Inhibition of Cytochrome P450 Enzymes by the *E*- and *Z*-Isomers of Norendoxifen

Jinzhong Liu, Peter J. Flockhart, Deshun Lu, Wei Lv, Wenjie Jessie Lu, Xu Han, Mark Cushman, and David A. Flockhart

Division of Clinical Pharmacology, Department of Medicine, Indiana University School of Medicine, Indiana Institute for Personalized Medicine, Indianapolis, Indiana (J.L., P.J.F., D.L., W.J.L., X.H., D.A.F.); and Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, and The Purdue Center for Cancer Research, Purdue University, West Lafayette, Indiana (W.L., M.C.)

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ABSTRACT

Aromatase catalyzes the conversion of testosterone to estradiol and is the main source of endogenous estrogen in postmenopausal women. Aromatase inhibitors (AIs) are used to treat postmenopausal women with hormone receptor–positive breast cancer. Norendoxifen [4-(1-(4-(2-aminoethoxy)phenyl)-2-phenylbut-1-en-1-yl)phenol], an active metabolite of the selective estrogen receptor modulator tamoxifen, has been shown to be a potent competitive AI, with an IC_{50} of 90 nM. To obtain data relevant to the clinical use of norendoxifen, the primary objective of this study was to investigate norendoxifen's inhibitory capability on enzymes related to drug-drug interactions. We determined the inhibitory ability of norendoxifen against important drug-metabolizing cytochrome P450 enzymes, including CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19, to establish the potency of norendoxifen as a potential

cause of drug-drug interactions. A second objective was to determine the effects of *E*- and *Z*-norendoxifen on the inhibition of these enzymes to further characterize the isomers' selectivity. The inhibitory abilities of *E*-, mixed, and *Z*-norendoxifen against recombinant aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5, and CYP2C19 were tested using microsomal incubations. Mixed norendoxifen inhibited these enzymes with K_i values of 70 ± 9 , 76 ± 3 , 375 ± 6 , 829 ± 62 , and 0.56 ± 0.02 nM, respectively. *E*-Norendoxifen had a 9.3-fold-higher inhibitory ability than *Z*-norendoxifen against CYP19, while *E*- and *Z*-norendoxifen had similar potencies against CYP1A2, CYP3A4, CYP3A5, and CYP2C19. These results suggest that norendoxifen is able to act as a potent AI, and that its *E*-isomer is 9.3-fold more potent than the *Z*-isomer.

Introduction

Drugs used to treat hormone receptor–positive breast cancer can be divided into two classes (Pickar et al., 2010; Yu et al., 2012): the selective estrogen receptor modulators (SERMs) and the aromatase inhibitors (AIs). Both classes of drugs are designed to interfere with the effects of estrogen, which plays an important role in the development and proliferation of breast cancer. SERMs reduce estrogenic effects by antagonism of estrogen binding to the estrogen receptors (ERs), whereas AIs act by reducing the generation of estrogens from androgens via CYP19 (Ziller et al., 2009). The conversion of testosterone to estradiol by CYP19 is the main source of endogenous estrogen in postmenopausal women (Subbaramaiah et al., 2008).

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Tamoxifen is the most widely used SERM that is approved by the US Food and Drug Administration. It has a complex metabolic profile involving both active and inactive metabolites (Jin et al., 2005). Previous studies have shown that two tamoxifen metabolites, *N*-desmethyltamoxifen (*N*-DMT) and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), can act as AIs in vitro (Lu et al., 2012a). Recently, *N*, *N*-didesmethyl-4-hydroxy-tamoxifen (norendoxifen), another active metabolite of tamoxifen, has also been shown to be a potent AI. Norendoxifen inhibited recombinant CYP19 via a competitive mechanism with an IC_{50} of 90 nM and inhibited human liver CYP2C9 with an IC_{50} of 990 nM. In this first study published, the inhibition of CYP2C19 activity by norendoxifen in human liver microsomes using *R*-omeprazole as a substrate probe was very weak, with <25% enzyme activity reduced when the concentration of norendoxifen was 5 μ M. No inhibition of CYP2B6 or CYP2D6 by 5 μ M norendoxifen was detected (Lu et al., 2012b).

