In Vitro Inhibition and Induction of Human Liver Cytochrome P450 Enzymes by Milnacipran

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ABSTRACT:

Milnacipran (Savella) inhibits both norepinephrine and serotonin reuptake and is distinguished by a nearly 3-fold greater potency in inhibiting norepinephrine reuptake in vitro compared with serotonin. We evaluated the ability of milnacipran to inhibit and induce human cytochrome P450 enzymes in vitro. In human liver microsomes, milnacipran did not inhibit CYP1A2, 2B6, 2C8, 2C9, 2C19, or 2D6 (IC50 ≥ 100 μM); whereas, a comparator with dual reuptake properties [duloxetine (Cymbalta)] inhibited CYP2D6 (IC50 = 7 μM) and CYP2B6 (IC50 = 15 μM) with a relatively high potency. Milnacipran inhibited CYP3A4/5 in a substrate-dependent manner (i.e., midazolam 1' hydroxylation IC50 ~ 30 μM; testosterone 6β-hydroxylation IC50 ~ 100 μM); whereas, duloxetine inhibited both CYP3A4/5 activities with equal potency (IC50 = 37 and 38 μM, respectively). Milnacipran produced no time-dependent inhibition (<10%) of P450 activity, whereas duloxetine produced time-dependent inhibition of CYP1A2, 2B6, 2C19, and 3A4/5. To evaluate P450 induction, freshly isolated human hepatocytes (n = 3) were cultured and treated once daily for 3 days with milnacipran (3, 10, and 30 μM), after which microsomal P450 activities were measured. Whereas positive controls (omeprazole, phenobarbital, and rifampin) caused anticipated P450 induction, milnacipran had minimal effect on CYP1A2, 2B6, 2C9, or 2C19 activity. The highest concentration of milnacipran (30 μM; >10 times plasma Cmax) produced 2.6- and 2.2-fold increases in CYP2B6 and CYP3A4/5 activity (making it 26 and 34% as effective as phenobarbital and rifampin, respectively). Given these results, milnacipran is not expected to cause clinically significant P450 inhibition or induction.

Milnacipran (Savella), which was recently approved for the treatment of fibromyalgia, is a dual reuptake inhibitor of norepinephrine and serotonin, which is distinguished by an approximately 3-fold greater potency in inhibiting norepinephrine reuptake in vitro compared with serotonin reuptake (Vaishnavi et al., 2004). These two neurotransmitters have been shown to exert significant modulatory effects on peripheral and central pain processing (Dubner and Hargreaves, 1989). Selective serotonin-norepinephrine reuptake inhibitors (SNRIs) such as duloxetine and venlafaxine are more potent inhibitors of serotonin reuptake than norepinephrine reuptake, whereas the converse is true of milnacipran (Vaishnavi et al., 2004).

Milnacipran is well absorbed (85-90%) after oral administration and has linear pharmacokinetics (PK) over the therapeutic dose range (Delini-Stula, 2000). The terminal elimination half-life in plasma is 6 to 8 h, and steady-state levels can be predicted from single-dose PK data, indicating the absence of autoinhibition or autoinduction. Milnacipran is eliminated primarily by renal excretion of the unchanged drug (50–60%), conjugation to form a carbamoyl glucuronide (~20%), and N-dealkylation by cytochrome P450 (mainly CYP3A4) to N-desethyl milnacipran (~8%) (Pouzzo and Leonard, 1996; Delini-Stula, 2000; Tsuruta et al., 2000; Pouzzo et al., 2005; Forest Research Institute, personal communication). Metabolism by cytochrome P450 plays only a minor role in the elimination of milnacipran (Caccia, 1998; Grzesiak et al., 2000; Sawada and Ohtani, 2001; Pouzzo et al., 2002). Consequently, genetic polymorphisms in CYP2D6 and inhibition of this enzyme do not affect the pharmacokinetics of milnacipran (Pouzzo et al., 2005), in contrast to the situation with many selective serotonin-reuptake inhibitors and tricyclic antidepressants (Bertilsson et al., 2002; Preskorn et al., 2007). The PK profile of milnacipran is the same in both CYP2D6 poor metabolizers (PMs) and extensive metabolizers (EMs), and this finding also holds for CYP2C19 PMs and EMs (Pouzzo et al., 2005). Limited to no modification of the pharmacokinetic profile is expected when milnacipran is coadministered with fluoxetine (a strong CYP2D6 inhibitor) or carbamazepine (an inducer of CYP2B6, CYP3A4, and several other enzymes) (Pouzzo et al., 2002, 2005, 2006).

