

INHIBITION OF HUMAN DRUG METABOLIZING CYTOCHROMES P450 BY ANASTROZOLE, A POTENT AND SELECTIVE INHIBITOR OF AROMATASE

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ABSTRACT:

Anastrozole (2,2'[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis(2-methylpropionitrile)) is a potent third-generation inhibitor of aromatase, currently marketed as a treatment for postmenopausal women with advanced breast cancer. While its potency and selectivity for inhibition of estrogen synthesis has been established in both preclinical and clinical studies, this study used *in vitro* methods to examine the effects of anastrozole on several drug-metabolizing CYP enzymes found in human liver. Human liver microsomes were co-incubated with anastrozole and probe substrates for CYP1A2 (phenacetin), CYP2A6 (coumarin), CYP2C9 (tolbutamide), CYP2D6 (dextromethorphan), and CYP3A (nifedipine). The formation of the CYP-specific metabolites following co-incubation with various anastrozole concentrations was determined to establish IC_{50} and K_i values for these enzymes. While anastrozole did not

inhibit CYP2A6 and CYP2D6 activities at concentrations below 500 μ M, this compound inhibited CYP1A2, CYP2C9, and CYP3A activities with K_i values of 8, 10, and 10 μ M, respectively. Dixon plots used to determine the K_i values for the inhibition of CYP1A2 and CYP3A activities by anastrozole were biphasic, indicating additional lower affinity K_i values. Major metabolites of anastrozole did not retain the ability to inhibit the metabolism of nifedipine (CYP3A). The results of this study indicate that, although anastrozole can inhibit CYP1A2, 2C9, and 3A-mediated catalytic activities, this compound would not be expected to cause clinically significant interactions with other CYP-metabolized drugs at physiologically relevant concentrations achieved during therapy with Arimidex (Zeneca, Ltd., Macclesfield, UK) 1-mg.

Anastrozole (ZD1033), an achiral triazole derivative known as 2,2'[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis(2-methylpropionitrile) (fig. 1), is a potent inhibitor of aromatase (CYP19), which converts androgens to estrogens. This compound is currently marketed as a treatment for postmenopausal women with advanced breast cancer. Anastrozole exhibits high intrinsic potency demonstrated by the *in vitro* inhibition of human placental aromatase with an IC_{50} of 15 nM. Preclinical studies with anastrozole demonstrated its selectivity for aromatase *in vivo* as compared with inhibition of other enzyme activities responsible for steroid biosynthetic pathways. Cholesterol biosynthesis was minimally inhibited *in vitro* and plasma cholesterol concentrations were unchanged in preclinical species at doses that were at least 30 times higher than its maximally effective dose (0.1 mg/kg) required for aromatase inhibition (1). Anastrozole, at doses 100–200 times its maximally effective aromatase inhibitory dose, did not interfere with cholesterol side-chain cleavage nor did it affect plasma aldosterone levels, sodium and potassium excretion, or adrenal and prostate gland weights. Anastrozole was a comparatively weak inhibitor of bovine adrenal 11 β -hydroxylase *in vitro* ($IC_{50} \approx 12 \mu$ M), and *in vivo* changes in circulating 11-deoxycorticosterone or hypokalemia were not observed at 3 and 10 mg/kg doses in monkeys and dogs, respectively. Androgen synthesis was not affected in rats and monkeys at 10 times or, in dogs, at 100 times the effective aromatase inhibitory dose. The selectivity of anastrozole was also established in early clinical studies where doses of 1 to 10 mg in postmenopausal female volunteers produced maximal suppression of

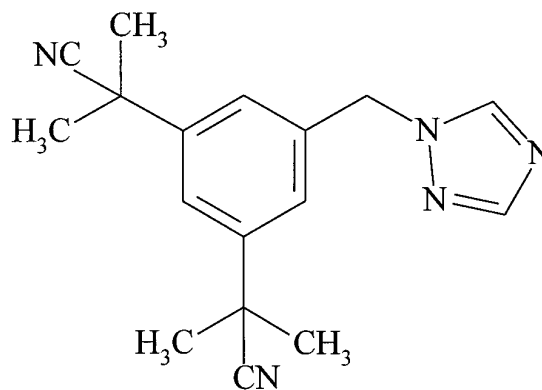


FIG. 1. Structure of anastrozole.

estradiol without affecting cortisol or aldosterone secretion (1, 2).

