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17-Hydroexemestane: A Potent Inhibitor of CYP19 (Aromatase) and Substrate of CYP3A

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Abstract

17-hydroexemestane is the major metabolite of exemestane *in vivo*. Previous studies have shown that 17-hydroexemestane is androgenic and bone protective. Due to structure similarities, we hypothesized that, like exemestane, 17-hydroexemestane is an inhibitor of aromatase (CYP19). Our aim was to assess the potency (IC_{50}) of 17-hydroexemestane toward CYP19 inhibition, and to determine the specific CYPs responsible for 17-hydroexemestane metabolism. Using recombinant human CYP19, we investigated the ability of exemestane and 17-hydroexemestane to block the formation of estradiol from testosterone. We found that 17-hydroexemestane potently inhibited aromatase. IC_{50} values for the inhibition of CYP19 by exemestane and 17-hydroexemestane were 1.5 μM and 3 μM, respectively. Furthermore, using recombinant human P450s, human liver microsomes, and HPLC analytical techniques, we identified one major metabolite (MIII) of 17-hydroexemestane in the human liver microsomal incubate. In a bank of 15 well-characterized HLMs, MIII formation rate was significantly correlated with the activity of CYP3A (r_s= 0.78, p=0.001).In a panel of baculovirus-expressed CYP enzymes, only CYP3A4 and CYP3A5 catalyzed MIII formation at the highest rate. In sum, these in *vitro* data suggest that 17-hydroexemestane is a potent inhibitor of CYP19 and that CYP3A plays a major role in its metabolism. Whether genetic polymorphisms and drug interactions involving these enzymes may contribute to the disposition and action of 17-hydroexemestane in breast cancer patients remains to be elucidated.

Introduction

Estrogens are important in the initiation and promotion of breast cancer [1,2]. Thus, inhibition of aromatase, a rate-limiting enzyme in the biosynthesis of estrogens, has been a key strategy for the management of women with breast cancer [3,4]. Exemestane, along with anastrozole and letrozole, belongs to the class of third-generation aromatase inhibitor drugs that effectively suppress the synthesis of estrogens and thus deprive breast cells from estrogens [5-7]. Exemestane has been approved by the United States Food and Drug Administration and is increasingly used in the treatment of women with advanced breast cancer. Exemestane and other third-generation aromatase inhibitors are relatively new to the market and a great deal remains to be learned in terms of factors that control their response. Emerging data suggest high interindividual variability in the biochemical (e.g. suppression of estrone sulphate) and possibly clinical effects of exemestane [6,8-10]. These effects of exemestane appear to be dose and/or concentration dependent, suggesting that inter-subject differences in the pharmacokinetics of exemestane may contribute to differences in biochemical and clinical effects of the drug. Indeed, some studies have documented large interindividual differences in the pharmacokinetics of exemestane [11-14].

Exemestane is predominantly cleared by hepatic metabolism. After absorption, exemestane is shunted to two different primary metabolic processes. One route involves its oxidation to 6-hydroxymethylexemestane primarily by CYP3A [15]. The other route involves its reduction by aldoketoreductases (AKR) and CYP4A11/CYP1A into 17-hydroexemestane, which represents the major metabolite of exemestane *in vivo* [11,13,15-17]. Preclinical studies have shown that 17-hydroexemestane is androgenic and bone protective. Preclinical studies have demonstrated that exemestane has a more favorable toxicity profile than non-steroidal aromatase inhibitors. These studies have also demonstrated that 17-hydroexemestane is responsible for exemestane-induced bone protection [18-20]. However, due to the significant interindividual variability observed with clinical response to exemestane, the validation of these preclinical findings ended with mixed and conflicting results. Some clinical studies have shown that women receiving non-steroidal aromatase inhibitors have significantly worse bone mineral densities than those treated with exemestane, while other studies found the exact opposite [21-25]. This discrepancy may be partly due to the significant inter-subject differences in the pharmacodynamics and pharmacokinetics of 17-hydroexemestane.