From the perspective of drug-drug interactions, drugs can be viewed as victims (objects) or perpetrators (precipitants) (Ogilvie et al., 2008). Milnacipran has low victim potential because its clearance is not heavily dependent on metabolism by a single drug-metabolizing enzyme; hence, its PK profile is not significantly affected by the genetic polymorphisms, P450 inhibitors, or P450 inducers that affect the disposition of other antidepressant drugs. More than half of the drug (50–60%) is eliminated unchanged in urine, which indicates that

ABBREVIATIONS: SNRI, selective serotonin-norepinephrine reuptake inhibitor; PK, pharmacokinetic(s); PMs, poor metabolizers; EMs, extensive metabolizers; P450, cytochrome P450; FDA, U.S. Food and Drug Administration; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; AUC, area under the curve; INR, international normalized ratio.
kidney function is the primary determinant of the elimination of milnacipran. The in vitro studies described in this report were designed to evaluate the perpetrator potential of milnacipran. The enzyme-inducing potential of milnacipran was evaluated in three preparations of freshly cultured human hepatocytes and focused on the major inducible human P450 enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4/5. CYP2D6 was not examined because this enzyme is recognized by the FDA as being noninducible (Draft Guidance for Industry: Drug Interaction Studies: Study Design, Data Analysis and Implications for Dosing and Labeling, 2006, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101). The ability of milnacipran to function as a direct-acting and metabolism-dependent inhibitor of P450 enzymes was evaluated with human liver microsomes. The enzymes evaluated included CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4/5 (with two substrates), as recommended by the FDA (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101; Huang et al., 2008). In the P450 inhibition study, the SNRI duloxetine (Cymbalta) was included as a comparator. The structures of milnacipran and duloxetine are shown in Fig. 1. The in vitro studies described herein were conducted in accordance with the FDA’s draft guidance document on the conduct of in vitro metabolism studies (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101) and the principles promulgated by Tucker et al. (2001), Bjorndsson et al. (2003), and Huang et al. (2008).

### Materials and Methods

#### Chemicals and Reagents.

Milnacipran and duloxetine were provided by Forest Research Institute (Jersey City, NJ). Stock solutions of milnacipran (10 and 50 mM) and duloxetine (10 mM) were prepared in high-purity water for P450 induction studies and in dimethyl sulfoxide (DMSO) for enzyme induction studies.

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): butyrophenon HCl, dextromethorphan, diclofenac, 4’-hydroxydiclofenac, (+)-4’-hydroxyphenytoin, 6’-hydroxytestosterone, midazolam, phencetic, and testosterone. Acetaminophen, N-desethylamodiaquine, dextrophan, and 1’-hydroxymidazolam were purchased from Cerillian Corporation (Round Rock, TX). Amodiaquine was purchased from U.S. Pharmacopeia (Rockville, MD). S-Mephenytoin was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Hydroxybutyropion, ITCs (insulin, human transferring, and selenic acid), and Matrigel were purchased from BD Biosciences (Bedford, MA). Dulbecco’s modified Eagle’s medium, GlutaMAX-1, insulin, minimum medium-essential noneutopic amino acids, modified Eagle’s medium (Dr. Chee’s modification), and liquid penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). PureCol was purchased from Inamed BioMaterials (Fremont, CA). Fetal bovine serum was purchased from SAFC Biosciences (Lenexa, KS). Loctite 4013 was purchased from the Loctite Corporation (Ro认可Hill, CT). The BCA Protein Assay Kit was purchased from Pierce Chemical (Rockford, IL). All other reagents were obtained from commercial sources, most of which have been described elsewhere (Robertson et al., 2000; Madan et al., 2003; Ogilvie et al., 2006).

#### Test System.

Pooled human liver microsomes (n = 16, mixed gender) were prepared and characterized at XenoTech, LLC (Lenexa, KS). Human hepatocytes from nontransplantable livers were prepared at XenoTech from three individual donors, all white men (ages 51, 74, and 77); initial cell viabilities were 83, 93, and 77%, respectively.

#### In Vitro P450 Inhibition.

The ability of milnacipran and duloxetine to inhibit the major drug-metabolizing P450 enzymes in a direct and time-dependent manner was investigated with a pool of human liver microsomes (pool of 16 individuals), as described by Ogilvie et al. (2006, 2008). In brief, duplicate incubations were conducted at 37 ± 1°C in 400-µl incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM, pH 7.4), an NADPH-generating system (consisting of 1 mM NADP, 5 mM glucose 6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase) and P450 marker substrate as indicated in Table 1. Reactions were initiated by the addition of the NADPH-generating system and terminated after 5 min by an equal volume of acetonitrile (v/v) containing an appropriate internal standard, as summarized in Table 1. Precipitated protein was removed by centrifugation (920g for 10 min at 10°C). Calibration and quality control metabolite standards were prepared in zero time incubations. The analytical procedures are summarized in Table 1.

To evaluate milnacipran and duloxetine as direct-acting inhibitors, pooled human liver microsomes (≤0.1 mg/ml) were incubated with P450 marker substrates (at concentrations approximately equal to the Km, as shown in Table 1) in the presence and absence of milnacipran or duloxetine (at concentrations ranging from 0.1 to 100 µM) to determine the IC50 value.

To examine their ability to act as time-dependent inhibitors, milnacipran or duloxetine (at the same concentrations used to evaluate direct inhibition) were preincubated at 37 ± 1°C, in duplicate, with human liver microsomes and an NADPH-generating system for 30 min. After the preincubation period, the marker substrate (at a concentration approximately equal to its Km) was added, and the incubation was continued for 5 min to measure residual P450 activity. Reactions were terminated after 5 min by the addition of an equal volume of acetonitrile (containing the appropriate internal standard) (Table 1). Known direct-acting and metabolism-dependent inhibitors were included as positive controls, most of which appear on the FDA’s list of recommended or accepted in vitro inhibitors (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101; Ogilvie et al., 2008). Samples were analyzed as described under Analytical Methods.

