Effect of Testosterone Suppression on the Pharmacokinetics of a Potent GnRH Receptor Antagonist

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Purpose. The expression of cytochrome P450 enzymes (CYPs) in animals and humans is under complex hormonal regulation. Chronic treatment with drugs that alter sex hormone levels such as GnRH receptor agonists or antagonists may affect the expression of hormone-dependent CYPs, and as a result the pharmacokinetics of drugs metabolized by them.

Methods. Enzyme kinetic parameters were obtained by incubating AG-045572 (0.1–30 μM) with human or rat liver microsomes, or expressed CYP3A4 and CYP3A5. The pharmacokinetics of AG-045572 (10 mg/kg i.v. or 20 mg/kg p.o.) were studied in intact male, female, castrated male and male rats pretreated with AG-045572 for 4 days.

Results. AG-045572 is metabolized by CYP3A in both rats and humans. The K_m values were similar in male and female human, female rat liver microsomes, and expressed CYP3A4 and CYP3A5 (0.39, 0.27, 0.28, 0.25, and 0.26 μM, respectively). The K_m in male rat liver microsomes was 1.5 μM, suggesting that in male and female rats AG-045572 is metabolized by different CYP3A isozymes. The oral bioavailability of AG-045572 in intact male rats was 8%, while in female or castrated male rats it was 24%. Pretreatment of intact male rats with AG-045572 i.m. for 4 days resulted in suppression of testosterone to castrate levels, accompanied by an increase in oral bioavailability of AG-045572 to 27%. In the same experiment, the male-specific pulsatile pattern of growth hormone remained unchanged with slightly elevated baseline levels.

Conclusions. The potent GnRH receptor antagonist AG-045572 is metabolized by hormone-dependent CYP3A. As a result, suppression of testosterone by pretreatment with AG-045572 "feminized" its own pharmacokinetics.

KEY WORDS: GnRH antagonist; cytochrome P450 3A; testosterone; hormonal regulation.

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; CYP, cytochrome P450; ACN, acetonitrile.

INTRODUCTION

The hypothalamic hormone, gonadotropin-releasing hormone (GnRH[1]) plays a key role in secretion of follicle-stimulating hormone and luteinizing hormone, and ultimately regulates the production of sex steroids. This makes the GnRH pathway an attractive target for treating hormone-dependent diseases such as prostate, ovarian, breast cancers, and endometriosis. GnRH receptor agents currently used clinically are peptides that are administered by injection due to their poor oral bioavailability. There are several GnRH antagonists, both peptide and nonpeptide, in pre-clinical or clinical development (1–3).

The chronic use of GnRH agonist or antagonist and resulting hormonal perturbations may affect the expression of several drug-metabolizing enzymes known to be under hormonal regulation. The expression of CYPs in various tissues can be directly or indirectly regulated by growth hormone, thyroid hormone, gonadal steroids, and glucocorticoids (4–9). Therefore, if GnRH agonists or antagonists are metabolized by the hormone-dependent CYPs one may expect the pharmacokinetics of these drugs to change after chronic administration.

In this paper we present the pharmacokinetics and metabolism of a small-molecule, orally-bioavailable GnRH receptor antagonist AG-045572 (Fig.1.) and the effect of multiple-dose treatment of male rats with this compound on its own pharmacokinetics.

MATERIALS AND METHODS

Materials

HPLC-grade acetonitrile, ethyl acetate and ammonium acetate was purchased from Fisher Scientific Products (Fair Lawn, NJ). Microsomes containing expressed human cytochrome P450s, Anti-Rat CYP3A2 serum, and monoclonal antibodies inhibitory to CYP3A4 (MAB3A4) were obtained from Gentest Corp. (Woburn, MA). Other chemicals were purchased from Sigma (St. Louis, MO).

In vitro Metabolism

Human and rat liver microsomes were prepared and characterized as described earlier (10). Specimens of human liver were obtained from the International Institute for the Advancement of Medicine (Scranton, PA).