Norendoxifen has been identified as a promising new class of AIs under development, and it should be evaluated for drug-drug interactions as a proposed new drug that undergoes significant metabolism by the cytochrome P450 (P450) system. In the development of any new therapeutic drug that undergoes metabolism, it is important to understand which enzyme is involved in the metabolic

ABBREVIATIONS: AI, aromatase inhibitor; BFC, 7-benzoyloxy-4-trifluoromethylcoumarin; CEC, 3-cyano-7-ethoxycoumarin; CHC, 3-cyano-7-hydroxycoumarin; CYP19, aromatase; *N*-DMT, *N*-desmethyltamoxifen; endoxifen, 4-hydroxy-*N*-desmethyltamoxifen; ER, estrogen receptor; HC, 7-hydroxycoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; 4HT, 4-hydroxytamoxifen; norendoxifen, *N,N*-didesmethyl-4-hydroxy-tamoxifen; MFC, 7-methoxy-4-trifluoromethylcoumarin; P450, cytochrome P450; SERM, selective estrogen receptor modulator.

disposition as this may influence our understanding of bioavailability of drug-drug interactions and of pharmacogenetic effects. The P450 superfamily consists of a large group of enzymes, including CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2B6, CYP2C9, CYP2C19, and CYP2D6, which are important in clinical drug-drug interactions and in drug metabolism (Stearns et al., 2003; Ward et al., 2004; Flockhart et al., 2008; Jeong et al., 2009; Kamdem et al., 2011). About 90% of human drug oxidation can be attributed to CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2C9, and CYP2C19 (Rendic, 2002). In this context, the inhibitory ability of norendoxifen against CYP1A2, CYP2A6, CYP3A4, and CYP3A5 has not been described. Since both IC_{50} and K_i values are important descriptions for drug-enzyme interactions, it follows that further studies are required to determine the effects of norendoxifen on these enzymes. The IC_{50} and K_i values of norendoxifen against recombinant CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19 were tested using microsomal incubations to determine the effects of norendoxifen as a P450 inhibitor for these enzymes. The double bond in norendoxifen leads to *E*- and *Z*-isomers, which may possess different inhibitory activities against different P450s due to their different chemical structures. The chemical structures of the *E*- and *Z*-isomers of norendoxifen are shown in Fig. 1. To investigate the inhibitory specificity of *E*- and *Z*-norendoxifen, we determined their IC_{50} and K_i values against recombinant CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19 in the same way using microsomal incubations. In this way, the selective effects of norendoxifen isomers were determined.

Materials and Methods

Chemicals and Reagents. The *E*-, mixed, and *Z*-norendoxifen were provided by Dr. Mark Cushman (Purdue University, West Lafayette, IN). The chemical makeup of the *E*-norendoxifen described in the studies is 100:1 *E/Z*, whereas the *Z*-isomer is 1:10 *E/Z*. They were stored at -20°C without light. P450 inhibitor screening kits of CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19 were purchased from BD Biosciences (San Jose, CA).

Inhibition of Recombinant Human P450 Enzymes by Microsomal Incubations. The activity of each recombinant human P450 enzyme was determined by measuring the conversion rate of a fluorometric substrate to its fluorescent metabolite. The activity of CYP19 was determined using the metabolism of 7-methoxy-4-trifluoromethylcoumarin (MFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). The activities of CYP1A2 and CYP2C19 were determined using the metabolism of 3-cyano-7-ethoxycoumarin (CEC) to 3-cyano-7-hydroxycoumarin (CHC). The activity of CYP2A6 was determined using the metabolism of coumarin to 7-hydroxycoumarin (HC). The activities of CYP3A4 and CYP3A5 were determined using the metabolism of 7-benzoyloxy-4-trifluoromethylcoumarin (BFC) to HFC. Experimental procedures were essentially as described previously (Lu et al., 2011). All the incubations were performed using incubation times and protein concentrations that were within the linear range for reaction velocity. All the substrates were dissolved in acetonitrile (25 mM final concentration for MFC, 20 mM final concentration for CEC, 1.1 mM final concentration for coumarin, and 50 mM final

