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

Rosa I. Sanchez, Regina W. Wang, Deborah J. Newton, Ray Bakhtiar, Ping Lu, Shueth-Hing Lee Chiu, David C. Evans, and Su-Er W. Huskey
Department of Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey (R.L.S., R.W.W., D.J.N., R.B., S.-H.L.C., D.C.E., S.W.H.); and Department of Drug Metabolism, Merck Research Laboratories West Point, Pennsylvania (P.L.)

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 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 isofom-specific substrates (IC50 > 70 μM). Aprepitant was a moderate inhibitor of CYP3A4, with K1 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 K1 values of 108 and 66 μM for the 7-hydroxylation of warfarin and the 4-hydroxylation of S-mephenytoin, 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]ethoxy]-3-(4-fluorophenyl)-4-morpholiny]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., 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, and the 4- and 7-hydroxylation of S-mephenytoin, 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; HPHH, 5-(4'-hydroxyphenyl)-5-phenylhydantoin; P450, cytochrome P450; TAO, troleandomycin; 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-mephentoin, 4-hydroxy-mephentoin, 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). [Morpholino-2-4C]aprepitant (specific activity 29.8 μCi/mg) and [4-fluorophenyl-3-14C]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 bicinchoninic acid method (Pierce Chemical, 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 was kept near or below their K_m 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 phenacitin 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 phenacitin (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 aprepitant (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 (phenacitin, 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 (phenacitin, 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 1000 g 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_i 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 μM solution of the internal standard. After centrifugation, aliquots (20 μl) of the supernatant were analyzed by LC-MS/MS as described before. 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′-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 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 (10–80 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 (HPHP) was added as an 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 (LC10ADvp), a diode array detector (SPD-10AVP), and a variable wavelength fluorometer (RF-10AXL). Separations were performed on a Zorbax SB-C8 analytical column (5 μm, 4.6 × 75 mm; Agilent Technologies) using linear gradients, 10 mM ammonium acetate in water as mobile phase A and 10 mM ammonium acetate in 9:1 acetonitrile/methanol (v/v) as mobile phase B. Analytes were eluted at a flow rate of 2 ml/min.

<table>
<thead>
<tr>
<th>Isobar</th>
<th>Probe</th>
<th>Gradient (% B)</th>
<th>Detection</th>
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</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacitin</td>
<td>10–50</td>
<td>UV, 250 nm</td>
</tr>
<tr>
<td>2D6</td>
<td>Bufuralol</td>
<td>15–45</td>
<td>Fluorescence, λ_ex = 252 nm, λ_em = 302 nm</td>
</tr>
<tr>
<td>2C9</td>
<td>Warfarin</td>
<td>35–45</td>
<td>Fluorescence, λ_ex = 320 nm, λ_em = 415 nm</td>
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<tr>
<td>2E1</td>
<td>Chlorzoxazone</td>
<td>8–65</td>
<td>UV, 287 nm</td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>15–45</td>
<td>UV, 230 nm</td>
</tr>
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ex, excitation; em, emission.
a controller (SCL10Avp), 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 scintillator 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 Scieix, 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 standard 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). Apparent K_m values were calculated using the Michaelis-Menten equation: v = V_max × S/(K_m + S).

For inhibition studies, replots of apparent K_m or apparent K_m/V_max (competitive or noncompetitive) versus concentrations of aprepitant were fitted to a linear regression where the intercept and slope represent K_m and K_m/K_c (competitive), or K_m/V_max and K_m/(V_max × K_c) (noncompetitive), respectively. IC_50 values were calculated according to the equation: V/V_0 = 1/(IC_50 + S), where V represents an array of the rates of the reaction measured in the presence of various concentrations of inhibitor, V_0 represents the rate of the reaction in the absence of inhibitor, and S represents a selected concentration of the substrate.

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 M-2. The presence of various concentrations of inhibitor, where V_max represents a selected concentration of the substrate.

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 K_m of 8.9 ± 1.3 μM and V_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 [^3H]aprepitant was examined in three preparations of human liver microsomes. The metabolism of [^3H]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 [^3H]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 conversion 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 (CYP2C9) 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 μM (Table 4), consistent with results obtained for testosterone 6β-hydroxylation (IC_50 = 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 K_i 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 2 Conditions for the LC-MS/MS quantitation of P450 substrates |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Substrate       | HPLC Column     | Flow Rate       | Mobile Phase    | MRM Transition  |
|                 |                 | ml/min          |                 | Internal Standard |
| Bupropion       | Zorbax 300 Extend C18, 5 μm, 4.6 × 50 mm | 1.5 | 20-80% B* (2-min linear gradient) | Hydroxybupropion 870 → 525 | rt-Propanolol 260 → 155 |
| Paclitaxel      | BDS Hypersil C8, 5 μm, 2 × 50 mm | 1.5 | 20-80% B* (1.9 min linear gradient) | 6β-Hydroxypaclitaxil 870 → 525 | Bscatin 857 → 405 |
| S-Mephenytoin   | Zorbax RxC8, 5 μm, 4.6 × 50 mm | 1 | 75% B* (isocratic) | 4-Hydroxymephenytoin 233 → 133 | HPPH 269 → 176 |
| Midazolam       | Zorbax RxC8, 5 μm, 4.6 × 50 mm | 1 | 75% B* (isocratic) | 1'-Hydroxymidazolam 342 → 203 | Aprepitant analog 503 → 259 |
|                 |                 |                 |                 |                 |                  |
| Diltiazem       | Zorbax RxC8, 5 μm, 4.6 × 50 mm | 1 | 75% B* (isocratic) | N-Demethyl diltiazem 401 → 178 | Aprepitant analog 503 → 259 |

*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).
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
was evidenced by the ability of CYP3A4 to catalyze both metabolic pathways of aprepitant and the complete inhibition observed in human liver microsomes when ketonazole 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 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 $= 6 \, \mu$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 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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