaction run simultaneously with solvent vehicle only.

Determination of the ICs<sub>50</sub> for the Inhibition of P450-Mediated Reactions by Aprepi

tant. Concentrations of substrates used in these experiments were kept near or below their K<sub>m</sub> values for bupropion and paclitaxel (Taxol) were determined in the pool of microsomes used for these experiments as 14.4 and 98 μM, respectively; bufuralol, chlorzoxazone, and testosterone concentrations were used based on literature values). For phenaceti

n and tolbutamide, concentrations of 100 μM were used based on a report by Von Moltke et al. (2001).

The reaction mixtures (500 μl except for paclitaxel and bupropion, which were done in 200 μl) containing phenaceti

n (100 μM), bupropion (100 μM), paclitaxel (15 μM), tolbutamide (100 μM), bufuralol (10 μM), chlorzoxazone (100 μM), or testosterone (10 μM), and various concentrations of aprepi

tant (1–100 μM) and human liver microsomes (0.25–1 mg/ml to obtain substrate turnovers of <15%) were incubated at 37°C for 20 min. The reactions (phenaceti

n, bufuralol, chlorzoxazone, testosterone) were quenched by the addition of 50 μl of methanol containing 1% (v/v) trifluoroacetic acid (TFA), 50 μl of 50% (v/v) TFA in water (tolbutamide), or 4 volumes of acetonitrile (bupropion, paclitaxel). The samples (phenaceti

n, bufuralol, tolbutamide, chlorzoxazone, and testosterone) were centrifuged and aliquots were analyzed by HPLC as described under "HPLC Methods". After addition of internal standard (see Table 2) and centrifugation at 1000g for 10 min, 500-μl aliquots of bupropion or paclitaxel reactions were dried under N2 and reconstituted in 100 μl of 30% (v/v) acetonitrile in water. Aliquots of 20 μl were analyzed by LC-MS/MS (see "LC-MS/MS Methods").

Determination of K<sub>i</sub> for the Inhibition of P450-Specific Reactions by Aprepi

tant. Midazolam. The reaction mixtures (250 μl) containing midazolam (2–250 μM), aprepi

tant (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 the addition of 4 volumes of acetonitrile and 10 μl of a 250 μM 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'-hy-

droxymidazolam and 0.05 to 10 μM for 4-hydroxymidazolam.

Diltiazem. Reaction mixtures (250 μl) containing diltiazem (5–200 μM), aprepi

tant (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-Desethyl diltiazem 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 aprepi

tant (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. Reacti

ons (200 μl) containing 10 to 200 μM S-mephenytoin, various concentrations of aprepi

tant (10–80 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 (Shimazu Scientific Instruments Inc., Columbia, MD) consisted of two pumps (LC10ADvp),
a controller (SCL10Awp), an autosampler ( SIL-10A), a UV detector (SPD-10AVP), and/or a fluorometer (model RF-10AXL).

**Aprepitant.** The separation of aprepitant from its metabolites was accomplished on a Zorbax RX-C8 analytical column (5 μm, 4.6 × 250 mm; Agilent Technologies, Palo Alto, CA) using a linear gradient from 35 to 80% B in 40 min at a flow rate of 1 ml/min. The mobile phase consisted of solvent A (10 mM ammonium acetate in water) and solvent B (10 mM ammonium acetate in 9:1 (v/v) acetonitrile/methanol). Radioactive aprepitant and its metabolites were monitored using an on-line radiometric detector (IN/US Systems Inc., Tampa, FL) and Ultima-Flow M (PerkinElmer Life and Analytical Sciences, Boston, MA) as scintillant at a flow rate of 3 ml/min.

**Metabolism of P450 Probes.** HPLC separations were performed on an SB-C8 analytical column (5 μm, 4.6 × 75 mm; Agilent Technologies) using the mobile phase described above. Analytes were eluted using 10-min gradients at a flow rate of 2 ml/min. Details for these methods are described in Table 1.