#### In Vitro P450 Induction.

The ability of milnacipran to induce or suppress the expression of P450 enzymes was investigated in primary cultures of freshly isolated human hepatocytes with a Matrigel overlay. After a 2-day adaptation period, three preparations of cultured human hepatocytes from three separate human livers were treated once daily for 3 consecutive days with milnacipran (3, 10, and 30 µM) or one of three prototype P450 inducers, namely omeprazole (100 µM), phenobarbital (750 µM), and rifampin (10 µM), at the final concentrations indicated. Milnacipran and the positive controls were dissolved in DMSO, and hepatocytes treated with DMSO (final concentration 0.1%, v/v) served as negative controls. The isolation, culturing, and treatment procedures were performed essentially as described by Madan et al. (2003). Human hepatocytes were harvested 24 h after the third treatment to prepare microsomes, as described by Madan et al. (2003). Microsomes (0.004–0.02 mg/ml) were incubated with phenacetin (80 µM), bupropion (500 µM), amodiaquine (20 µM), diclofenac (100 µM), S-mephenytoin (400 µM), and testosterone (250 µM) for 10 to 30 min to measure CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4/5 activity, respectively, essentially as described above (see In Vitro P450 Inhibition). Samples were analyzed as described under Analytical Methods (see below).

#### Analytical Methods.

All analyses of P450 enzyme activities were performed with validated high-performance liquid chromatography (HPLC)-tandem mass spectrometry methods. The mass spectrometry equipment was either an ABI Sciex (Applied Biosystems/MDS Sciex, Foster City, CA) API 2000, API 3000, or API 4000 mass spectrometer with Shimadzu HPLC pumps and autosampler systems (Shimadzu, Kyoto, Japan). The HPLC columns used were as follows: Waters Atlantis C18 (5-µm particle size, 50 × 2.1 mm) (Waters, Milford, MA) preceded by a Phenomenex Luna C-8 guard column (4.0 × 2.0 mm) (Phenomenex, Torrance, CA) or Waters Atlantis (5-µm particle size, 4.6 × 50 mm) (Waters, Milford, MA) preceded by a Phenomenex Luna C-8 guard column (4.0 × 2.0 mm) (Phenomenex, Torrance, CA).
100 × 2.1 mm) (Waters) preceded by a Phenomenex Luna C-8 guard column (4.0 × 2.0 mm) (Phenomenex). Formic acid or ammonium acetate-based mobile phases were used for all sample analyses and flow rates ranged from approximately 0.55 to 0.90 ml/min. All columns were maintained at ambient temperature during analysis. Metabolites were quantified by back calculation of a weighted (1/x) linear, least-squares regression. The regression fit was based on analyst/internal standard peak area ratios calculated from calibration standard samples, which were prepared from authentic metabolic standards. Peak areas were integrated with an Analyst data system (version 1.4.1 or 1.4.2; Applied Biosystems/MDS Sciex).

**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>P450 Activity</th>
<th>Substrate Concentration</th>
<th>Protein</th>
<th>Ionization Mode*</th>
<th>Mass Transition Monitored</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>40</td>
<td>100</td>
<td>ESI+</td>
<td>152−110</td>
<td>d₂-Acetaminophen</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>50</td>
<td>100</td>
<td>ESI+</td>
<td>236−238</td>
<td>d₂-Hydroxybupropion</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>7</td>
<td>100</td>
<td>ESI+</td>
<td>328−293</td>
<td>d₂-N-Desethylaminodiolquine</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin</td>
<td>40</td>
<td>100</td>
<td>ESI−</td>
<td>310−266</td>
<td>d₄-4'-Hydroxydiclofenac</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>7.5</td>
<td>100</td>
<td>ESI+</td>
<td>233−190</td>
<td>d₄-4'-Hydroxymephenytoin</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone</td>
<td>100</td>
<td>100</td>
<td>ESI+</td>
<td>305−269</td>
<td>d₆-6β-Hydroxytestosterone</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam 1'-hydroxylation</td>
<td>100</td>
<td>50</td>
<td>ESI+</td>
<td>342−324</td>
<td>d₆-1'-Hydroxymidazolam</td>
</tr>
</tbody>
</table>

amu, atomic mass units; ESI, electrospray ionization.

* Indicates the type of ionization (i.e., ESI) and the polarity (+ or −).

The positive controls for CYP1A2 and CYP2B6 induction were omeprazole and phenobarbital, respectively. For all other P450 enzymes the positive control was rifampin.