Little is known about 17-hydroexemestane pharmacokinetics and drug metabolizing enzymes involved in its elimination. Although Sun et al. have recently shed some light into the phase two 17-hydroexemestane drug metabolizing enzymes [26]; specific CYPs responsible for its metabolism remain unknown. The goal of this study was to assess the potency of 17-hydroexemestane toward aromatase and to determine the specific CYPs responsible for 17-hydroexemestane metabolism using *in vitro* hepatic microsomal systems.

Materials and Methods

Chemicals

17-hydroexemestane was a kind gift of Pfizer (New York, NY, USA). Exemestane and norgestrel (internal standard) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Testosterone, (beta)-estradiol, glucose 6-phosphate, glucose-6-

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phosphate dehydrogenase, magnesium chloride, and NADP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents which were all of HPLC grade were purchased from reliable commercial sources.

Human Liver Microsomes (HLMs)

HLMs (catalog numbers 452132-452138, 452141-452144, 452161, 452165, 452172, 452183) used were obtained from a commercial source (BD Bioscience/Gentest, Bedford, MA, USA). Gentest states that the human materials were prepared from human liver donor tissues that were obtained with informed consent in conformance with the guidelines promulgated by the USA Uniform Anatomical Gift Act (1987). Baculovirus-insect cell-expressed human CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP19, and CYP4A11) and FMO3 were purchased from BD Gentest Corp. (Woburn, MA, USA). All microsomal preparations were stored at -80°C until used. The total P450 content, protein concentrations, and specific activity of each P450 isoforms were as supplied by the manufacturer.

Incubation Conditions

Microsomal incubations were carried out as described previously [15]. Briefly, pilot experiments were carried out in HLMs to identify 17-hydroeexemestane primary metabolites and to optimize conditions for incubation and HPLC analysis. 17-hydroexemestane (500 μM, initial concentration) was prepared in methanol and serially diluted with methanol to the required concentration. Stability studies conducted in our laboratory (data not shown) indicated that methanolic solutions of 17-hydroexemestane or exemestane were stable at -20°C for at least 12 months. Prior to metabolic incubation, the methanol was evaporated to dryness under reduced pressure using a Speedvac SC110 Model RH40-12 (Savant Instruments, Holbrook, NY, USA). The metabolism of 17-hydroexemestane was assessed in HLMs and a panel of recombinant CYP isoforms (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP19, and CYP4A11). For studies conducted in HLMs, duplicate mixtures of exemestane reconstituted in a phosphate reaction buffer (pH, 7.4) and a NADPH-generating system (1.3 mM beta-NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCL_2$, and 0.4 U/ml glucose 6-phosphate dehydrogenase) were pre-incubated at 37°C for 5 min. For studies conducted in expressed P450s, duplicate mixtures of exemestane and a NADPH-generating system (same composition as above) were used. After the reaction was initiated by adding 25 μL of HLMs (2.5 mg protein/ml) or 25 μL of P450 (25 pmol) and incubated for 10 min at 37°C (final volume, 250 μL), the reaction was terminated by placing tubes on ice and immediately adding 500 μL of acetonitrile. Norgestrel (100 μL of 50 μM) was added as an internal standard to the incubation sample, vortexed for 30 seconds, and centrifuged at 14000 rpm for 5 min in an Eppendorf model 5415D centrifuge (Brinkmann Instruments, Westbury, NY, USA). The supernatant was removed to a clean tube and extracted using ethyl acetate under alkaline pH (0.5 mL of 0.5 M NaOH/ Glycine Buffer, adjusted to pH 10). The sample was centrifuged for 15 minutes at 2500 rpm in a Beckman GS-6R centrifuge (Global Medical Instrumentation, Inc., Ramsey, Minnesota, USA). The organic layer was removed after centrifugation and evaporated to dryness by speed vacuum and reconstituted with 150 μl of the mobile phase from which 100 μl was injected into the HPLC system. Negative control incubations were run in parallel and included no incubation, no substrate, no cofactor, or no HLMs (bovine serum albumin was used instead). The formation of MIII was linear with time between 0 and 15 min and with protein over the range from 0 to 1 mg/ml (data not shown). The end concentrations of 17-hydroexemestane ranged from 0 to 300 μM. The substrate consumption was less than 15% over the incubation time (10 min).