Incubation mixtures consisted of 0.05 mg/ml microsomal protein (3 male and 3 female individual liver microsomal preparations from both human and rat), 2 mM NADPH and 0.1–30 μM AG-045572. The solubility of the compound prevented incubations at higher concentrations. In incubations with expressed CYPs 5–20 pmol CYP/ml was used. Incubations were conducted in duplicate in a volume of 0.4 ml. The reaction was terminated at 3–15 min by the addition of 4 volumes of ACN containing internal standard (a compound with a similar chemical structure). Samples were centrifuged, and the supernatant dried under nitrogen. The residue was reconstituted in 80 μl of mobile phase and 50 μl analyzed by HPLC as described below.

The enzyme kinetic parameters were calculated for the total metabolism of AG-045572 using initial rates. Prelimi-

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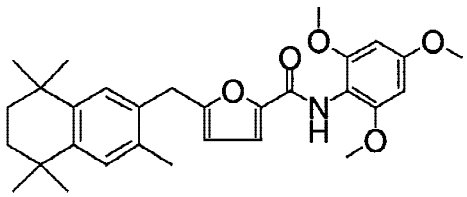

Fig. 1. Chemical structure of AG-045572

nary kinetic analysis was performed using Eadie-Hofstee linearization. When metabolism is biphasic, the rate of a reaction is described by Equation (1):

$$v = \frac{V_{max1}\,S}{K_{m1} + S} + \frac{V_{max2}\,S}{K_{m2} + S} \quad (1)$$

Subscript 1 represents high affinity and subscript 2 the low affinity enzyme and S is the concentration of substrate. When solubility prevented the saturation of low affinity enzyme, the data were fit into equation (2), assuming that we can observe only linear part of the second Michaelis term. The fit was performed using GraphPad Prism software (GraphPad Software, version 3.0, Inc. San Diego, CA).

$$v = \frac{V_{max1}\,S}{K_{m1} + S} + \frac{V_{max2}}{K_{m2}}\,S \quad (2)$$

For identification of metabolites, the compound was incubated at 10 μM concentration with rat or human liver microsomes (0.5 mg/ml protein, 1 h, 37°C). The reaction was terminated by the addition of ACN. Samples were vortexed, centrifuged, and the supernatant was dried under nitrogen. The residue was reconstituted in MeOH/H_2O 1/1 (v/v) and metabolites were analyzed as described below.

For immunoinhibition studies, human liver microsomes pooled from four donors (2 males and 2 females) were preincubated with monoclonal antibodies inhibitory to CYP3A (0–10 mg IgG/mg microsomal protein) for 15 min on ice followed by the addition of AG-045572 (10 μM) and NADPH. The samples were incubated for 40 min at 37°C and analyzed for total metabolism as described below. For immunoinhibition of AG-045572 metabolism in rat liver microsomes, a mixture (0.5 mg/ml protein and 0–20 μl of anti-rat CYP3A2 serum) was preincubated for 30 min at room temperature followed by the addition of 10 μM AG-045572 (pooled male liver microsomes) or 1 μM AG-045572 (pooled female rat liver microsomes) and NADPH. The samples were incubated for 10 min at 37°C and analyzed as described below.

Animals

Adult male (250–325 g) or female (200–230 g) Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (San Diego, CA). Animals were maintained in a temperature-controlled room (22 ± 2°C) with a photoperiod of 12 h light/12 h dark. Rat chow (Teklad rat diet) and tap water were provided *ad libitum*. Experiments followed the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985)

Pharmacokinetic Studies

Rats were prepared with indwelling jugular vein catheters as described earlier (11) and allowed to recover overnight with free access to standard rat chow and water. Male rats were surgically castrated via scrotal approach under halothane anesthesia and allowed 14 days post-operative recovery prior to study. AG-045572 was administered acutely at 10 mg/kg (i.v.) or 20 mg/kg (p.o.) as 10 mg/ml solution in 10% DMSO, 10% Cremophor EL, 80% saline. For multiple-dose pretreatment, the compound was given to male rats at 40 mg/kg (20 mg/ml in 20% EtOH, 20% Cremophor EL, 60% saline) i.m. twice a day for 3 days. On the 4th day, rats received a single dose in the morning. The pharmacokinetics of AG-045572 in pretreated rats was studied 24h after the last dose. Immediately before the experiment a sample of blood was taken to ensure that there was no residual compound in plasma. The blood samples were withdrawn at specific times, plasma was immediately separated (100 μl) and compound was extracted with ethyl acetate (1.4 ml) containing internal standard. The supernatant was dried under nitrogen, the residue reconstituted in 80 μl of mobile phase and 50 μl was analyzed by HPLC. The pharmacokinetic parameters were calculated using WinNonlin software (Scientific Consulting, Inc.). The bioavailability was calculated as AUCp.o./AUCi.v., where AUCp.o. and AUCi.v. are areas under the plasma concentration—time curve after oral and i.v. administration, respectively.