Variousazole-containing compounds have been shown to be potent inhibitors and inducers of cytochrome P450-mediated drug metabolism both *in vitro* and *in vivo*. Lipophilic compounds containing nitrogen heterocycles such as phenylimidazoles and phenylpyridines inhibit rat liver monooxygenase activities with varying microsomal binding affinities depending on the position of heterocycle substitution (3). Many investigators have demonstrated that *N*- and *C4*-substituted imidazole drugs such as ketoconazole and cimetidine inhibit CYP¹ activity and

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¹ Abbreviations used are: CYP, cytochrome P450; BSA, bovine serum albumin; BMPN-benzoic acid, 3,5-bis-(2-methyl-propionitrile)-benzoic acid; PMSF, phenylmethyl-sulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; HCl, hydrochloric acid; MTBE, methyl-tert-butyl ether; C_{max} , maximum concentration.

drug clearance in both animals and man (4–8). The potency of inhibition by these agents varies greatly and is determined by their hydrophobic nature and the effect of steric hindrance on the strength of the bond between their heteroatomic lone pair of electrons and the CYP heme iron. Although manyazole-containing compounds are generally thought to be nonspecific inhibitors of monooxygenase activity, the selectivity of CYP inhibition by compounds such as ketoconazole has been shown to be concentration-dependent in human *in vitro* systems (9).

Information on potential drug:drug interactions is necessary during the development of all compounds intended for therapeutic use. *In vitro* methodologies are now routinely used to study the potential of a drug candidate to inhibit specific CYP enzymes and classify the drugs most likely to interact during clinical use. This study examined the effects of anastrozole on several of the major drug metabolizing CYP enzymes in human liver using *in vitro* approaches. Comparisons of the inhibitory effects were made to other known inhibitors.

Materials and Methods

Chemicals. Anastrozole, BMPN-benzoic acid, dehydronifedipine, and dextrorphan were synthesized by Zeneca Pharmaceuticals (Macclesfield, UK or Wilmington, DE). Phenacetin and acetaminophen were obtained from US Pharmacopoeia Convention, Inc. (Rockville, MD). Cimetidine, coumarin, dextromethorphan, ketoconazole, nifedipine, tolbutamide, and umbelliferone were obtained from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxytolbutamide was obtained from Ultrafine Chemicals, Ltd. (Manchester, UK). All other reagents and supplies were obtained from standard commercial sources.

Microsome preparations. Transplant quality samples of human liver were obtained from the International Institute for the Advancement of Medicine (Exton, PA). Microsomes were prepared from liver tissue immediately upon receipt by differential centrifugation using previously described methods (10). Microsomal suspensions were stored for subsequent use at -80°C in 10 mM Tris-acetate (pH 7.4) containing 0.1 mM EDTA, 0.1 mM PMSF, and 20% glycerol (v/v). The total protein content of each microsomal sample was determined using the bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

Determination of Human CYP Activities. Phenacetin deethylation to acetaminophen was used to assess CYP1A2 activity in human liver microsomes. Incubation mixtures of 0.5 ml contained 0.1 mg microsomal protein, 50 mM HEPES (pH 7.5), 15 mM MgCl_2 , 1 mM NADPH, and phenacetin (50, 100, and 200 μM). Reactions were stopped after 15 min by the addition of 2 ml isopropanol:ethyl acetate (1:9 v/v). The samples were vortexed and then centrifuged to separate aqueous and organic layers. The organic layer was transferred to a clean tube and evaporated under nitrogen to dryness. Samples were reconstituted in HPLC mobile phase (10:90 acetonitrile: 0.1% TFA in water). The concentration of acetaminophen produced in the microsomal incubation was determined by HPLC with UV detection at 248 nm. Separation of acetaminophen from phenacetin and other reaction components was accomplished using a Zorbax SB-C8 column (MacMod Analytical, Inc., Chadds Ford, PA), 4.6×75 mm, with a precolumn 0.5 μm filter (Upchurch Scientific, Oak Harbor, WA). Acetaminophen ($rt = 1.6$ min) was eluted from the column using acetonitrile: 0.1% TFA in HPLC grade water (10:90 v:v) at a flow of 1.5 ml min^{-1} . Phenacetin ($rt = 4.8$ min) was washed from the column following a step gradient to 25% acetonitrile.