concentration for BFC). *E*- and mixed norendoxifen were dissolved in methanol/dichloromethane [1:1 (v/v), 10 mM final concentration stock]. *Z*-Norendoxifen was dissolved in methanol (10 mM final concentration stock). During serial dilutions, all the *E*-, mixed, and *Z*-norendoxifen were diluted in methanol to required concentrations. A series of concentrations of *E*-, mixed, and *Z*-norendoxifen in a volume of 2 μl were mixed with 98 μl of NADPH-cofactor mix (16.25 μM NADP⁺, 825 μM MgCl₂, 825 μM glucose 6-phosphate, and 0.4 units/ml glucose-6-phosphate dehydrogenase) and were prewarmed for 10 minutes at 37°C . The enzyme/substrate mix was prepared with fluorometric substrate, recombinant human P450 enzymes, and 0.1 M potassium phosphate buffer (pH 7.4). Reactions were initiated by adding 100 μl enzyme/substrate mix to bring the incubation volume to 200 μl , and incubated for 30 minutes. All the reactions were stopped by adding 75 μl of 0.1 M Tris base dissolved in acetonitrile. The amount of fluorescent product was determined immediately by measuring fluorescence response using a BioTek (Winooski, VT) Synergy 2 fluorometric plate reader. Excitation-emission wavelengths for MFC metabolite and BFC metabolite were 409–530 nm, for CEC metabolite were 410–460 nm, for coumarin metabolite were 390–460 nm. Standard curves were constructed using the appropriate fluorescent metabolite standards. Quantification of samples was performed by applying the linear regression equation of the standard curve to the fluorescence response. The limits of quantification for the metabolites of MFC, CEC, coumarin, and BFC were 24.7, 66.7, 74.1, and 222.2 pmol in a final volume of 200 μl , respectively, with intra- and inter-assay coefficients of variations <10%.

Kinetic Analysis of Recombinant Human P450 Enzymes. The rates of metabolite formation in the presence of test inhibitors were compared with those in the control incubation, in which the inhibitor was replaced with vehicle. The extent of enzyme inhibition was expressed as the percentage of remaining enzyme activity compared with the control. IC_{50} was determined as the inhibitor concentration that brought about a 50% reduction in enzyme activity by fitting all the data to a one-site competition equation using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). To characterize the inhibitory mechanism of norendoxifen against CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19, all inhibitory data for norendoxifen at different substrate concentrations were plotted as Lineweaver-Burk and Dixon plots. The inhibitory constant K_i values were determined by nonlinear least-squares regression analysis using GraphPad Prism 5.0. Before modeling the data using nonlinear models, initial information about the inhibitory mechanism was obtained by visual inspection of Lineweaver-Burk and Dixon plots. Final decisions on the mechanism of inhibition were made on model-derived parameters, such as R^2 and absolute sum of squares.

Results

To test the potency of mixed norendoxifen against CYP19 and understand whether the inhibitory ability of norendoxifen on CYP19 extends to other important drug-drug interactions and drug-metabolizing enzymes in the P450 superfamily, the inhibition of P450 enzymes by norendoxifen, including CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19, was tested using microsomal incubations. CEC was used as a substrate probe for CYP2C19 to further explore the possibility of substrate probe-dependent inhibition. Figure 2 and Table 1 show the inhibitory potency of norendoxifen against these enzymes. The order of inhibitory potency of norendoxifen against these enzymes was as follows: recombinant CYP2C19 > CYP19 > CYP1A2 > CYP3A4 > CYP3A5 > CYP2A6. Norendoxifen inhibited recombinant CYP2C19, CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2A6 with IC_{50} values of 2.80 ± 0.29 , 131 ± 54 , 207 ± 26 , 285 ± 81 , 723 ± 27 , and 6373 ± 983 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for these recombinant enzymes was 0.9853, 0.9734, 0.9866, 0.9372, 0.9962, and 0.9866, respectively.