HPLC Analysis

An analytical liquid chromatography instrument combined with an ultraviolet detection system was used to quantify 17-hydroexemestane and its major metabolite MIII. Briefly, ethyl acetate extracts of incubation samples were injected onto an Agilent Zorbax analytical C18 column (150 × 4.6 mm i.d.; 5 μm, 100A; Phenomenex, Torrance, CA, USA) guarded with a Luna 10- × 3 mm C18 guard column (Phenomenex, Torrance, CA, USA) to a UV detection lamp (Advanced Separation Technologies, Whippany, NJ, USA) at a flow rate of 0.8 ml/min with a mobile phase composed of 40% acetonitrile and 10% methanol in 10 mM monobasic potassium phosphate. 17-hydroexemestane and its major metabolite MIII were detected by absorbance at 245 nm. The HPLC system consisted of a Waters Assoc. model 515 pump (Milford, MA, USA), a Waters Assoc. Model 717 plus auto sampler, and a Waters Assoc. model 486 tunable absorbance detector. The ratio of the area under the curve for the metabolite to the area under the curve for each internal standard was calculated. Concentrations of metabolites were measured by standard curves obtained with 17-hydroexemestane, as authentic samples of the synthetic metabolite was not available to us. The possibility that the ultraviolet intensity might differ between the metabolite and 17-hydroexemestane could not be excluded. The metabolite formation rates normally presented as pmol/mg × min (or pmol/ pmol P450 × min) should be viewed more appropriately as apparent velocities (arbitrary unit) where an arbitrary unit = 1000 × (metabolite peak area/internal standard peak area)/slope of the 17-hydroexemestane standard curve. Intra- and inter-day coefficients of variation of the assays were less than 15%.

Kinetic Analysis in HLMs and Expressed P450s

The enzyme kinetics of 17-hydroexemestane were characterized in three different HLMs. 17-hydroexemestane (0 μM to 300 μM) was allowed to incubate for 10 min at 37°C at a protein concentration of 0.25 mg/ml. The metabolite concentration was measured by standard curves obtained with 17-hydroexemestane, as authentic samples of the synthetic metabolite was not available. V_{max} normally presented as pmol/mg × min should be viewed more appropriately as apparent velocities (arbitrary unit) where an arbitrary unit=1000 × (metabolite peak area/internal standard peak area)/slope of the standard curve.

Correlation Experiments in a Panel of HLMs

17-hydroexemestane (10 μM) was incubated for 10 min at 37°C with microsomes from 15 different human livers (0.25 mg protein/ml) and NADPH-generating system. Values for the activity of each P450 were provided by the supplier of the HLMs studied (see http://www.gentest.com).

Data Analysis

Metabolite formation versus 17-hydroexemestane concentrations was fit to hyperbolic and nonhyperbolic enzyme kinetic models to estimate apparent kinetic parameters using the Enzyme Kinetics Module of Sigma Plot (Systat Software, Inc., San Jose, CA). Intrinsic clearance, CL_{int} was determined using the following equation: $CL_{int} = V_{max}/K_m$.

Correlation analysis was performed using a Spearman's rank correlation test. *P<0.05* was considered statistically significant. All experiments were performed in duplicate. Data are presented as the mean of duplicate measurements.

Aromatase Activity Assay

The ability of exemestane and 17-hydroexemestane to block the formation of estradiol from testosterone was used as an aromatase activity assay. This assay was carried out as described previously [27]. Specifically, a reaction mixture containing 25 pmol CYP19, testosterone (50 μM) and a NADPH generating system consisting of 1.3 mM NADP, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCL_2$, and 0.4 U/ml glucose 6-phosphate dehydrogenase in potassium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. After incubation, the reaction was stopped by addition of acetonitrile containing norgestrel as the internal standard. The supernatant was removed, evaporated to dryness, and reconstituted with mobile phase from which an aliquot was injected onto HPLC-UV system for analysis. The product was detected by absorbance at 200 nm and quantitated by comparing the absorbance to a standard curve of (beta)-estradiol. To measure the effectiveness (IC50: 50% inhibitory concentration) of 17-hydroexemestane versus exemestane (which was used as a positive control) in inhibiting aromatase enzyme, testosterone was incubated with aromatase in the absence and presence of varying concentrations of the inhibitor (0.03 μM, 0.3 μM, 3 μM, 30 μM and 300 μM).