Coumarin hydroxylation to umbelliferone (hydroxycoumarin) was used to assess CYP2A6 activity in human liver microsomes using methods modified from published procedures (11). Incubation mixtures of 0.5 ml contained 50 μg microsomal protein, 25 mM phosphate buffer (pH 7.4), 2.5 mM MgCl_2 , 1 mM NADPH, and 50 μM coumarin. Reactions were stopped after 15 min by the addition of 0.5 ml 6% TCA (w/v). The samples were vortexed and then centrifuged to remove precipitated protein. A 0.5 ml aliquot of the supernatant was transferred to a tube containing 3 ml 0.8M Tris/0.8M glycine (pH 9.0). Umbelliferone concentrations in samples and standards were analyzed by fluorescence detection at excitation and emission wavelengths set at 380 nm and 460 nm, respectively.

Tolbutamide methyl-hydroxylation was used to assess CYP2C9 activity in human liver microsomes. Incubation mixtures of 0.5 ml contained 0.5 mg

microsomal protein, 50 mM HEPES (pH 7.5), 15 mM MgCl_2 , 1 mM NADP^+ , 10 mM glucose-6-phosphate, 0.33 U glucose-6-phosphate dehydrogenase, and tolbutamide (100, 200, and 1000 μM). Reactions were stopped after 30 min by the addition of 25 μl 1N HCl. The samples were extracted by vortexing with 2 ml MTBE. After centrifugation, the organic layer was transferred to a clean tube and evaporated under nitrogen to dryness. Samples were reconstituted in HPLC mobile phase (47:53 methanol: 0.1% TFA in water, pH 2.6 with NH_4OH). The concentration of hydroxytolbutamide in each sample was determined by HPLC using a Zorbax SB-C8 column, 4.6×150 mm, and UV detection at 230 nm. At a flow rate of 1.5 ml min^{-1} , hydroxytolbutamide, chlorpropamide (internal standard), and tolbutamide eluted at approximately 3, 7, and 11 min.

CYP2D6 activity in human liver microsomes was assessed by determining dextrorphan formed from dextromethorphan using methods modified from published procedures (12). Incubation mixtures of 0.15 ml contained 50 μg microsomal protein, 50 mM HEPES (pH 7.5), 15 mM MgCl_2 , 2 mM NADPH, and 100 μM dextromethorphan. Reactions were stopped after 15 min by the addition of a drop of 10% NH_4OH . The samples were extracted by vortexing with 1 ml MTBE. The MTBE layer was transferred after centrifugation to a clean tube and evaporated under nitrogen to dryness. Samples were reconstituted in HPLC mobile phase (25:75 acetonitrile: 30 mM ammonium acetate, pH 4.0). The concentration of dextrorphan in each sample was determined by HPLC using a Zorbax SB-C8 column, 4.6×150 mm, and UV detection at 230 nm. At a flow rate of 1.5 ml min^{-1} , dextrorphan and dextromethorphan eluted at approximately 3 and 10 min.