**LC-MS/MS Methods.** All methods were developed on a PerkinElmer HPLC apparatus linked to a triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) with an atmospheric pressure chemical ionization source in the positive ion mode. Metabolites were quantified by multiple reaction monitoring (MRM). LC conditions as well as analyte and internal standards transitions monitored are described in Table 2.

**Kinetic Calculations.** Kinetic constants for the inhibition of specific P450 isoform-mediated reactions were calculated using KaleidaGraph software (Abelbeck/Synergy, Reading PA) as scintillant at a flow rate of 3 ml/min.

**Results**

**Metabolism of Aprepitant by Recombinant P450s.** CYP3A4 catalyzed the N-dealkylation reaction of aprepitant to the primary metabolite M-1 and the subsequent oxidation of M-1 to the imine M-2. The presence of metabolites M-3 and M-4 in incubations of metabolite M-1 and the subsequent oxidation of M-1 to the imine indicated that this enzyme also catalyzed the O-dealkylation of aprepitant. CYP1A2 and CYP2C19 catalyzed the O-dealkylation of aprepitant to M-3 and M-4 (Fig. 1).

The pathways for aprepitant metabolism are outlined in Fig. 2.

**Kinetics for the Metabolism of Aprepitant in Human Liver 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 \( V_{\text{max}} \) of 89 ± 1.3 μM and \( V_{\text{max}} \) of 127 ± 6 pmol/min were obtained. Based on these results, subsequent studies were performed using a 10 μM 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 \[^{[3]}H\]aprepitant was examined in three preparations of human liver microsomes. The metabolism of \[^{[3]}H\]aprepitant was inhibited >98% by the CYP3A inhibitors ketoconazole (1 μM) and TAO (25 μM), which suggested that CYP3A4 was responsible, primarily, for the metabolism of \[^{[3]}H\]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-mephentoin (CYP2C19) were used as substrates in these studies. The IC₅₀ values of aprepitant on the metabolic reactions of midazolam (hydroxylation) or diltiazem (N-demethylation), were estimated to be approximately 10 μM (Table 4), consistent with results obtained for testosterone 6β-hydroxylation (IC₅₀ = 6.3 ± 2.8 μM at 10 μM 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-mephentoin 4’-hydroxylation with Ki values >50 μM (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>TABLE 2</th>
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<tr>
<td><strong>Conditions for the LC-MS/MS quantification of P450 substrates</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HPLC Column</th>
<th>Flow Rate</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxybupropion</td>
<td>Bupropion</td>
<td>Zorbax 300 Extend C18, 5 μm, 4.6 × 50 mm</td>
<td>1.5</td>
</tr>
<tr>
<td>Hydroxydiltiazem</td>
<td>Diltiazem</td>
<td>BDS Hypersil C8, 5 μm, 2 × 50 mm</td>
<td>1.5</td>
</tr>
<tr>
<td>Hydroxydiltiazem</td>
<td>Diltiazem</td>
<td>Zorbax RxC8, 5 μm, 4.6 × 50 mm</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxydiltiazem</td>
<td>Diltiazem</td>
<td>Zorbax RxC8, 5 μm, 4.6 × 50 mm</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxydiltiazem</td>
<td>Diltiazem</td>
<td>Zorbax RxC8, 5 μm, 4.6 × 50 mm</td>
<td>75% B* (isocratic)</td>
</tr>
</tbody>
</table>

* 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.

** Notes **

1. Mobile phase consisted of 75:25 acetonitrile/water (v/v) with 10 mM ammonium acetate and 0.1% TFA.
2. Internal standard described by Constanzer et al. (2004).
tions were eliminated in feces or further metabolized by either conjugation or oxidation, while products of O-dealkylation were readily eliminated in urine (Huskey et al., 2004). Similar results were obtained when [14C]aprepitant was administered to human subjects (Huskey et al., unpublished data).

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

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 μM (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 chemotherapeutic 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 CYP2C9 is not likely to be clinically relevant for most CYP2C9 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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