Results

Identification of MIII (primary 17-hydroexemestane metabolite) in microsomal incubates

Using HPLC with UV detection, one major chromatographic peak that was unique to incubations consisting of 17-hydroexemestane, HLMs, and cofactors was noted. A typical chromatogram of 17-hydroexemestane and its main metabolite (MIII) is shown in (Figure 1). The retention times for MIII, 17-hydroexemestane, and the internal standard (norgestrel) were 4.9 min, 12.9 min, and 19.5 min, respectively. The formation of this metabolite was not observed in the negative control experiments; and depended on the NADPH-generating system, duration of incubation, microsomal protein concentration, and substrate concentration (data not shown).

Kinetic analysis in HLMs

Kinetic analysis for the formation of MIII from 17-hydroexemestane was studied in pooled HLM preparations. A representative Michaelis-Menten plot for MIII formation in pooled HLM is shown in (Figure 2). The formation rate of MIII revealed Michaelis-Menten saturation curve. The K_m, V_{max}, and CL_{int} values of MIII formation from 17-hydroexemestane for pooled HLM were 36.63 μM, 2225.91 pmol/mg x min, and 60.76 μl/mg × min, respectively.

Correlation analysis

(Figure 3) shows the rates of MIII formation from

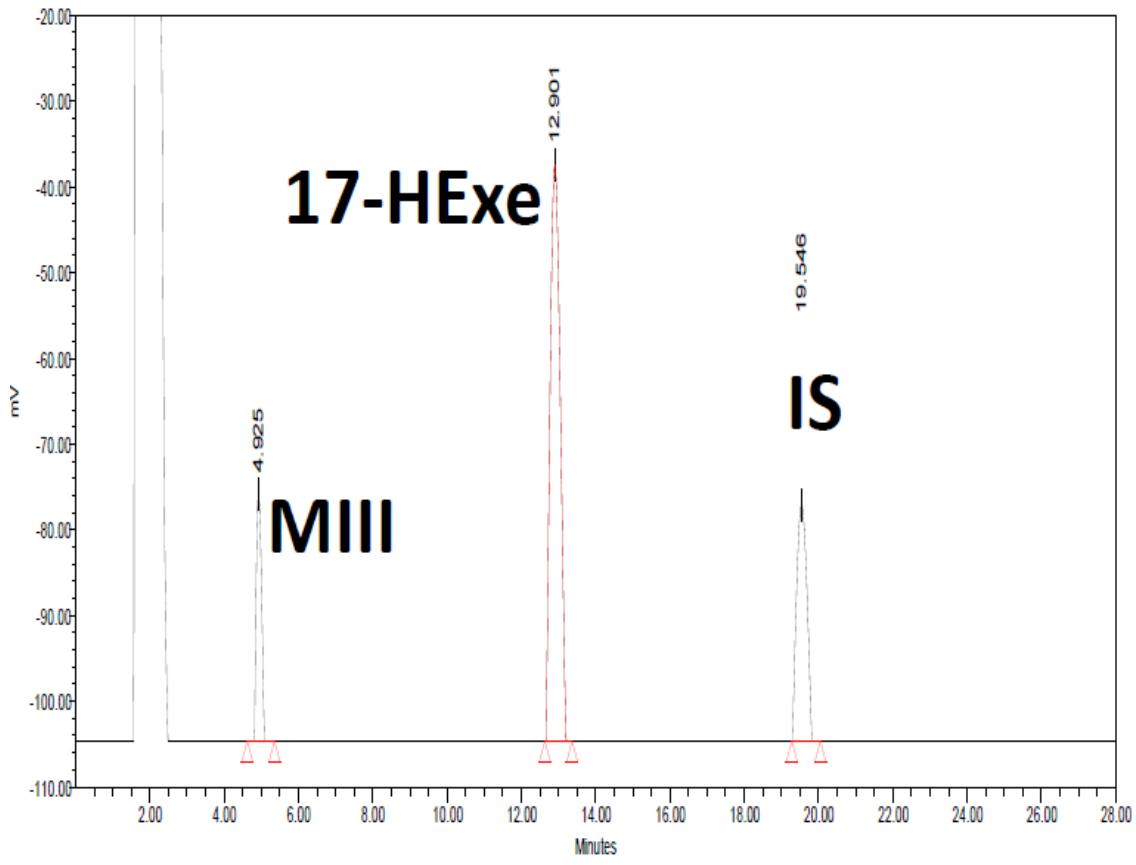

Figure 1: Representative Chromatogram of 17-hydroexemestane main metabolite (MIII) extracted from human liver microsomal incubates. Norgestrel was used as an internal standard (IS). The retention times for MIII, 17-hydroexemestane (17-HExe), and norgestrel (IS) were 4.9 min, 12.9 min, and 19.5 min, respectively.

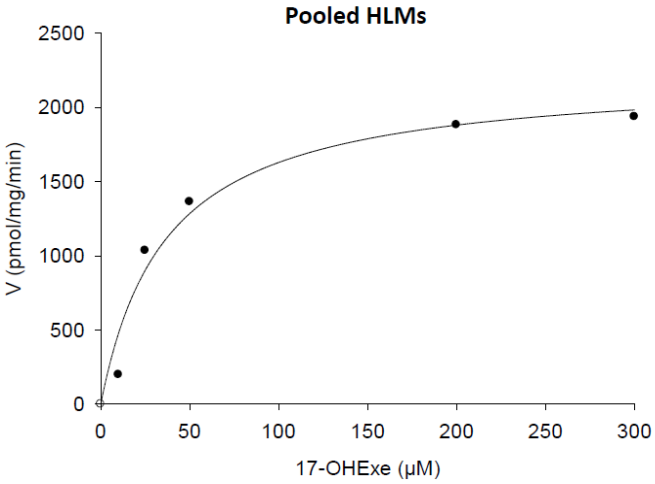

Figure 2: Representative enzyme kinetic plot of the velocities of MIII formation rate by pooled HLMs as a function of 17-hydroexemestane concentration (dose response curve).

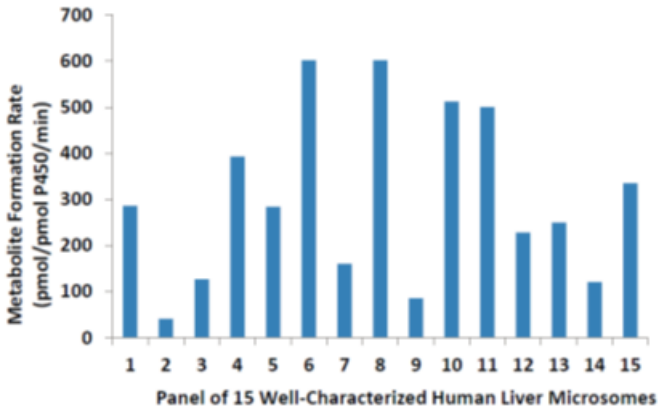

Figure 3: Formation rate of MIII from 17-hydroexemestane in a panel of well-characterized human liver microsomal preparations. Incubations were performed with 0.25 mg/ml microsomal protein and 10 μM 17-hydroexemestane for 10 min at 37°C. Data represent means of duplicate determinations from a single experiment.

17-hydroexemestane in a panel of fifteen characterized HLMs. The average apparent formation rate of MIII at 10 μM 17-hydroexemestane was 301.64 ± 185.19 pmol/mg × min (range 40.93-601.95, 14.7-fold). As shown in Table 1, MIII formation rate significantly correlated with CYP2A6 (spearman r_s=0.54, p =0.036) and CYP3A (spearman r_s= 0.78, p = 0.001) isoform-specific activities.

