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

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Abbreviations:

CYP, cytochrome P450; CYP19, aromatase; AI, aromatase inhibitor; SERM, selective estrogen receptor modulator; N-DMT, N-desmethyltamoxifen; 4HT, 4-hydroxytamoxifen; Endoxifen, 4-hydroxy-N-desmethyltamoxifen; Norendoxifen, N,N-didesmethyl-4-hydroxy-tamoxifen; IC₅₀, the half maximal inhibitory concentration; Kᵢ, the equilibrium dissociation constant of the inhibitor; MFC, 7-methoxy-4-trifluoromethylcoumarin; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; CEC, 3-cyano-7-ethoxycoumarin; CHC, 3-cyano-7-hydroxycoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; HC, 7-hydroxycoumarin.
Abstract

Aromatase catalyzes the conversion of testosterone to estradiol, and is the main source of endogenous estrogen in post-menopausal women. Aromatase inhibitors (AIs) are used to treat post-menopausal women with hormone receptor-positive breast cancer. Norendoxifen, an active metabolite of the selective estrogen receptor modulator (SERM), tamoxifen, has been shown to be a potent competitive AI with an IC<sub>50</sub> of 90 nM. In order 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 ability 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<sub>i</sub> values of 70 ± 9 nM, 76 ± 3 nM, 375 ± 6 nM, 829 ± 62 nM and 0.56 ± 0.02 nM, respectively. E-norendoxifen had a 9.3-fold higher inhibitory ability than Z-norendoxifen against aromatase (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), while AIs act by reducing the generation of estrogens from androgens via aromatase (CYP19) (Ziller et al., 2009). The conversion of testosterone to estradiol by aromatase (CYP19) is the main source of endogenous estrogen in post-menopausal women (Subbaramaiah et al., 2008).

Tamoxifen is the most widely used SERM that is approved by the FDA (the United States 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 endoxifen, can act as AIs in vitro (Lu et al., 2012a). Recently, norendoxifen, another active metabolite of tamoxifen, has also been shown to be a potent AI. Norendoxifen inhibited recombinant aromatase (CYP19) via a competitive mechanism with an IC50 of 90 nM and inhibited human liver CYP2C9 with an IC50 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 less than 25% enzyme activity reduced when the concentration of norendoxifen was 5 μM. No inhibition of CYP2B6 or CYP2D6 by 5 μM norendoxifen was detected (Lu et al., 2012b).

Norendoxifen has been identified as a promising new class of aromatase inhibitors (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 system. In the development of any
new therapeutic drug that undergoes metabolism, it is important to understand which enzyme is involved in the metabolic disposition. Since this may influence our understanding of bioavailability of drug-drug interactions and of pharmacogenetic effects. The cytochrome P450 superfamily consists of a large group of enzymes, including aromatase (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 IC₅₀ and Kᵢ 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 IC₅₀ and Kᵢ values of norendoxifen against recombinant aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19 were tested using microsomal incubations to determine the effects of norendoxifen as a cytochrome P450 inhibitor for these enzymes. The double bond in norendoxifen leads to E- and Z-isomers, which may possess different inhibitory activities against different cytochromes P450 due to their different chemical structures. The chemical structures of the E- and Z-isomers of norendoxifen are shown in Figure 1. In order to investigate the inhibitory specificity of the E- and Z-norendoxifen, we determined their IC₅₀ and Kᵢ values against recombinant aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 in the same way using microsomal incubations. In this way, the isomers’ selective effects of norendoxifen 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. Cytochrome P450 (CYP) inhibitor screening kits of aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19 were purchased from BD Biosciences (San Jose, CA).

Inhibition of Recombinant Human Cytochrome P450 Enzymes by Microsomal Incubations.