Testosterone Measurements

Male rats were single-housed and allowed to acclimate for 1 week. Three days before study, animals were anesthetized with halothane and instrumented with indwelling jugular vein cannulae. On study day, animals were allowed to acclimate to the procedure room while residing in their home cage. Basal blood samples were drawn from all animals between 8:15 and 8:30 am. Immediately following basal sampling, test compound (40 mg/kg) or vehicle was administered by intramuscular injection. Test compound was formulated at 20 mg/ml in 20 % EtOH, 20% Cremophor EL, and 60% physiological saline. Blood samples were drawn into heparin containing tubes at multiple time points post treatment. The blood was centrifuged immediately, plasma collected and stored in −20°C until assayed.

Plasma samples were analyzed using DSL-4000 ACTIVE Testosterone coated-tube radioimmunoassay kit from Diagnostic Systems Laboratories, Inc. Webster, TX. Testosterone levels (ng/ml) were expressed as means ± SEM. Significant differences between groups of animals were determined by two-way analysis of variance with *post hoc* Tukey multiple comparisons at each particular time point (SPSS software, Chicago, IL). Differences were considered significant if $P < 0.05$.

Growth Hormone Measurements

Rats were prepared as described above for testosterone measurements. Blood samples were withdrawn every 20 min for 8 h beginning at 9:00 a.m. To study the effect of multiple doses of AG-045572 male rats received the compound at 40 mg/kg (20 mg/ml in 20% EtOH, 20% Cremophor EL, 60% saline) i.m. twice a day for 3 days. On the 4th day, rats received a single dose in the morning followed by blood sampling. Plasma was immediately separated and stored at −20°C

until assayed for growth hormone or testosterone (as described above).

Growth hormone in plasma was measured using Rat growth hormone enzyme immunoassay system, code RPN 2561 from Amersham Pharmacia Biotech Inc., Piscataway, NJ.

HPLC Analysis

Samples were analyzed on a Betabasic C18 column, 3 μm, 4.6 × 50 mm (Western Analytical Products, Inc., Murrieta, CA) using a Hewlett-Packard HPLC system (model 1100, Palo Alto, CA). A linear gradient of 50 to 70 % ACN in 10 mM ammonium phosphate buffer pH 7 over 10 min was used with UV detection at 260 nm. The flow rate was 1 ml/min. The instrumental detection limit for the assay of AG-045572 plasma concentrations was 10 ng/ml.

LC/MS/MS Analysis of Metabolites

The analysis was performed using Hewlett-Packard HPLC system (model 1100, Palo Alto, CA) connected to a Finnigan LCQ ion trap (Classic model, San Jose, CA) mass spectrometer. The metabolites were separated on a Betabasic C18 column, 3 μm, 2x30 mm (Western Analytical Products, Inc., Murrieta, CA) using a linear gradient of acetonitrile (10 to 95% over 15 min) in 50 mM ammonium acetate. The flow rate was 1 ml/min.

For ion formation the standard Finnigan atmospheric pressure chemical ionization (APCI) source was used in the positive ion mode. The MS/MS spectra were acquired using the data-dependent scanning feature, whereby the Xcalibur instrument control software automatically selects the ion with the highest abundance, isolates it, and then performs the MS/MS experiment. Dissociation was performed with wide-band activation at a relative dissociation energy of 45%, and an isolation window of ± m/z 2.5. The automatic gain control (AGC) mode of operation was enabled.