Metabolism of 17-hydroexemestane by recombinant P450s

17-hydroexemestane was incubated with a panel of recombinant P450s at 10 μM at 37°C for 10 min. Formation rates of MIII from 17-hydroexemestane (10 μM) are shown in (Figure 4). CYP3A4 and CYP3A5 were the major enzymes capable of MIII formation from 17-hydroexemestane with rates of 6.5 pmol/pmol P450 × min and 2.83 pmol/pmol P450 × min, respectively. CYP2A6, CYP2B6, CYP2C8/9, CYP2D6, and CYP19 also contributed to MIII formation although their contributions were 15-20-fold lower.

Inhibition of CYP19 (aromatase) by 17-hydroexemestane

Because 17-hydroexemestane and exemestane are structurally similar and differ only by the absence or presence of 2H at C17 position, it was hypothesized that 17-hydroexemestane, like exemestane potently inhibit aromatase. To test this hypothesis, an aromatase activity assay based on the ability of aromatase to produce estradiol from testosterone was developed. As shown in (Figure 5), the results showed that like exemestane, 17-hydroexemestane potently inhibits aromatase (17-hydroexemestane IC_{50}=3 μM, exemestane IC_{50}=1.5 μM).

Discussion

The goal of this *in vitro* study was to assess the ability and potency of 17-hydroexemestane toward aromatase inhibition and to identify the specific CYP enzymes responsible for its metabolism. Using the CYP19-mediated formation of beta-estradiol from testosterone as an aromatase activity assay and a panel of well characterized human liver microsomes with known isoform-specific activities along with a panel of cDNA-expressed enzymes, we demonstrated that 17-hydroexemestane

	MIII	
	Pearson, r (*p*)	**Spearman, r_s (*p*)**
P450	0.61 (0.014*)	0.525 (0.045*)
b5	0.076 (0.78)	0.039 (0.88)
CYP1A2	0.395 (0.14)	0.209 (0.454)
CYP2A6	0.579 (0.024*)	0.545 (0.036*)
CYP2B6	0.565 (0.028*)	0.489 (0.064)
CYP2C8	0.234 (0.40)	0.227 (0.41)
CYP2C9	0.027 (0.92)	0.136 (0.629)
CYP2C19	0.469 (0.078)	0.246 (0.376)
CYP2D6	-0.577(0.024*)	-0.543 (0.036*)
CYP2E1	-0.073 (0.79)	-0.068 (0.80)
CYP3A	**0.837 (0.0001*)**	**0.785 (0.001*)**
CYP4A11	-0.062 (0.82)	-0.045 (0.874)

Table 1: Correlation between the formation rate of MIII from 17-hydroexemestane and individual P450 isoform-specific activities in a panel of well characterized HLMs.

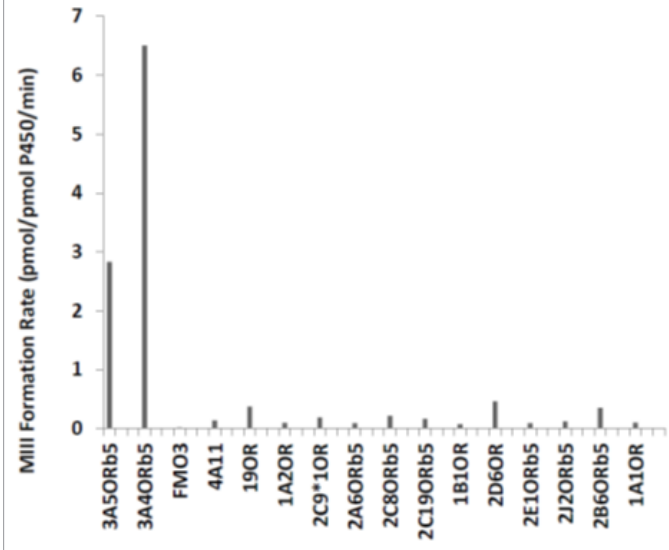

Figure 4: Formation rate of MIII from 17-hydroexemestane in a panel of cDNA baculovirus-expressed P450 enzymes.