The activity of each recombinant human cytochrome P450 enzyme was determined by measuring the conversion rate of a fluorometric substrate to its fluorescent metabolite. The activity of aromatase (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-benzyloxy-4-trifluoromethyl-coumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (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).
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 \( \mu L \) were mixed with 98 \( \mu L \) of NADPH-Cofactor Mix (16.25 \( \mu M \) NADP*, 825 \( \mu M \) MgCl₂, 825 \( \mu M \) glucose-6-phosphate and 0.4 Units/mL glucose-6-phosphate dehydrogenase), and were pre-warmed for 10 minutes at 37°C. The Enzyme/Substrate Mix was prepared with fluorometric substrate, recombinant human cytochrome P450 enzymes and 0.1 M potassium phosphate buffer (pH 7.4). Reactions were initiated by adding 100 \( \mu L \) Enzyme/Substrate Mix to bring the incubation volume to 200 \( \mu L \), and incubated for 30 minutes. All the reactions were stopped by adding 75 \( \mu L \) of 0.1 M tris base dissolved in acetonitrile. The amount of fluorescent product was determined immediately by measuring fluorescent 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 pmol, 66.7 pmol, 74.1 pmol and 222.2 pmol in a final volume of 200 \( \mu L \), respectively, with intra- and inter-assay coefficients of variations less than 10%.

**Kinetic Analysis of Recombinant Human Cytochrome 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 percentage of remaining enzyme activity compared to the control. IC\(_{50}\) was determined as the inhibitor concentration which 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., San Diego, CA). To characterize the inhibitory mechanism of norendoxifen against aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19, all inhibitory data of norendoxifen at different substrate concentrations were plotted as Lineweaver-Burk and Dixon plots. The inhibitory constant $K_i$ values were determined by nonlinear least square regression analysis using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). 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 Square ($R^2$) and absolute sum of squares.
Results

To test the potency of mixed norendoxifen against aromatase (CYP19) and understand whether the inhibitory ability of norendoxifen on aromatase (CYP19) extends to other important drug-drug interaction and drug-metabolizing enzymes in the cytochrome P450 superfamily, the inhibition of cytochrome P450 enzymes by norendoxifen, including aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19, were tested using microsomal incubations. 3-Cyano-7-ethoxycoumarin (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 > aromatase (CYP19) > CYP1A2 > CYP3A4 > CYP3A5 > CYP2A6. Norendoxifen inhibited recombinant CYP2C19, aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2A6 with IC₅₀ values of 2.80 ± 0.29 nM, 131 ± 54 nM, 207 ± 26 nM, 285 ± 81 nM, 723 ± 27 nM and 6373 ± 983 nM, respectively. The goodness of fit (R²) between the inhibition model and the data for these recombinant enzymes were 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 aromatase (CYP19), CYP1A2, CYP2A6 and CYP2C19 via a competitive mechanism, while 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.

In order to determine and characterize the isomers’ selective effects of norendoxifen against these enzymes, the inhibitory activities of E- and Z-norendoxifen against recombinant
aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 were further tested in the same way using microsomal incubations (Table 2, Figure 4). The IC\textsubscript{50} values of \(E\)- and \(Z\)-norenodoxifen against recombinant aromatase (CYP19) were 98 ± 40 nM and 1053 ± 185 nM, respectively (Figure 4A), the goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant aromatase (CYP19) were 0.9895 and 0.9917, respectively. While the IC\textsubscript{50} values of \(E\)- and \(Z\)-norenodoxifen against recombinant CYP1A2 were 160 ± 22 nM and 285 ± 43 nM, respectively (Figure 4B), the goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant CYP1A2 were 0.9966 and 0.9960, respectively. The IC\textsubscript{50} values of \(E\)- and \(Z\)-norenodoxifen against recombinant CYP3A4 were 182 ± 79 nM and 925 ± 145 nM, respectively (Figure 4C), the goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant CYP3A4 were 0.9699 and 0.9657, respectively. The IC\textsubscript{50} values of \(E\)- and \(Z\)-norenodoxifen against recombinant CYP3A5 were 930 ± 66 nM and 655 ± 27 nM, respectively (Figure 4D), the goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant CYP3A5 were 0.9931 and 0.9825, respectively. The IC\textsubscript{50} values of \(E\)- and \(Z\)-norenodoxifen against recombinant CYP2C19 were 1.90 ± 0.35 nM and 3.88 ± 0.79 nM, respectively (Figure 4E), the goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant CYP2C19 were 0.9812 and 0.9636, respectively.