RESULTS

In vitro Metabolism

The metabolism of AG-045572 (Fig.1) in human and rat liver microsomes was NADPH dependent. AG-045572 was hydroxylated at the 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethyl-naphthyl moiety (M1) followed by either introduction of a second hydroxyl group, or further oxidation to a keto form (M2). A product of O-demethylation at the N-(2,4,6-trimethyxyphenyl) moiety (M3) was also observed. All major metabolites appear to be similar in human (male/female), and rat (male/female) microsomes and expressed CYP3A4 and CYP3A5 (Fig. 2). The LC-MS/MS analysis performed here did not allow us to assign the exact atomic position of modifications. However, it was decided that this was sufficient, as the aim of this work was to show the similarity of metabolic pathways between the specimens tested, which was supported by the identical retention times and masses of the products (Fig. 2).

Examination of the rates of total metabolism using Eadie-Hofstee linearization suggested that kinetics of AG-045572 metabolism was biphasic in both male and female human liver microsomes (Fig. 3). Solubility of the compound prevented saturation of the low affinity enzyme and therefore, the data were fit into equation (2). The high-affinity K_m values were similar in male and female liver microsomes (Table I). The V_{max} was characterized by high interindividual variability.

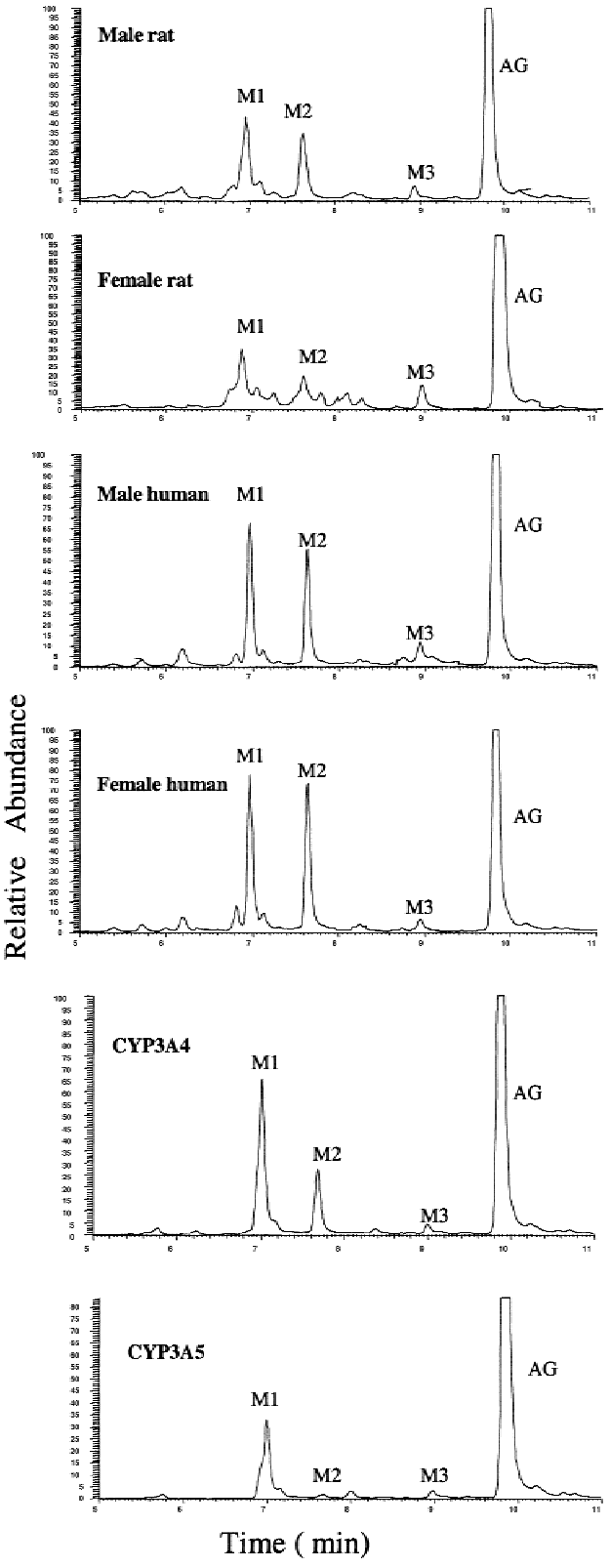

Fig. 2. Patterns of metabolites of AG-045572 formed in incubations with liver microsomes or recombinant CYP3As.