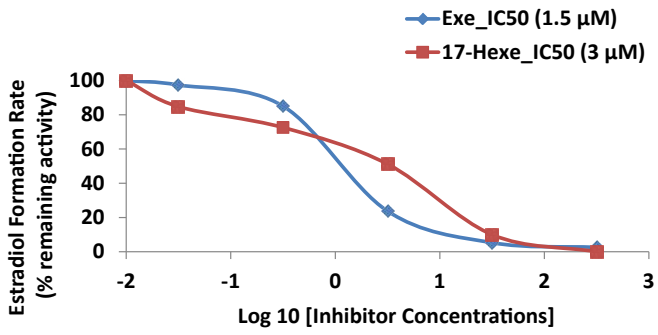

Figure 5: IC_{50} plots depicting the effects of exemestane (Exe, squares) and 17-hydroexemestane (17-Hexe, diamonds) on the formation of estradiol from testosterone. Incubations were performed with cDNA-expressed CYP19 (aromatase) and 50 µM testosterone for 20 min at 37°C. Data represent means of duplicate determinations from a single experiment.

is a potent inhibitor of aromatase (CYP19) and that it is predominantly metabolized to MIII by CYP3A.

To the authors' knowledge, two independent *in vitro* preclinical studies demonstrated that 17-hydroexemestane is a potent inhibitor of aromatase. The first study which was conducted by Buzzetti et al. in 1993 used microsomes from human placenta as the aromatase enzyme source [28]. In their study, aromatase activity was tested by measuring the release of ^3H_2O from [1β-^{3}H] androstenedione. They found that both exemestane and 17-hydroexemestane are potent inhibitors of aromatase with IC_{50}values of 27 nM and 69 nM, respectively. According to their finding, 17-hydroexemestane was found to be 2.6-fold less potent than exemestane *in vitro*. 17 years later, Sun et al. found similar results [26]. Using homogenates from a HEK293 aromatase overexpressing cell line and the formation of estrone from androst-[4-^{14}C]-ene-3,17-dione as the aromatase activity assay, they showed that 17-hydroexemestane (IC_{50} =2.3 µM) is approximately 2.0-fold less potent than exemestane (IC_{50}=1.4 µM). Our finding is in agreement with the works of Buzzetti and Sun. Our result shows that like exemestane, 17-hydroexemestane potently inhibits aromatase (17-hydroexemestane IC_{50} =3 µM, exemestane IC_{50}=1.5 µM). Altogether, these data generated from three different laboratories (with distinct i*n vitro* activity assays and sources of aromatase) strongly suggest that 17-hydroexemestane is an active anti-estrogenic metabolite of exemestane that could play an important role in overall exemestane action and clinical response.

Given the potential pharmacological importance of 17-hydroexemstane, we decided to elucidate the primary enzymes involved in its metabolism. Previously we have shown that exemestane is a substrate of CYP3A [15]. In this study, we also demonstrate that 17-hydroexemestane, its major metabolite, is also a CYP3A substrate. This is evidenced by the following: i) the formation of MIII (the major metabolite of 17-hydroexemestane) is predominantly catalyzed by CYP3A4 and CYP3A5 cDNA-expressed enzymes purchased from BD Gentest (Figure 4), ii) the formation rate of MIII correlates significantly with CYP3A-isoform specific activities in a panel of 15 well characterized human liver microsomes obtained from BD Gentest (Table 1, spearman r_s =0.78, p=0.001), and iii) the formation rate of MIII correlates significantly with the formation rate of MII (data not shown). MII (6-hydroxymethylexemestane) is the oxidative metabolite of exemestane methylene group at position C6. Previously, this reaction has been shown to be mediated by CYP3A. These findings suggest that 17-hydroexemestane is probably also oxidized at the same position to MIII by CYP3A.