To explore the inhibitory mechanism employed by norenodoxifen, the inhibitory effects of \(E\)-, mixed and \(Z\)-norenodoxifen were tested against recombinant aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 across a range of fluorometric substrate concentrations (Table 3). \(E\)-, mixed and \(Z\)-norenodoxifen inhibited recombinant aromatase (CYP19) via a competitive mechanism with \(K_i\) values of 48 ± 3 nM, 70 ± 9 nM and 445 ± 6 nM, respectively. The goodness of fit (R\textsuperscript{2}) between the inhibition model and the data for recombinant aromatase (CYP19) were 0.9880, 0.9447 and 0.9625, respectively. \(E\)-, mixed and \(Z\)-norenodoxifen inhibited recombinant CYP1A2 via a competitive mechanism with \(K_i\) values of 49 ± 3 nM, 76 ± 3 nM and
96 ± 8 nM, respectively. The goodness of fit ($R^2$) between the inhibition model and the data for recombinant CYP1A2 were 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 nM, 375 ± 6 nM and 910 ± 59 nM, respectively. The goodness of fit ($R^2$) between the inhibition model and the data for recombinant CYP3A4 were 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 nM, 829 ± 62 nM and 707 ± 17 nM, respectively. The goodness of fit ($R^2$) between the inhibition model and the data for recombinant CYP3A5 were 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 nM, 0.56 ± 0.02 nM and 0.70 ± 0.11 nM, respectively. The goodness of fit ($R^2$) between the inhibition model and the data for recombinant CYP2C19 were 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 post-menopausal women (ATAC, IES, BIG-98). Their efficacy is limited, however, by toxicities that reduce quality of life and compromise compliance (Santoro et al., 2011; Thompson et al., 2011). Obviously, new aromatase inhibitors (AIs) with less toxicity are needed to allow better treatment of hormone receptor-positive breast cancer in post-menopausal women. We identified a new aromatase inhibitor (AI), namely norendoxifen. Norendoxifen becomes an attractive lead compound for new class of aromatase inhibitors (AIs) for two unique features. On one hand, the potent inhibitory ability of norendoxifen is efficiently able to reduce estrogen biosynthesis in the breast to inhibit the tumor growth, while, its estrogen receptor modulatory ability make it possible to ameliorate the side effects in bone and other tissues caused by estrogen depletion. On the other hand, since norendoxifen is a metabolite of the most widely used selective estrogen receptor modulator (SERM), tamoxifen, lots of patients have already been exposed to it (Lv et al., 2013). The bioavailability and relative safety of norendoxifen hold more confidence than other lead compounds under development.

The metabolism of tamoxifen results in two main metabolites, N-desmethy tamoxifen (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 selective estrogen receptor modulators (SERMs), serving as antagonists of estrogen binding to the estrogen receptors (ERs) (Lim et al., 2005). N-DMT and endoxifen act as AIs with IC\textsubscript{50} values of 6.1 μM 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 aromatase (CYP19), CYP2B6, CYP2D6, human liver CYP2C9 and CYP2C19. Other members of the cytochrome P450 superfamily, including CYP1A2, CYP2A6, CYP3A4 and CYP3A5, also play important roles. In human liver, CYP1A2 accounts for 13% of the cytochrome 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 cytochrome 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 about 50% of the cytochrome P450 content and are the predominant cytochrome 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 cytochrome P450 system should be evaluated for drug-drug interactions. In this study, we tested mixed norendoxifen’s effects on the inhibition of important cytochrome P450 enzymes, which were not studied before, including CYP1A2, CYP2A6, CYP3A4 and CYP3A5. To verify the inhibition profiles of CYP2C19 are dependent on a specific inhibitor-substrate interaction, 3-cyano-7-ethoxycoumarin (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 \textit{in vitro} inhibition of CYP2C19, while, 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 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 aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19. Norendoxifen has high isomer's selectivity against aromatase (CYP19). $E$-norendoxifen had 9.3-fold higher inhibitory ability than $Z$-norendoxifen against aromatase (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 double bond of norendoxifen does have a significant impact on the inhibitory activities of its $E$- and $Z$-isomers against aromatase (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 aromatase (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 estrogen receptors (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 showed 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 aromatase (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 post-menopausal 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 activity and estrogen receptor modulatory activity. The high selectivity of \(E\)-norendoxifen also provides new information for the development of potent AIs. While 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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Authorship Contributions