To determine their inhibitory effects on CYP3A activity, various concentrations of anastrozole, BMPN-benzoic acid, triazole, ketoconazole, or cimetidine were co-incubated *in vitro* as potential inhibitors with nifedipine using modified procedures from those described by Guengerich *et al.* (13). Incubation mixtures of 0.5 ml contained 0.1 mg microsomal protein, 50 mM HEPES (pH 7.5), 15 mM MgCl_2 , 1 mM NADP^+ , 10 mM glucose-6-phosphate, 0.33 U glucose-6-phosphate dehydrogenase, and nifedipine (10, 25, and 50 μM). Reactions were stopped after 20 min by the addition of 0.1 ml Na_2CO_3 . The samples were extracted by vortexing with 2 ml MTBE. The MTBE layer was transferred after centrifugation to a clean tube and evaporated under nitrogen to dryness. Samples were reconstituted in HPLC mobile phase (60:40 methanol:water). The concentration of dehydronifedipine in each sample was determined by HPLC using a Zorbax SB-C8 column, 4.6×250 mm, and UV detection at 260 nm. At a flow rate of 1.5 ml min^{-1} , dehydronifedipine and nifedipine eluted at approximately 6 and 9 min. Incubations, extractions, and HPLC analyses of samples and standards containing nifedipine were conducted while minimizing the exposure to light.

Data Analysis. Incubation of the CYP-marker substrates with microsomes from 1 to 3 individual donors in the presence or absence of various concentrations of anastrozole was used to determine IC_{50} values. IC_{50} values for the inhibition of the CYP activities were determined by nonlinear regression analysis (Origin, version 3.0, Microcal Software, Inc., Northampton, MA) using the following equation:

$$y = 100 - (100 - E_0) \times (I / (I + \text{IC}_{50}))$$

where I is the initial concentration of inhibitor in a microsomal incubation, E_0 is the per cent enzyme activity that is not inhibited at $I = \infty$ and, IC_{50} is the inhibitor concentration that inhibits enzyme activity by 50%.

Rates of metabolite formation from pooled human liver microsomes following co-incubation of three concentrations of CYP-marker substrate with multiple inhibitor concentrations were used to determine K_i values using the method of Dixon (14). The type of inhibition was assessed by transforming the data to give S /metabolite formation rate vs. inhibitor concentration (15).

Results

The potential inhibitory effects of the aromatase inhibitor, anastrozole, on drug metabolism were evaluated by determining IC_{50} and K_i values for metabolic reactions that are selectively catalyzed by five different cytochrome P450 forms in human hepatic microsomes (table 1). Anastrozole inhibited less than 10% of the control coumarin

TABLE 1

Effects of anastrozole on the cytochrome P450 activities in human liver microsomes

CYP	Marker Substrate	Test Substance	IC ₅₀ (μM) ^a	K _i (μM)
1A2	Phenacetin	Anastrozole	30	8/80
2A6	Coumarin	Anastrozole	>500 ^b	—
2C9	Tolbutamide	Anastrozole	48	10
2D6	Dextromethorphan	Anastrozole	>500 ^b	—
3A	Nifedipine	Anastrozole	27	10/55
3A	Nifedipine	Triazole	>250 ^b	—
3A	Nifedipine	BMPN-benzoic acid	>250 ^b	—
3A	Nifedipine	Ketoconazole	0.02	—
3A	Nifedipine	Cimetidine	650	—

^aIC₅₀ values were determined using the following concentrations of the specific CYP-marker substrates: phenacetin, 100 μM; coumarin, 50 μM; tolbutamide, 1 mM; dextromethorphan, 100 μM; nifedipine, 25 μM.

^bAnastrozole and its metabolites (products formed by *N*-dealkylation) were not tested for CYP inhibition above 250–500 μM. Because little inhibition was observed at high concentrations, K_i values were not determined.