**Results**

**In Vitro P450 Inhibition.** Milnacipran and duloxetine (0.1–100 μM) were evaluated for their abilities to inhibit P450 activity in pooled human liver microsomes with P450-selective substrates at concentrations approximately equal to the Kᵅᵣ. In the case of CYP3A4/5, two substrates were used (testosterone and midazolam), as recommended by the FDA (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101). Before measuring P450 activity, the test articles were preincubated with NADPH-fortified human liver microsomes for 0 or 30 min to assess the potential for time-dependent inhibition. The results are summarized in Figs. 2 and 3 and Table 2.

Milnacipran did not inhibit CYP1A2, 2B6, 2C8, 2C9, 2C19, or 2D6 (IC₅₀ ≥ 100 μM), as shown in Fig. 2. Duloxetine inhibited some of these enzymes more potently than milnacipran, inhibiting CYP2D6 with an IC₅₀ of 7 μM and CYP2B6 with an IC₅₀ of 15 μM. As shown in Fig. 2, preincubation of milnacipran with NADPH-fortified human liver microsomes did not increase its inhibitory effect on CYP1A2, 2B6, 2C8, 2C9, 2C19, or 2D6. In contrast, duloxetine produced time-dependent inhibition of CYP1A2, 2B6, and 2C19 as indicated by the leftward shift in IC₅₀ curves after the 30-min preincubation period. The 30-min preincubation period slightly decreased the inhibitory effect of duloxetine on CYP2D6 (Fig. 2).

As shown in Fig. 3, milnacipran inhibited CYP3A4/5 in a substrate-dependent manner inasmuch as it inhibited midazolam 1'-hydroxylation (IC₅₀ = 28 μM) more potently than it inhibited testosterone 6β-hydroxylation (IC₅₀ = ~100 μM). Inhibition of midazolam 1'-hydroxylation was also evaluated with a wider range of milnacipran concentrations (up to 500 μM), which indicated an IC₅₀ value of 31 μM, thereby confirming the original estimate of 28 μM (data not shown). Duloxetine also directly inhibited CYP3A4/5, with IC₅₀ values of 37 and 38 μM for the 1'-hydroxylation of midazolam and the 6β-hydroxylation of testosterone, respectively, suggesting that the inhibition is not dependent on substrate. Milnacipran did not produce time-dependent inhibition (<10%) of CYP3A4/5 activity, whereas duloxetine did produce time-dependent inhibition of CYP3A4/5 activity toward testosterone and midazolam (although the time-dependent inhibition toward midazolam was to a lesser extent) (Fig. 3).

**In Vitro P450 Induction.** To evaluate the enzyme-inducing potential of milnacipran, three preparations of freshly isolated human hepatocytes were cultured and treated once daily for 3 consecutive days with milnacipran (3, 10, or 30 μM) or one of three prototypical...
enzyme inducers, namely, omeprazole (100 µM), phenobarbital (750 µM), and rifampin (10 µM). Microsomes were prepared 24 h after the final treatment and assayed for CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4/5 activity. Under the conditions examined, milnacipran caused no cell toxicity based on light microscopic evaluation. Throughout the treatment period, the cultured hepatocytes were free of detectable autophagic and lipid vesicles, were cuboidal in shape, and contained intact cell membranes and granular cytoplasm with one or two centrally located nuclei.

As shown in Table 3 and Fig. 4, all three preparations of human hepatocytes responded as expected to treatment with prototypical P450 inducers. Treatment with omeprazole produced a marked increase in CYP1A2 (37-fold), whereas treatment with phenobarbital or rifampin produced an increase in CYP2B6 (6–10-fold), CYP2C8 (4–5-fold), CYP2C9 (~2-fold), CYP2C19 (3–6-fold), and CYP3A4 (~4-fold).

Treatment of human hepatocytes with up to 30 µM milnacipran for 3 consecutive days had little or no effect on CYP1A2, CYP2C8, CYP2C9, or CYP2C19 activity (Table 3; Fig. 4). Milnacipran produced a concentration-dependent increase in CYP2B6 activity with the highest concentration tested (30 µM), effecting a statistically significant increase (2.59-fold; p < 0.05) in CYP2B6 activity. At concentrations of 1, 10, and 30 µM, milnacipran was 1, 12, and 26% as effective as phenobarbital as a CYP2B6 inducer (Fig. 5). In one of the three preparations of hepatocytes treated with the highest concentration of milnacipran (30 µM), which is almost 1 order of magnitude greater than the highest plasma Cmax value observed clinically (3.1 µM) (Forest Research Institute, personal communication), the relative
effectiveness for CYP2B6 induction exceeded the FDA’s cutoff value of 40% (measured value was 51.9%; individual data not shown).

Milnacipran produced a concentration-dependent increase in CYP3A4/5 activity (Table 3; Fig. 4). The highest concentration of milnacipran (30 μM) produced a 2.15-fold increase in CYP3A4/5 activity, which was not statistically significant. At concentrations of 1, 10, and 30 μM, milnacipran was ~6, 20, and 34% as effective as rifampin as a CYP3A4/5 inducer (Fig. 5). In one of the three preparations of hepatocytes treated with the highest concentration of milnacipran (30 μM), which is almost 1 order of magnitude greater than the highest plasma C_{max} value observed clinically (3.1 μM), the relative effectiveness for CYP3A4 induction exceeded the FDA’s cutoff value of 40% (the measured value was 42.4%; individual data not shown).
### Discussion

The FDA provides the following guidance to design and interpret in vitro studies to evaluate the victim and perpetrator potential of a new drug: 1) identify the role of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 in the metabolism of the drug and further clarify its victim potential by identifying any other pathways that contribute 25% or more to the drug’s clearance; 2) evaluate the potential for direct inhibition of P450 enzymes based on the ratio of $[I]$, the plasma $C_{\text{max}}$, of total (bound and free) drug, and the inhibition constant $K_i$ with a cutoff value of $[I]/K_i = 0.1$, below which it is reasonable to assume.