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

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

Contributed new reagents or analytic tools: Lv and Cushman

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

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


Footnotes

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Figure Legends

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

**Figure 2.** Inhibition of cytochrome P450 enzymes by norendoxifen. In the presence of a range of concentrations of mixed norendoxifen, the remaining enzyme activity of recombinant aromatase (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 percentage of remaining enzyme activity compared to the control. Each point represents the mean of four independent incubations and error bars represent the standard deviations of four independent points.

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

**Figure 4.** Inhibition of aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 by E- and Z-norendoxifen. (A) The remaining enzyme activity of recombinant aromatase (CYP19) in
the presence of a range of concentrations of \textit{E}-norendoxifen (●) and \textit{Z}-norendoxifen (◆) were determined by measuring the formation rates from MFC to HFC. (B) The remaining enzyme activity of recombinant CYP1A2 in the presence of a range of concentrations of \textit{E}-norendoxifen (●) and \textit{Z}-norendoxifen (◆) were determined by measuring the formation rates from CEC to CHC. (C) The remaining enzyme activity of recombinant CYP3A4 in the presence of a range of concentrations of \textit{E}-norendoxifen (●) and \textit{Z}-norendoxifen (◆) were determined by measuring the formation rates from BFC to HFC. (D) The remaining enzyme activity of recombinant CYP3A5 in the presence of a range of concentrations of \textit{E}-norendoxifen (●) and \textit{Z}-norendoxifen (◆) were determined by measuring the formation rates from BFC to HFC. (E) The remaining enzyme activity of recombinant CYP2C19 in the presence of a range of concentrations of \textit{E}-norendoxifen (●) and \textit{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 standard deviations of four independent points.
### Tables

#### Table 1. IC$_{50}$ and $K_i$ values (nM) of norendoxifen against important cytochrome P450 enzymes

<table>
<thead>
<tr>
<th>Important Enzymes of Cytochrome P450 Superfamily</th>
<th>IC$_{50}$ (nM)</th>
<th>$K_i$ (nM)</th>
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<tbody>
<tr>
<td>Recombinant Aromatase (CYP19)</td>
<td>131 ± 54</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>Recombinant CYP1A2</td>
<td>207 ± 26</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>Recombinant CYP2A6</td>
<td>6373 ± 983</td>
<td>2176 ± 256</td>
</tr>
<tr>
<td>Recombinant CYP3A4</td>
<td>285 ± 81</td>
<td>375 ± 6</td>
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<tr>
<td>Recombinant CYP3A5</td>
<td>723 ± 27</td>
<td>829 ± 62</td>
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<tr>
<td>Recombinant CYP2C19</td>
<td>2.80 ± 0.29</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>Recombinant CYP2B6</td>
<td>No Inhibition$^a$</td>
<td></td>
</tr>
<tr>
<td>Recombinant CYP2D6</td>
<td>No Inhibition$^a$</td>
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</tbody>
</table>

$^a$ No inhibition of CYP2B6 or CYP2D6 by norendoxifen was observed at the concentration of 5 μM.
Table 2. IC\textsubscript{50} values (nM) of \(E\)-, mixed, \(Z\)-norendoxifen against aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19