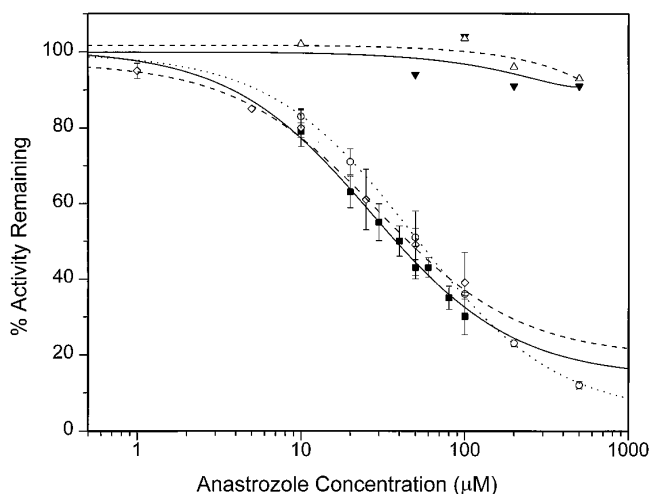


FIG. 2. Inhibition of phenacetin *O*-deethylase (◇), coumarin hydroxylase (Δ), tolbutamide hydroxylase (○), dextromethorphan *O*-demethylation (▼), and nifedipine oxidation (■) in human liver microsomes by anastrozole.

Data for phenacetin, tolbutamide, and nifedipine metabolism represent mean ± SE (*N* = 3 individual microsomes samples). Data for coumarin and dextromethorphan represent the average of duplicate incubations.

hydroxylase (CYP2A6) and dextromethorphan *O*-demethylase (CYP2D6) activities in the human microsomes samples at concentrations up to 500 μM (fig. 2).

The *O*-deethylation of phenacetin as a marker activity for CYP1A2 was inhibited by anastrozole at concentrations between 10 and 100 μM with an IC₅₀ of 30 μM. Dixon plots of the reciprocal deethylation rates when 50, 100, or 200 μM phenacetin was co-incubated with 0 to 500 μM anastrozole exhibited considerable nonlinearity (fig. 3A).²

The nonlinearity in the results was also evident in the Cornish-Bowden plot (fig. 3B) when the data generated at 500 μM anastrozole

² The kinetics of phenacetin *O*-deethylation by the microsomes used in this investigation was best fit to a 2-enzyme model with a K_m of 59 μM for the high affinity site. This result suggests that more than one enzyme contributes to this *O*-deethylation reaction and may potentially explain the appearance of biphasicity in the Dixon plot. In contrast and although other CYP3A enzymes are known to metabolize nifedipine, the kinetics of dehydronifedipine formation was fitted best to a 1-enzyme Michaelis-Menten model (K_m ~25 μM).

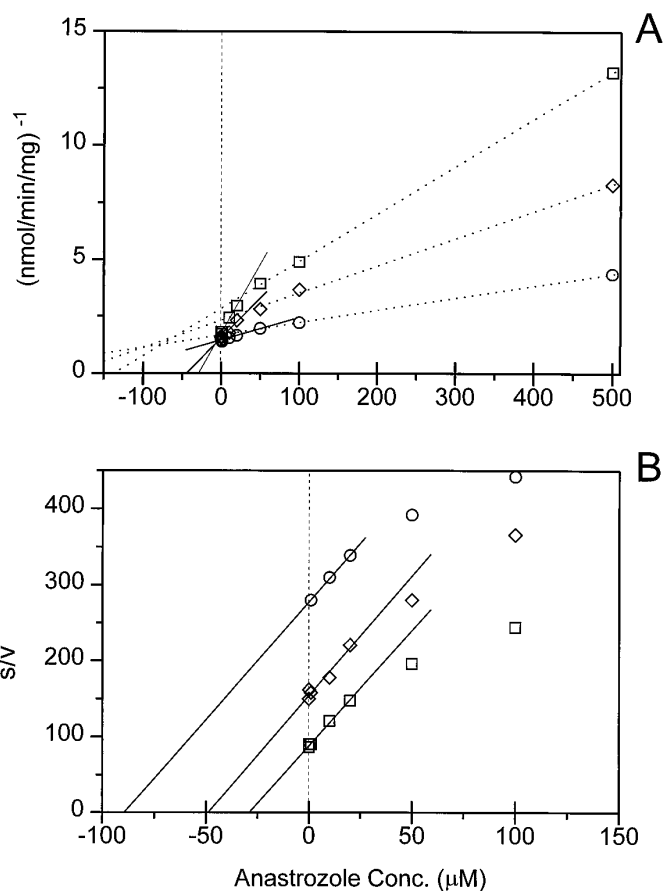


FIG. 3. Dixon (A) and Cornish-Bowden (B) plots of the effect of anastrozole co-incubation on the rate of acetaminophen formation from phenacetin in human liver microsomes.