![Graphs showing enzyme activity](image)

**FIG. 4.** Effects of treating cultured human hepatocytes with DMSO, milnacipran, or prototypical inducers on microsomal P450 activity.
that a drug will not cause clinically significant P450 inhibition; 3) evaluate the potential for time-dependent inhibition of P450 enzymes and conduct clinical studies to assess the in vivo significance of positive in vitro findings; and 4) evaluate the potential for enzyme induction in three preparations of human hepatocytes and conduct clinical enzyme induction studies when, at pharmacologically relevant concentrations, a drug is 40% or more as effective as a suitable positive control (http://www.fda.gov/downloads/Drugs/Guidance-ComplianceRegulatoryInformation/Guidances/ucm072101; Huang et al., 2008).

Previous studies have established that milnacipran has low victim potential with respect to metabolism by cytochrome P450. More than half (50–60%) of the drug is eliminated unchanged in urine, 20% is conjugated to a carbamoyl-glucuronide, and 8% is metabolized by cytochrome P450 (Delini-Stula, 2000; Tsuruta et al., 2000; Puozzo et al., 2005; Forest Research Institute, personal communication). The latter is most likely catalyzed by CYP3A4, which converts milnacipran to one hydroxylated and two N-dealkylated metabolites (Puozzo and Leonard, 1996; Tsuruta et al., 2000). N-Desethyl-milnacipran is the major circulating oxidative metabolite of milnacipran, accounting for approximately 10% of the dose excreted in urine (Puozzo et al., 2005). In contrast, the SNRI duloxetine has high victim potential with respect to metabolism by cytochrome P450. Duloxetine is extensively metabolized by CYP1A2 and CYP2D6, and clinical studies with 14C-duloxetine have shown that the parent drug accounts for only 3% of systemic exposure (plasma AUC) to 14C-duloxetine-derived radioactivity (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021427s027s028lbl.pdf). Inhibition of CYP1A2 by fluvoxamine results in a 5- to 6-fold increase in duloxetine AUC in CYP2D6 PM subjects, whereas inhibition of CYP2D6 by paroxetine increases plasma AUC by 60% in EMs (Cymbalta package insert, http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021427s021s027s028lbl.pdf). Cigarette smoking, which induces CYP1A2, decreases the plasma AUC of duloxetine by approximately one-third (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021427s021s027s028lbl.pdf). In contrast to the situation with duloxetine and several other antidepressants (Bertilsson et al., 2002; Preskorn et al., 2007), genetic polymorphisms in CYP2D6 and CYP2C19 have no impact on the PK of milnacipran (Puozzo et al., 2005) nor does inhibition of CYP2D6 and CYP3A4 by fluoxetine (DeVane et al., 2004; Puozzo et al., 2006). Enzyme induction by carbamazepine is associated with a small decrease (20%) in milnacipran plasma steady-state concentrations (Puozzo et al., 2002).

The present in vitro study was designed to evaluate the perpetrator potential of milnacipran. At the pharmacologically relevant concentration of 3 μM and even at 10 μM milnacipran, which is 4 to 5 times the mean steady-state plasma Cmax of 2.2 μM, milnacipran produced no significant induction of CYP1A2, 2B6, 2C8, 2C9, 2C19, or CYP3A4 in cultured human hepatocytes under conditions in which the positive controls exerted their anticipated inductive effects. At 30 μM (~14 times Cmax), milnacipran was 26% as effective as phenobarbital at inducing CYP2B6 and was 34% as effective as rifampin at inducing CYP3A4. These in vitro results indicate that, at pharmacologically relevant concentrations, milnacipran is not 40% or more as effective as phenobarbital or rifampin at inducing P450 enzymes, which, based on the relevant 2006 FDA Guidance for Industry (http://www.fda.gov/downloads/Drugs/Guidance-ComplianceRegulatoryInformation/Guidances/ucm072101) suggests that milnacipran will not produce clinically significant enzyme induction.