<table>
<thead>
<tr>
<th></th>
<th>(E)-Norendoxifen (nM)</th>
<th>Mixed Norendoxifen (nM)</th>
<th>(Z)-Norendoxifen (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} against CYP19</td>
<td>98 ± 40</td>
<td>131 ± 54</td>
<td>1053 ± 185</td>
</tr>
<tr>
<td>IC\textsubscript{50} against CYP1A2</td>
<td>160 ± 22</td>
<td>207 ± 26</td>
<td>285 ± 43</td>
</tr>
<tr>
<td>IC\textsubscript{50} against CYP3A4</td>
<td>182 ± 79</td>
<td>285 ± 81</td>
<td>925 ± 145</td>
</tr>
<tr>
<td>IC\textsubscript{50} against CYP3A5</td>
<td>930 ± 66</td>
<td>723 ± 27</td>
<td>655 ± 27</td>
</tr>
<tr>
<td>IC\textsubscript{50} against CYP2C19</td>
<td>1.90 ± 0.35</td>
<td>2.80 ± 0.29</td>
<td>3.88 ± 0.79</td>
</tr>
</tbody>
</table>

CYP19, aromatase
Table 3. $K_i$ values (nM) of $E$-, mixed, $Z$-norendoxifen against aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19

<table>
<thead>
<tr>
<th></th>
<th>$E$-Norendoxifen (nM)</th>
<th>Mixed Norendoxifen (nM)</th>
<th>$Z$-Norendoxifen (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ against CYP19</td>
<td>48 ± 3</td>
<td>70 ± 9</td>
<td>445 ± 6</td>
</tr>
<tr>
<td>$K_i$ against CYP1A2</td>
<td>49 ± 3</td>
<td>76 ± 3</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>$K_i$ against CYP3A4</td>
<td>242 ± 9</td>
<td>375 ± 6</td>
<td>910 ± 59</td>
</tr>
<tr>
<td>$K_i$ against CYP3A5</td>
<td>859 ± 76</td>
<td>829 ± 62</td>
<td>707 ± 17</td>
</tr>
<tr>
<td>$K_i$ against CYP2C19</td>
<td>0.48 ± 0.05</td>
<td>0.56 ± 0.02</td>
<td>0.70 ± 0.11</td>
</tr>
</tbody>
</table>

CYP19, aromatase
Figure 2

% Remaining Enzyme Activity vs. log10 [Norendoxifen, nM] for enzymes Aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, and CYP2C19.
Figure 3

A

\[
\frac{1}{v} \text{ (pmol/min/pmol CYP1A2)}
\]

vs.

Norendoxifen (nM)

- CEC
- 10 \mu M
- 5 \mu M
- 2.5 \mu M

B

\[
\frac{1}{v} \text{ (pmol/min/pmol CYP2A6)}
\]

vs.

Norendoxifen (\mu M)

- Coumarin
- 7 \mu M
- 5 \mu M
- 3 \mu M

C

\[
\frac{1}{v} \text{ (pmol/min/pmol CYP3A4)}
\]

vs.

Norendoxifen (nM)

- BFC
- 100 \mu M
- 75 \mu M
- 25 \mu M

D

\[
\frac{1}{v} \text{ (pmol/min/pmol CYP3A5)}
\]

vs.

Norendoxifen (nM)

- BFC
- 100 \mu M
- 50 \mu M
- 25 \mu M

E

\[
\frac{1}{v} \text{ (pmol/min/pmol CYP2C19)}
\]

vs.

Norendoxifen (nM)

- CEC
- 75 \mu M
- 25 \mu M
- 10 \mu M
Figure 4

(A) Remaining Aromatase (CYP19) Activity vs. log10 (Norendoxifen, nM)

(B) Remaining CYP1A2 Activity vs. log10 (Norendoxifen, nM)

(C) Remaining CYP3A4 Activity vs. log10 (Norendoxifen, nM)

(D) Remaining CYP3A5 Activity vs. log10 (Norendoxifen, nM)

(E) Remaining CYP2C19 Activity vs. log10 (Norendoxifen, nM)

- E-Norendoxifen
- Z-Norendoxifen