Phenacetin was incubated at initial concentrations of 50 (□), 100 (○), and 200 (◇) μM.

was excluded. The Dixon plot could be divided into two distinct linear phases. Therefore, when the data corresponding to the two phases were subjected to linear regression analysis, two apparent K_i values of 8 and 80 μM were determined for the inhibition of phenacetin metabolism to acetaminophen. The high affinity K_i of 8 μM estimated in this *in vitro* investigation was used to estimate conservatively the *in vivo* interaction potential towards CYP1A2 substrates. Parallel plots in the Cornish-Bowden transformation of the data indicate that the high affinity inhibition is competitive in nature.

Tolbutamide methyl-hydroxylase activity, a marker of CYP2C9 activity in human liver microsomes, was inhibited by anastrozole with an IC₅₀ of 48 μM when tolbutamide was co-incubated at 1000 μM (fig. 2). At this concentration of tolbutamide, CYP2C8 most likely contributes to the methylhydroxylase activity (16, 17) whereas at lower concentrations, the contribution by this CYP2C form would be negligible. An apparent K_i of 10 μM was determined based on plots of the reciprocal hydroxylation rates when 100, 200, and 1000 μM tolbutamide was co-incubated with 0 to 100 μM anastrozole (fig. 4A). The inhibition of tolbutamide hydroxylase activity by anastrozole exhibited linear behavior on Dixon plots and Cornish-Bowden plots demonstrated parallel best-fit lines indicating competitive-type inhibition (fig. 5B).

Anastrozole inhibited nifedipine oxidation in human liver microsomes with an IC₅₀ of 27 μM. By comparison, ketoconazole was a

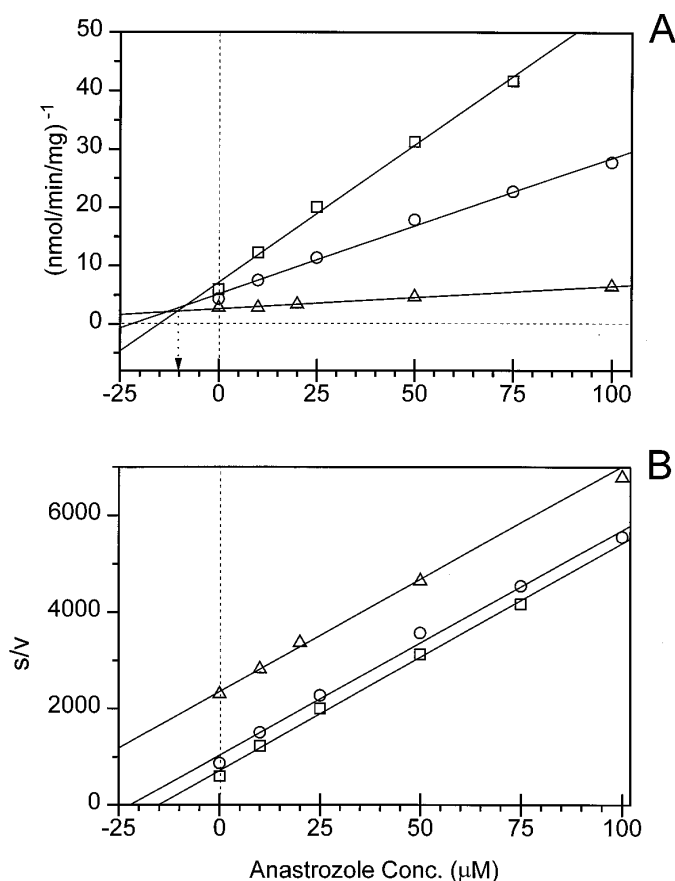


FIG. 4. Dixon (A) and Cornish-Bowden (B) plots of the effect of anastrozole co-incubation on the rate of 4-hydroxytolbutamide formation from tolbutamide in human liver microsomes.