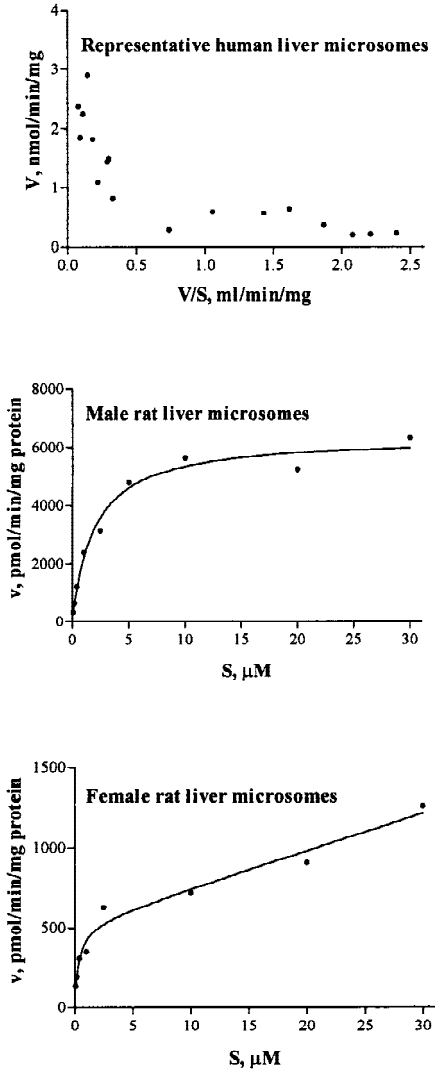

Fig. 3. Representative kinetic curves of metabolism of AG-045572 by human (Eadie-Hofstee plot), male, and female rat liver microsomes.

In contrast to human liver microsomes, there was a significant difference in metabolism of AG-045572 by male and female rat liver microsomes (Table I, Fig. 3). Female rats showed kinetics similar to that observed in humans with high affinity K_m similar to that in humans. In male rats, the metabolism of AG-045572 followed the classical Michaelis-Menten kinetics suggestive of a single enzyme. Moreover, the K_m value for this enzyme was ~10-fold higher than the high-affinity K_m in human and female rat liver microsomes. The rate of metabolism by female rat liver microsomes was significantly lower than by all other specimens tested.

The K_m for AG-045572 metabolism by CYP3A4 and CYP3A5 was similar to the high affinity constant observed with human and female rat liver microsomes (Table I). The rate of metabolism by CYP3A7 was similar to CYP3A5. CYP2D6, CYP2C9, and CYP2E1 did not show any activity toward metabolism of AG-045572 at 10μM concentration of the compound (data not shown).

The metabolism of AG-045572 in human liver microsomes was compeletely suppressed by monoclonal antibodies inhibitory to CYP3A added at 10:1 (w:w) ratio to microsomal protein. Our data suggest that the contribution of low affinity enzyme is small at 10 μM concentration, which also means that it would not play a significant role in the elimination of the compound *in vivo*. Anti-rat CYP3A2 serum completely suppressed metabolism of AG-045572 in male and female liver microsomes. These data suggest that this compound is metabolized by CYP3A in both male and female rat liver microsomes.

Table I. Enzyme Kinetic Parameters of Total metabolism of AG-045572

Sample	K_{ml}, μM	V_{max1}, pmol/min/mg
Male human	0.39 ± 0.16	787 ± 488
Female human	0.27 ± 0.06	1317 ± 563
Male rat	1.51 ± 0.33	8649 ± 2067
Female rat	0.28 ± 0.09	374 ± 144
CYP3A4	0.25[a]	6.7[b]
CYP3A5	0.26[a]	1.7[b]

Values are the mean of three different microsomal preparations. Variations are ± SD [a]mean of two determinations; [b]pmol/min/pmol CYP.

Pharmacokinetics

When AG-045572 was given to intact male rats, it showed medium $T_{1/2}$, CL and V_{ss} but oral bioavailability was low (Table II). However, in female rats the bioavailability was much higher (24%). In castrated male rats the pharmacokinetics of AG-045572 was similar to that in female rats (Table II).

Pretreatment of intact male rats with AG-045572 for 4 days resulted in a change of its pharmacokinetics. The parameters became similar to those in female and castrated male rats (Table II).