The potential for milnacipran to inhibit P450 enzymes in human liver microsomes was compared with that of duloxetine. The only P450 enzyme potentially inhibited by milnacipran was CYP3A4, the enzyme implicated in its N-dealkylation and hydroxylation, which are minor pathways of milnacipran clearance (Tsurtu et al., 2000). Milnacipran inhibited the 1'-hydroxylation of midazolam with an IC50 of approximately 30 μM. The concentration of midazolam was 4 μM, which is approximately equal to its Km, hence, the Kc value would be ~15 or ~30 μM depending on whether the inhibition of CYP3A4 by milnacipran was competitive or noncompetitive, respectively (Ogilvie et al., 2008). After a dosage of 100 mg b.i.d., the mean Cmax at steady state is 2.2 μM, and ranges from 1.3 to 3.1 μM (Forest Research Institute, personal communication). Based on a conservative estimate of Kc (~15 μM) and the mean Cmax of 2.2 μM, the value of [I]/Kc for CYP3A4 inhibition by milnacipran is ~0.15, which slightly exceeds the FDA’s cutoff of 0.1. When testosterone was used to measure CYP3A4 activity, the Kc for milnacipran was conservatively estimated to be 51 μM (based on an IC50 of ~102 μM). Accordingly, [I]/Kc is 0.043 (based on the mean Cmax of 2.2 μM), which falls below the FDA’s cutoff value of 0.1.

Milnacipran has been reported to have no effect on the urinary excretion of 6β-hydroxy corticosterol or the PK of carbamazepine, which, despite being an imperfect marker of CYP3A4 activity, provides some evidence that milnacipran does not cause clinically significant inhibition of CYP3A4 (Table 4) (Puozzo et al., 2005). For all other P450 enzymes, estimates of [I]/Kc are less than 0.1, and there was no evidence of metabolism-dependent inhibition. Based on this cutoff, the lack of time-dependent inhibition, and the rank order approach to extrapolating in vitro findings to the in vivo situation (Obach et al.,
N-demethylated and are major duloxetine metabolites in human plasma and urine, whereas metabolites (as well as dihydroxylated metabolites and conjugates) and by CYP2D6 (in the 4- and 5-positions). These hydroxylated of duloxetine is catalyzed by CYP1A2 (in the 4-, 5-, and 6-positions) Lobo et al. (2008) reported that the hydroxylation of the naphthyl ring 3A4. Based on experiments with recombinant human P450 enzymes, metabolism-dependent inhibition of P450 enzymes by duloxetine was 3). This study did not establish the effects of NADPH or whether the dependent inhibition of CYP1A2, 2B6, 2C19, and 3A4/5 (Figs. 2 and 4). With caffeine as the in vivo probe substrate, CYP2C19 clinical drug-drug interaction studies, (summarized in Table 4), milnacipran would not be expected to cause clinically significant inhibition of CYP1A2, 2B6, 2C8, 2C9, 2C19, or 2D6. In clinical drug-drug interaction studies, (summarized in Table 4), milnacipran has been shown to cause no inhibition (or induction) of CYP1A2 (with caffeine as the in vivo probe substrate), CYP2C19 (racemic mephenytoin), and CYP2D6 (sparteine) (Puozzo et al., 2005). Overall, there is good correspondence between the in vitro results and the available in vivo clinical findings. Compared with milnacipran, duloxetine was a more potent inhibitor of all of the P450 enzymes examined and showed evidence of time-dependent inhibition of CYP1A2, 2B6, 2C19, and 3A4/5 (Figs. 2 and 3). This study did not establish the effects of NADPH or whether the metabolism-dependent inhibition of P450 enzymes by duloxetine was due to the formation of metabolites that are more potent reversible inhibitors or are irreversible inhibitors of CYP1A2, 2B6, 2C19, and 3A4. Based on experiments with recombinant human P450 enzymes, Lobo et al. (2008) reported that the hydroxylation of the naphthyl ring of duloxetine is catalyzed by CYP1A2 (in the 4-, 5-, and 6-positions) and by CYP2D6 (in the 4- and 5-positions). These hydroxylated metabolites (as well as dihydroxylated metabolites and conjugates) are major duloxetine metabolites in human plasma and urine, whereas N-demethylated and O-dealkylated metabolites are minor in vivo metabolites (Lantz et al., 2003). N-Demethylation of duloxetine would be expected to produce a more potent direct-acting P450 inhibitor than the parent drug, whereas dihydroxylation of duloxetine to a catechol metabolite on the naphthyl ring may potentially lead to irreversible inhibition of one or more P450 enzymes. In this regard, it is interesting that CYP1A2 (which showed evidence of time-dependent inhibition) catalyzes the 5- and 6-hydroxylation of duloxetine. Consequently, hydroxylation at both these sites (which appear on the same ring and are adjacent to each other) would lead to catechol formation. In contrast, CYP2D6 (which showed no evidence of time-dependent inhibition) catalyzes the 4- and 5-hydroxylation of duloxetine. Hydroxylation at both these sites would not produce a catechol because these two sites are not adjacent to each other but appear on different rings of the naphthyl moiety (Lantz et al., 2003; Lobo et al., 2008). Duloxetine also contains a thiophene ring. Although metabolites involving thiophene oxidation have not been reported for duloxetine, this particular functional group is associated with several cases of irreversible P450 inhibition, as in the case of tienilic acid, ticlopidine, and clopidogrel (Fontana et al., 2005; Ogilvie et al., 2008; Parkinson and Ogilvie, 2008).