Here is an update of the known primary and secondary metabolic pathways of exemestane. As shown in (Figure 6), after absorption, exemestane is shunted to two different primary metabolic processes. One route involves its oxidation to 6-hydroxymethylexemestane (MII) mainly by CYP3A. The other route involves its reduction by aldoketoreductases and CYP4A11/CYP1A into an active anti-estrogenic and androgenic metabolite, 17-hydroexemestane (MI), which represents the major metabolite of exemestane in humans *in vivo* [11,13,15-17]. This active metabolite is further secondarily deactivated by two main drug metabolizing enzymes, CYP3A and UGT2B17, to MIII and 17-hydroexemestane glucuronide [26], respectively.

CYP3A and UGT2B17 expression and activity are known to vary dramatically between individuals both *in vitro* and *in vivo*. In humans, CYP3A4 and CYP3A5 are responsible for the metabolism of 45%-60% of currently used drugs [29]. CYP3A5 is polymorphically expressed among different ethnic groups, with CYP3A5×3 (inactive gene form) allele frequencies of 30%, 50%, and 90% in African, Asian, and Caucasian, respectively [29]. CYP3A4 variability is mostly governed by environmental factors, age, gender, and concomitant drug administration. Studies have shown that drug-drug interactions can expand the range of variability of the area under the curve (AUC) for CYP3A4 substrates to about 400-fold [30]. Like CYP3A5, UGT2B17 is polymorphically expressed among several ethnic groups, with UGT2B17×2 (inactive deleted gene form) allele frequencies of 21%, 83%, and 33% in African, Asian, and Caucasian, respectively [31-33].

There is a wide interindividual variability in the plasma concentrations of 17-hydroexemestane among people taking exemestane for the adjuvant treatment of hormone-positive breast cancer [13]. One study showed that the ratio of 17-hydroexemestane/exemestane in the plasma of 30% of patients was 5-fold that observed in the other 70% [11]. Our present study and that of Sun et al. [26] suggest that decreased expression of CYP3A and UGT2B17 may be associated with higher circulating plasma concentrations of 17-hydroexemestane. This in turn could increase exemestane androgenic-related bone protective activity and anti-aromatase activity. A recent study showed that approximately

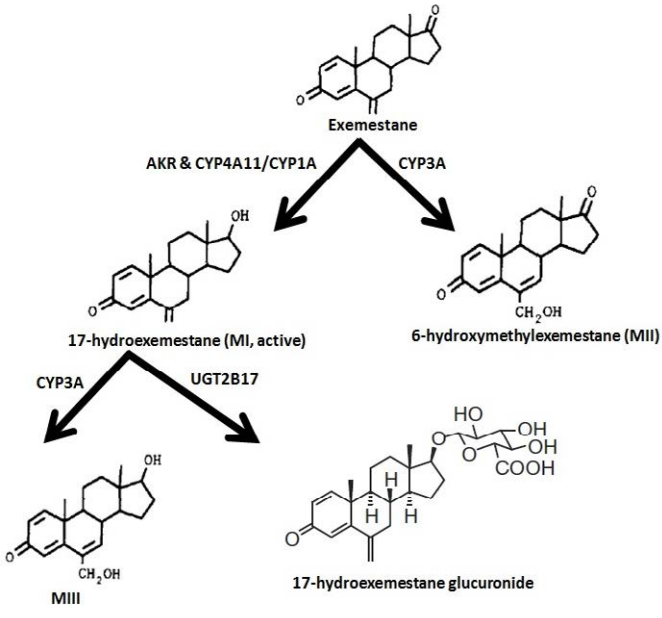

Figure 6: Update: Known primary and secondary metabolic pathways of exemestane.

25% of patients taking exemestane discontinue specifically because of musculoskeletal symptoms [34]. Future studies focusing on elucidating the impact of CYP3A activity status and UGT2B17 gene deletion on the pharmacokinetics and pharmacodynamics of exemestane may provide some insights into the mechanisms underlying exemestane-associated adverse effects and treatment discontinuation.

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