Figure 3 shows the Dixon plots of inhibition of CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19 by norendoxifen. Norendoxifen inhibited CYP19, CYP1A2, CYP2A6, and CYP2C19 via a competitive

Fig. 1. Chemical structures of the *E*- and *Z*-isomers of norendoxifen.

Fig. 2. Inhibition of P450 enzymes by *E,Z*-norendoxifen. In the presence of a range of concentrations of mixed norendoxifen, the remaining enzyme activities of recombinant CYP19, CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19 were determined by measuring the conversion rates from specific fluorometric substrates to their fluorescent metabolites. The extent of enzyme inhibition was expressed as the percentage of remaining enzyme activity compared with the control. Each point represents the mean of four independent incubations, and error bars represent the S.D.'s of four independent points.

mechanism, whereas norendoxifen inhibited CYP3A4 and CYP3A5 via a noncompetitive mechanism. Norendoxifen showed significantly different inhibitory activity against CYP2C19 when CEC was used as a substrate relative to that seen when *R*-omeprazole was used.

To determine and characterize the selective effects of norendoxifen isomers against these enzymes, the inhibitory activities of *E*- and *Z*-norendoxifen against recombinant CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19 were further tested in the same way using microsomal incubations (Fig. 4; Table 2). The IC_{50} values of *E*- and *Z*-norendoxifen against recombinant CYP19 were 98 ± 40 and 1053 ± 185 nM, respectively (Fig. 4A); the goodness of fit (R^2) between the inhibition model and the data for recombinant CYP19 was 0.9895 and 0.9917, respectively. The IC_{50} values of *E*- and *Z*-norendoxifen against recombinant CYP1A2 were 160 ± 22 and 285 ± 43 nM, respectively (Fig. 4B); the goodness of fit (R^2) between the inhibition model and the data for recombinant CYP1A2 was 0.9966 and 0.9960, respectively. The IC_{50} values of *E*- and *Z*-norendoxifen against recombinant CYP3A4 were 182 ± 79 and 925 ± 145 nM, respectively (Fig. 4C); the goodness of fit (R^2) between the inhibition model and the data for recombinant CYP3A4 was 0.9699 and 0.9657, respectively. The IC_{50} values of *E*- and *Z*-norendoxifen against recombinant CYP3A5 were 930 ± 66 and 655 ± 27 nM, respectively (Fig. 4D); the goodness of fit (R^2) between the inhibition model and the data for recombinant CYP3A5 was 0.9931 and 0.9825, respectively.

TABLE 1

IC_{50} and K_i values of *E,Z*-norendoxifen against important P450 enzymes

Important Enzymes of P450 Superfamily	IC_{50}	K_i
	nM	
Recombinant CYP19	131 ± 54	70 ± 9
Recombinant CYP1A2	207 ± 26	76 ± 3
Recombinant CYP2A6	6373 ± 983	2176 ± 256
Recombinant CYP3A4	285 ± 81	375 ± 6
Recombinant CYP3A5	723 ± 27	829 ± 62
Recombinant CYP2C19	2.80 ± 0.29	0.56 ± 0.02
Recombinant CYP2B6	No inhibition ^a	
Recombinant CYP2D6	No inhibition ^a	

^a No inhibition of CYP2B6 or CYP2D6 by norendoxifen was observed at the concentration of 5 μ M.

The IC_{50} values of *E*- and *Z*-norendoxifen against recombinant CYP2C19 were 1.90 ± 0.35 and 3.88 ± 0.79 nM, respectively (Fig. 4E); the goodness of fit (R^2) between the inhibition model and the data for recombinant CYP2C19 was 0.9812 and 0.9636, respectively.