Tolbutamide was incubated at initial concentrations of 100 (\square), 200 (\circ), and 1000 (Δ) μM .

much more potent inhibitor of this CYP3A-catalyzed reaction with an IC_{50} of 0.02 μM , while cimetidine was a weaker inhibitor ($\text{IC}_{50} = 650 \mu\text{M}$) when co-incubated with nifedipine. Dixon plots of the reciprocal oxidation rates exhibited similar biphasicity to that observed for the inhibition of CYP1A2 by anastrozole (fig. 5A). Two distinct intercepts were obtained by linear regression analysis of the data and thus, two apparent K_i values of 10 and 55 μM were determined for the inhibition of CYP3A-catalyzed nifedipine oxidation by anastrozole. Cornish-Bowden plots suggested that the CYP3A inhibition was mixed (competitive/noncompetitive) in nature (fig. 5B).

Because CYP3A4 appears to be the most abundant cytochrome P450 enzyme in both human liver and intestine and this enzyme is involved in the metabolism of many drug substrates, triazole and BMPN-benzoic acid were tested for their ability to inhibit nifedipine oxidation. These two compounds are major metabolites of anastrozole that are produced by *N*-dealkylation (cleavage of the methylene bridge). When co-incubated with nifedipine at concentrations up to 250 μM , these metabolites of anastrozole did not inhibit nifedipine oxidation.

Discussion

Many, if not most, clinically relevant drug interactions are caused by inhibition of drug metabolizing enzymes leading to a decreased metabolic clearance and increased exposure to the inhibited drug. Cytochrome P450 enzymes mediate the rate limiting step of the

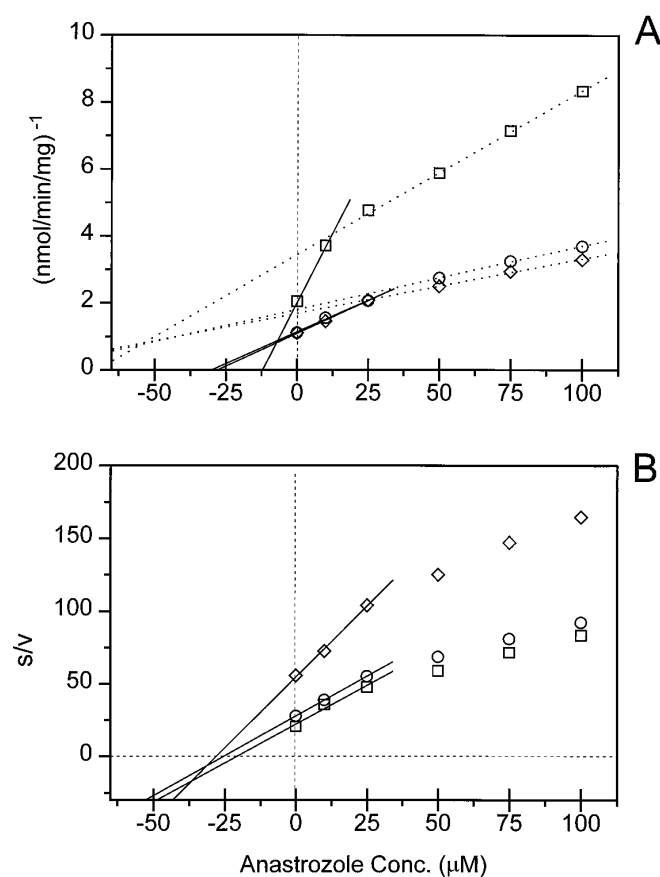


FIG. 5. Dixon (A) and Cornish-Bowden (B) plots of the effect of anastrozole co-incubation on the rate of dehydronifedipine formation from nifedipine in human liver microsomes.