Duloxetine inhibited both of the enzymes implicated in its metabolism, namely CYP1A2 and CYP2D6. Duloxetine inhibited CYP1A2 with an IC₅₀ of 50 μM without any preincubation and an IC₅₀ of 18 μM with a 30-min preincubation. Lobo et al. (2008) also evaluated duloxetine as a direct-acting inhibitor of CYP1A2 and reported that duloxetine causes competitive inhibition of CYP1A2 with a Kᵢ value of 18 μM. When incubated with marker substrate at a concentration equal to the Kᵢ, the Kᵢ value for a competitive inhibitor is half its IC₅₀ value; hence, the Kᵢ of 18 μM reported by Lobo et al. (2008) translates to an IC₅₀ value of 36 μM, which is comparable to our value of 50 μM (determined without a preincubation period) and 18 μM (determined with a preincubation period). Lobo et al. (2008) do not specifically report having evaluated duloxetine as a time-dependent inhibitor of CYP1A2. Clinical interaction studies with theophylline, a CYP1A2 substrate, established that duloxetine is a weak inhibitor of CYP1A2 in vivo; it increased the plasma AUC of theophylline by 7% (1–15%) in one study and 20% (13–27%) in another (Table 5). With no preincubation, the enzyme most potently inhibited by duloxetine

<table>
<thead>
<tr>
<th>Victim Drug</th>
<th>n a</th>
<th>Enzyme b</th>
<th>Treatment</th>
<th>Change in Pharmacokinetics of the Victim Drug</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sparteine</td>
<td>25</td>
<td>CYP2D6</td>
<td>Milnacipran: 50 mg single dose (day 1), then 50 mg b.i.d. (days 2–8) Sparteine: 100 mg q.d. (days –2, 1, 8, 20)</td>
<td>19.5% Increase in sparteine/metabolite ratio in CYP2C19 EMs c</td>
<td>Puozzo et al., 2005</td>
</tr>
<tr>
<td>Mephenytoin</td>
<td>25</td>
<td>CYP2C19</td>
<td>Milnacipran: 50 mg single dose (day 1), then 50 mg b.i.d. (days 2–8) Mephenytoin: 100 mg q.d. (days –2, 1, 8, 20)</td>
<td>No increase in S/R-mephenytoin ratio d</td>
<td>Puozzo et al., 2005</td>
</tr>
<tr>
<td>Caffeine</td>
<td>25</td>
<td>CYP1A2</td>
<td>Milnacipran: 50 mg single dose (day 1), then 50 mg b.i.d. (days 2–8) Caffeine: 200 mg q.d. (days –2, 1, 8, 20)</td>
<td>No increase in the caffeine/paraxanthine AUC ratio e</td>
<td>Puozzo et al., 2005</td>
</tr>
<tr>
<td>Warfarin</td>
<td>25</td>
<td>CYP2C9 for S-warfarin and CYP1A2/3A4 for R-warfarin e</td>
<td>Milnacipran: 25 mg bid (days 1–3), 50 mg bid (days 4–6), and 100 mg bid (days 7–11) Warfarin: 25 mg single dose on days 11 and 25 or Milnacipran: 25 mg b.i.d. (days 15–17), 50 mg b.i.d. (days 18–20), and 100 mg b.i.d. (days 21–25) Warfarin: 25 mg single dose on days 1 and 25</td>
<td>No change in S- or R-warfarin pharmacokinetics or pharmacodynamics (INR)</td>
<td>FDA drug label; FRI, personal communication</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>25</td>
<td>CYP3A4</td>
<td>Milnacipran: 50 mg b.i.d. (days 1–4) and days (29–35) Carbamazepine: 100 mg b.i.d. (days 8–11); 200 mg b.i.d. (days 12–35)</td>
<td>No change in the pharmacokinetics of carbamazepine or its epoxide metabolite</td>
<td>FDA drug label; FRI, personal communication</td>
</tr>
</tbody>
</table>

a Number of subjects completing each study.
b Principal metabolizing enzyme.
c Before milnacipran treatment, the sparteine/metabolite ratio was 0.51 and 14.6 in CYP2C19 EMs and PMs, respectively.
d Before milnacipran treatment, the S/R-mephenytoin ratios were 0.066 and 1.56 in CYP2C19 EMs and PMs, respectively.
e Before milnacipran treatment, the caffeine/paraxanthine AUC ratio was 2.3.

This study did not establish the effects of NADPH or whether the metabolism-dependent inhibition of P450 enzymes by duloxetine was due to the formation of metabolites that are more potent reversible inhibitors or are irreversible inhibitors of CYP1A2, 2B6, 2C19, and 3A4. Based on experiments with recombinant human P450 enzymes, Lobo et al. (2008) reported that the hydroxylation of the naphthyl ring of duloxetine is catalyzed by CYP1A2 (in the 4-, 5-, and 6-positions) and by CYP2D6 (sparteine) (Puozzo et al., 2005). Overall, there is good correspondence between the in vitro results and the available in vivo clinical findings. Compared with milnacipran, duloxetine was a more potent inhibitor of all of the P450 enzymes examined and showed evidence of time-dependent inhibition of CYP1A2, 2B6, 2C19, and 3A4/5 (Figs. 2 and 3).