To explore the inhibitory mechanism employed by norendoxifen, the inhibitory effects of *E*-, mixed, and *Z*-norendoxifen were tested against recombinant CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19 across a range of fluorometric substrate concentrations (Table 3). *E*-, mixed, and *Z*-norendoxifen inhibited recombinant CYP19 via a competitive mechanism with K_i values of 48 ± 3 , 70 ± 9 , and 445 ± 6 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for recombinant CYP19 was 0.9880, 0.9447, and 0.9625, respectively. *E*-, mixed, and *Z*-norendoxifen inhibited recombinant CYP1A2 via a competitive mechanism with K_i values of 49 ± 3 , 76 ± 3 , and 96 ± 8 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for recombinant CYP1A2 was 0.9790, 0.9620, and 0.9618, respectively. *E*-, mixed, and *Z*-norendoxifen inhibited recombinant CYP3A4 via a noncompetitive mechanism with K_i values of 242 ± 9 , 375 ± 6 , and 910 ± 59 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for recombinant CYP3A4 was 0.9459, 0.8707, and 0.9399, respectively. *E*-, mixed, and *Z*-norendoxifen inhibited recombinant CYP3A5 via a noncompetitive mechanism with K_i values of 859 ± 76 , 829 ± 62 , and 707 ± 17 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for recombinant CYP3A5 was 0.9474, 0.9719, and 0.9621, respectively. *E*-, mixed, and *Z*-norendoxifen inhibited recombinant CYP2C19 via a competitive mechanism with K_i values of 0.48 ± 0.05 , 0.56 ± 0.02 , and 0.70 ± 0.11 nM, respectively. The goodness of fit (R^2) between the inhibition model and the data for recombinant CYP2C19 was 0.8562, 0.8993, and 0.8989, respectively.

Discussion

The current third-generation AIs, including letrozole, anastrozole, and exemestane, are demonstrably superior to tamoxifen as agents to reduce the recurrence of breast cancer in postmenopausal women (Howell et al., 2005; Fallowfield et al., 2006; Giobbie-Hurder et al., 2009). Their efficacy is limited, however, by toxicities that reduce quality of life and compromise compliance (Santoro et al., 2011;

Fig. 3. Dixon plots of inhibition of CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19 by *E,Z*-norendoxifen. (A) CEC (2.5–10 μ M) was incubated with 2.5 nM recombinant CYP1A2 in the presence of increasing norendoxifen concentrations (0–90 nM). (B) Coumarin (3–7 μ M) was incubated with 5 nM recombinant CYP2A6 in the presence of increasing norendoxifen concentrations (0–11 μ M). (C) BFC (25–100 μ M) was incubated with 5 nM recombinant CYP3A4 in the presence of increasing norendoxifen concentrations (0–1600 nM). (D) BFC (25–100 μ M) was incubated with 5 nM recombinant CYP3A5 in the presence of increasing norendoxifen concentrations (0–1150 nM). (E) CEC (10–75 μ M) was incubated with 5 nM recombinant CYP2C19 in the presence of increasing norendoxifen concentrations (0–4 nM). Each point represents one incubation, and two independent incubations were conducted for each concentration of norendoxifen and substrate.

Thompson et al., 2011). Obviously, new AIs with less toxicity are needed to allow better treatment of hormone receptor-positive breast cancer in postmenopausal women. We identified a new AI, namely norendoxifen. Norendoxifen becomes an attractive lead compound for

a new class of AIs for two unique features. On one hand, the potent inhibitory ability of norendoxifen may reduce estrogen biosynthesis in the breast to inhibit the tumor growth, while its potential SERM activity may make it possible to ameliorate the side effects in bone and

Fig. 4. Inhibition of CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19 by *E*- and *Z*-norendoxifen. (A) The remaining enzyme activities of recombinant CYP19 in the presence of a range of concentrations of *E*-norendoxifen and *Z*-norendoxifen were determined by measuring the formation rates from MFC to HFC. (B) The remaining enzyme activities of recombinant CYP1A2 in the presence of a range of concentrations of *E*-norendoxifen and *Z*-norendoxifen were determined by measuring the formation rates from CEC to CHC. (C) The remaining enzyme activities of recombinant CYP3A4 in the presence of a range of concentrations of *E*-norendoxifen and *Z*-norendoxifen were determined by measuring the formation rates from BFC to HFC. (D) The remaining enzyme activities of recombinant CYP3A5 in the presence of a range of concentrations of *E*-norendoxifen and *Z*-norendoxifen were determined by measuring the formation rates from BFC to HFC. (E) The remaining enzyme activities of recombinant CYP2C19 in the presence of a range of concentrations of *E*-norendoxifen and *Z*-norendoxifen were determined by measuring the formation rates from CEC to CHC. Each point represents the mean of four independent incubations, and error bars represent the S.D.'s of four independent points.