Nifedipine was incubated at initial concentrations of 10 (\square), 25 (\circ), and 50 (224) μM .

primary Phase I metabolic reactions for the vast majority of drug compounds as well as the biosynthesis or catabolism of a number of endogenous substances with potent physiological and pathophysiological functions. Specific cytochrome P450 enzymes in the CYP1A, 2A, 2C, 2D, and 3A subfamilies are responsible for the oxidative metabolism of most drugs in humans (18, 19). Toxicities and other adverse events may therefore develop during clinical therapy with drugs that have narrow therapeutic margins when the concomitant administration of CYP inhibitors causes potent metabolic inhibition of drug clearance. Probably the most prominent example of this type of potentially harmful interaction is the cardiotoxicity that can be produced following co-administration of CYP3A4 inhibitors such as ketoconazole and erythromycin with terfenadine, a prodrug metabolized exclusively by CYP3A enzyme(s) (20). *In vitro* studies are now being used routinely to investigate the interaction potential for drugs resulting from inhibition of CYP-mediated metabolism (21, 22). The experiments reported here examined the ability of anastrozole to inhibit the metabolism of substrates for CYP1A2, CYP2A6, CYP2C9, CYP2D6, and CYP3A. The results were used 1) to compare the inhibition of drug metabolizing CYPs by anastrozole to its *in vitro* ability to inhibit estrogen synthesis mediated by human aromatase and 2) to predict the potential for anastrozole to cause metabolic drug interactions.

Anastrozole inhibited specific reactions catalyzed by CYP1A2, CYP2C9, and CYP3A but did not inhibit CYP2A6 or CYP2D6-mediated pathways. The inhibition of both CYP1A2 and CYP3A

activities were biphasic in nature although the basis of the nonlinear results has not been established. Two apparent K_i values were exhibited by anastrozole for inhibition of CYP1A2 and CYP3A metabolism. The low K_i values for these two enzymes was similar to the single apparent K_i determined for inhibition of CYP2C9 activity by anastrozole ($K_i = 8-10 \mu\text{M}$ corresponds to 2.3 to $2.9 \mu\text{g ml}^{-1}$). The low K_i values were used to estimate conservatively the potential for inhibitory drug interactions with anastrozole during clinical use of the drug. Average steady-state C_{max} concentrations in patients chronically administered the 1-mg marketed dose of Arimidex were approximately $0.08 \mu\text{g ml}^{-1}$ ($0.3 \mu\text{M}$). These plasma concentrations are 30-fold lower than the apparent K_i values determined in these *in vitro* studies, suggesting that anastrozole would cause little or no inhibition *in vivo* of the metabolism of drugs that are substrates for CYP1A2, CYP2C9, and CYP3A enzymes (per cent inhibition *in vivo* predicted to be approximately 3%). Although a lack of inhibition potential can be predicted from these *in vitro* results, any effect of intracellular binding or accumulation of anastrozole in hepatocytes on the *in vivo* inhibition have not been accounted for in these investigations.³

Nifedipine oxidation in human liver microsomes was not decreased by two major metabolites (triazole and BMPN-benzoic acid) of anastrozole at concentrations up to $250 \mu\text{M}$. The lack of CYP inhibition by these metabolites is consistent with requirement for both hydrophobic character for active site access and the heteroatomic lone pair of electrons in triazole for Type II binding to the heme iron (23).

The results herein also show that the ability of anastrozole to inhibit drug metabolizing CYP enzymes in liver microsomes is much weaker than the inhibition of aromatase. When comparing the IC_{50} for inhibition of human placental aromatase activity (15 nM) with the inhibition of the hepatic microsomal CYP produced by anastrozole *in vitro*, a margin of selectivity of at least 500-fold is obtained.

In conclusion, the results of the *in vitro* experiments performed in human liver microsomes indicate that, although anastrozole can inhibit CYP1A2, 2C9, and 3A-mediated catalytic activities, the level of inhibition *in vivo* during clinical therapy with Arimidex (Zeneca, Ltd., Macclesfield, UK) 1-mg would not be expected to cause clinically significant interactions with other CYP-metabolized drugs.

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³ The plasma protein binding of anastrozole was determined *in vitro* to be 40% (unpublished results). Therefore, the free (unbound) concentration of anastrozole would be approximately 0.6 times the total concentrations in plasma.