TABLE 4

Summary of clinical drug-drug interactions studies to evaluate the effect of milnacipran on the disposition of coadministered drugs
was CYP2D6 (IC$_{50}$ = 7 μM). With a preincubation, the enzyme most potently inhibited by duloxetine was CYP2B6 (IC$_{50}$ = ~5 μM). Duloxetine produces clinically significant inhibition of CYP2B6 based on its ability to cause up to a 3-fold increase in the plasma AUC of desipramine, a sensitive CYP2D6 in vivo probe drug (Table 5). The enzyme most potently inhibited by duloxetine in vitro was CYP2D6, and the most pronounced clinical drug-drug interaction reported for duloxetine is its interaction with desipramine, a drug whose clearance is largely dependent (80–90%) on metabolism by CYP2D6. However, the inhibition of CYP2D6 observed in vivo would not be predicted from the in vitro inhibition data based on [I]/K$_i$. The average maximum plasma concentrations of duloxetine at steady state (C$_{max}$) is 20.7 ng/ml (assuming a conservative estimate of 3.5 μM) and a principal metabolizing enzyme.

**TABLE 5**

*Summary of clinical drug-drug interaction studies to evaluate the effect of duloxetine on the disposition of coadministered drugs*

<table>
<thead>
<tr>
<th>Victim Drug</th>
<th>n$^a$</th>
<th>Enzyme$^b$</th>
<th>Treatment</th>
<th>Arithmetic Mean AUC Change</th>
<th>AUC Geometric Mean Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theophylline</td>
<td>28</td>
<td>CYP1A2</td>
<td>Duloxetine: 60 mg b.i.d. (days 1–4), then 60 mg q.d. (day 5)</td>
<td>1.13</td>
<td>1.07–1.18$^c$</td>
<td>Lobo et al., 2008</td>
</tr>
<tr>
<td>Temazepam</td>
<td></td>
<td>UGT</td>
<td>Duloxetine: 20 mg q.d. (days 1–6)</td>
<td>8.6</td>
<td>1.11–1.21</td>
<td>Duloxetine NDA (21-427)$^d$</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>16</td>
<td>UGT</td>
<td>Duloxetine: 60 mg b.i.d. (days 1–6)</td>
<td>8.2</td>
<td>1.08–1.16</td>
<td>Duloxetine NDA (21-427)$^d$</td>
</tr>
<tr>
<td>Desipramine</td>
<td>17</td>
<td>CYP2D6</td>
<td>Duloxetine: 30 mg b.i.d. (days 6–15)</td>
<td>107</td>
<td>2.22–2.51</td>
<td>Patroneva et al., 2008</td>
</tr>
<tr>
<td>Desipramine</td>
<td>13</td>
<td>CYP2D6</td>
<td>Duloxetine: 40 mg b.i.d. (days 8–13), then 60 mg b.i.d. (days 14–27)</td>
<td>168</td>
<td>2.92–3.34</td>
<td>Skinner et al., 2003</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>16</td>
<td>CYP2D6</td>
<td>Duloxetine: 30 mg q.d. (day 1), then 60 mg q.d. (days 2–17)</td>
<td>180$^e$</td>
<td></td>
<td>Preskorn et al., 2007</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>16</td>
<td>CYP2D6, 3A4</td>
<td>Duloxetine: 40 mg b.i.d. (days 1–4), then 40 mg q.d. (day 5)</td>
<td>94.5$^f$</td>
<td>1.71–2.23$^g$</td>
<td>Hua et al., 2004</td>
</tr>
</tbody>
</table>

CI, confidence interval; Unk., unknown; UGT, UDP-glucuronosyltransferase; NDA, new drug application.

$^a$ Number of subjects completing each study.

$^b$ Principal metabolizing enzyme.

$^c$ Theophylline AUC increased 20% in women (n = 18; statistically significant) but only 7% in men (n = 10; statistically insignificant).

$^d$ Duloxetine NDA (21-427); http://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/021427_s000_Cymbalta.cfm.

$^e$ Original value reported in Table 5 in Preskorn et al. (2007).

$^f$ Recalculated value based on AUC values reported in Table 4 in Preskorn et al. (2007). The recalculated value agrees with that reported in the University of Washington Metabolism and Transport Drug Interaction Database (MTDI database: http://www.druginteractioninfo.org).

$^g$ Mean 90% CI (all other values are 90% CI).

$^h$ Value represents 95% CI (all other values are 90% CI).

In summary, the results of this in vitro study established that duloxetine inhibits CYP2D6 and other P450 enzymes and has been shown to cause clinically significant inhibition of CYP2D6. In contrast, the only human P450 enzyme inhibited by milnacipran is CYP3A4, which milnacipran inhibited weakly and in a substrate-dependent manner (midazolam, but not testosterone). Milnacipran would not be expected to produce clinically significant inhibition of P450 enzymes, which is consistent with clinical data demonstrating a lack of interaction between milnacipran and drugs metabolized by
CYP1A2, 2C9, 2C19, 2D6, or 3A4. In addition, the results of the present study suggest that milnacipran will not produce clinically significant induction of P450 enzymes.

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References


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