TABLE 2

IC₅₀ values of *E*-, mixed, and *Z*-norendoxifen against CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19

Target Enzyme	IC ₅₀ of:		
	<i>E</i> -Norendoxifen	Mixed Norendoxifen	<i>Z</i> -Norendoxifen
	<i>nM</i>		
Aromatase (CYP19)	98 ± 40	131 ± 54	1053 ± 185
CYP1A2	160 ± 22	207 ± 26	285 ± 43
CYP3A4	182 ± 79	285 ± 81	925 ± 145
CYP3A5	930 ± 66	723 ± 27	655 ± 27
CYP2C19	1.90 ± 0.35	2.80 ± 0.29	3.88 ± 0.79

other tissues caused by estrogen depletion. On the other hand, because norendoxifen is a metabolite of the most widely used SERM, tamoxifen, lots of patients have already been exposed to it (Lv et al., 2013). The bioavailability and relative safety of norendoxifen instill more confidence than other lead compounds under development.

The metabolism of tamoxifen results in two main metabolites, *N*-DMT and *Z*-4-hydroxytamoxifen (*Z*-4HT). *N*-DMT is converted into endoxifen, a minor metabolite, mainly by CYP2D6, while *Z*-4HT is converted into endoxifen mainly by CYP3A4 and CYP3A5 (Del Re et al., 2012). Endoxifen is demethylated to norendoxifen via a metabolic route that is still unknown. Among tamoxifen metabolites, 4HT and endoxifen have been shown to be SERMs, serving as antagonists of estrogen binding to the ERs (Lim et al., 2005). *N*-DMT and endoxifen act as AIs with IC₅₀ values of 6.1 and 20.7 μM, respectively, via noncompetitive mechanisms (Lu et al., 2012a). In previous studies, we have characterized the inhibitory ability of norendoxifen against recombinant CYP19, CYP2B6, CYP2D6, human liver CYP2C9, and CYP2C19. Other members of the P450 superfamily, including CYP1A2, CYP2A6, CYP3A4, and CYP3A5, also play important roles. In human liver, CYP1A2 accounts for 13% of the P450 content and catalyzes the primary metabolic route for a number of important drugs, including caffeine, clozapine, flutamide, lidocaine, olanzapine, and zolmitriptan (Zhou et al., 2010). CYP2A6 is a major P450 isoform in human liver, specifically involved in the oxidative metabolism of nicotine. It is also involved in the metabolism of pharmaceutical agents such as methoxyflurane, halothane, losigamone, letrozole, valproic acid, disulfiram, and fadrozole (Ridderstrom et al., 2001; Desta et al., 2011). CYP3A4 and CYP3A5 account for ~50% of the P450 content and are the predominant P450 contributors to metabolism in human liver, accounting for 40%–60% of the oxidative metabolism of marketed drugs (Pearson et al., 2007).

Any proposed new drug that undergoes significant metabolism by the P450 system should be evaluated for drug-drug interactions. In this study, we tested mixed norendoxifen's effects on the inhibition

of important P450 enzymes that were not studied before, including CYP1A2, CYP2A6, CYP3A4, and CYP3A5. To verify that the inhibition profiles of CYP2C19 are dependent on a specific inhibitor-substrate interaction, CEC was used as another substrate probe compared with *R*-omeprazole.

A previous study showed substrate-dependent inhibition of CYP2C19 when *S*-mephenytoin, *R*-omeprazole, *S*-omeprazole, and *S*-fluoxetine were used as substrates (Foti and Wahlstrom, 2008). Our studies with *R*-omeprazole and CEC confirmed that inhibition of CYP2C19 is substrate-dependent. CEC has high sensitivity to in vitro inhibition of CYP2C19, whereas no inhibition was observed using *R*-omeprazole as substrate. An analysis of homology models of CYP2C enzymes reveals more than one binding region within the active site, and this may be the mechanism that underlies the substrate-dependent inhibition seen (Ridderstrom et al., 2001). It is also possible that the differences in incubation conditions, such as enzyme sources, incubation times, and measurement methods, may also contribute to the different inhibition profiles that we observed.

Further study was conducted on testing the inhibitory activities of *E*- and *Z*-norendoxifen against CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19. Norendoxifen has high isomer selectivity against CYP19. *E*-Norendoxifen had 9.3-fold-higher inhibitory ability than *Z*-norendoxifen against CYP19. *E*-Norendoxifen inhibited CYP1A2 and CYP3A4 2.0-fold and 3.7-fold, respectively, more potently than *Z*-norendoxifen. *E*- and *Z*-norendoxifen had similar inhibitory ability against CYP3A5 and CYP2C19. The presence of the double bond in norendoxifen leads to the different inhibitory activities observed in the *E*- and *Z*-isomers against CYP19.

As a minor metabolite of tamoxifen, norendoxifen turns out to be the most potent AI among the known metabolites that we have tested. The data reveal a complex metabolism of tamoxifen that may lead to some metabolites that are able to inhibit CYP19 or act as a SERM or combine both of these activities. Since endoxifen functions both as a SERM and an AI, norendoxifen may also be an antagonist to ERs due to their similar chemical structures. Also, the contribution of norendoxifen to the overall effects of tamoxifen remains unknown, and its concentration in patients is not well defined. Studies have shown that endoxifen has low concentration in plasma; however, the tissue concentrations of endoxifen are higher, appearing to be 10–100 times more (Lien et al., 1991). The concentration of norendoxifen in the tissue may be higher, as demonstrated with endoxifen. Because of norendoxifen's potent inhibitory ability against CYP19, it may significantly increase the effects of tamoxifen in vivo. All of these possibilities deserve more study and are important for better understanding of tamoxifen's function and novel drug development for breast cancer.

As we know, potent and selective AIs with limited side effects are required to improve the treatment of hormone receptor-positive breast cancer in postmenopausal women. Norendoxifen has the potential to be a therapeutically useful AI with fewer side effects, and in the meantime, it provides a new lead compound for the rational design of a series of novel compounds with dual aromatase-inhibitory and ER-modulatory activity. The high selectivity of *E*-norendoxifen also provides new information for the development of potent AIs. Although the main form of norendoxifen as the metabolite of tamoxifen in human body is the *Z*-isomer, the toxicity and metabolism of the *E*-isomer deserve more study.

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TABLE 3

K_i values of *E*-, mixed, and *Z*-norendoxifen against CYP19, CYP1A2, CYP3A4, CYP3A5, and CYP2C19

Target Enzyme	K _i of:		
	<i>E</i> -Norendoxifen	Mixed Norendoxifen	<i>Z</i> -Norendoxifen
	<i>nM</i>		
CYP19	48 ± 3	70 ± 9	445 ± 6
CYP1A2	49 ± 3	76 ± 3	96 ± 8
CYP3A4	242 ± 9	375 ± 6	910 ± 59
CYP3A5	859 ± 76	829 ± 62	707 ± 17
CYP2C19	0.48 ± 0.05	0.56 ± 0.02	0.70 ± 0.11

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Authorship Contributions

Participated in research design: Liu, D. Lu, D. A. Flockhart.

Conducted experiments: Liu, P. J. Flockhart, Han.

Contributed new reagents or analytic tools: Lv, Cushman.

Performed data analysis: Liu, D. Lu, W. J. Lu, P. J. Flockhart, D. A. Flockhart.

Wrote or contributed to the writing of the manuscript: Liu, D. Lu, W. J. Lu, Cushman, D. A. Flockhart.

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Address correspondence to: Jinzhong Liu, Division of Clinical Pharmacology, Department of Medicine, Indiana University School of Medicine, Indiana Institute for Personalized Medicine, 1001 West 10th Street, Room 7123, Wishard Memorial Hospital Myers Building, Indianapolis, IN 46202. E-mail: jinzliu@iu.edu