Suppression of Testosterone

Testosterone levels were measured in rats after a single i.m. dose of AG-045572 and after the last dose in the multiple-dose study (Fig. 4) The data suggests that this treatment regimen provided suppression of testosterone to castrate levels throughout the 4-day pretreatment experiment.

Growth Hormone Measurements

The secretory patterns of growth hormone in intact male and female rats were typical pulsatile and more continuous, respectively. After pretreatment of male rats with AG-045572 for 4 days, the male-specific pattern of growth hormone re-

Table II. Pharmacokinetic Parameters of AG-045572 in Rats after Administration at 10 mg/kg i.v. and 20 mg/kg p.o.

Animals	$T_{1/2}$, h	CL, L/h/kg	V_{ss}, L/kg	C_{max}, μM	T_{max}, h	$F_{p.o.}$ %
Male	1.4 ± 0.1	2.2 ± 0.5	2.1 ± 0.1	0.61 ± 0.21	1	8
Female	1.7 ± 0.1	1.5 ± 0.1	2.7 ± 0.4	2.31 ± 0.57	1	24
Castrated male	1.7 ± 0.4	1.5 ± 0.3	3.7 ± 1.5	1.98 ± 0.51	1	23
Pretreated male	1.9 ± 0.2	1.5 ± 0.2	2.0 ± 0.6	1.89 ± 0.41	1	27

Values are the mean of three rats. Variations are ± SD.

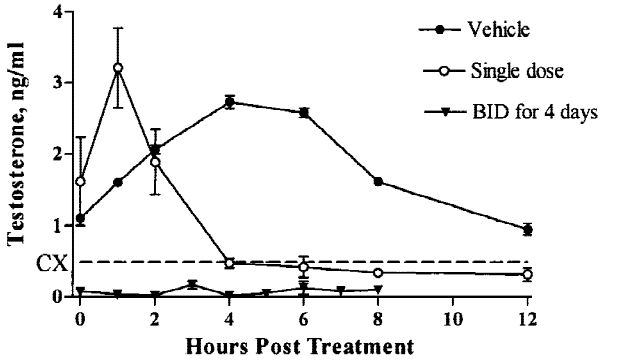

Fig. 4. Plasma testosterone levels in male rats received AG-045572 (40 mg/kg i.m.) as a single dose or BID for 4 days. CX – castrate level of testosterone.

mained unchanged, but with slightly elevated baseline levels. (Fig. 5). Overall concentrations of growth hormone measured in our study were higher than previously reported which could be attributed to the different assay system used.

DISCUSSION

The expression of CYP enzymes in animals and humans is under complex hormonal regulation. The presence of hormones such as growth hormone, thyroid hormone, testosterone, and estrogen during development imprints the expression patterns of CYPs in adults (9,12,13).

One of the most studied CYP subfamilies under hormonal regulation is CYP3A. Although the neuroendocrine mechanisms regulating CYP3A expression is not fully understood, it has been shown that removal of hormones at different stages of development results in different expression patterns of this CYP. Neonatal hypophysectomy or monosodium glutamate treatment of male rats results in suppression of male-specific CYP3A2 at adulthood (12,13). In contrast, hypophysectomy of adult male and female rats results in overexpression of CYP3A2 and CYP3A18 mRNA but has no effect on CYP3A9 (9,14–16).

It is believed that growth hormone plays the most important role in regulation of gender-specific CYPs. This is attributed to its characteristic secretory patterns: high amplitude pulses every 3–5 h in male rats and lower amplitude higher frequency pulses in female (17). However, recently it was shown that treatment of male rats hypophysectomized at adulthood with growth hormone, had no effect on expression of CYP3A2, CYP3A9, and CYP3A18 (16). Moreover, suppression of CYP3A2 in adult hypophysectomized male and female rats requires the presence of thyroid hormone (7,15,18).

Testosterone, in addition to imprinting the adult expression of CYPs during neonatal period, is also required during adulthood for full expression of male-specific CYPs (19). Castration of adult male rats causes reduction in CYP3A2 mRNA (4,19) and administration of testosterone restores physiological levels of this enzyme (4). A similar role is played by estrogen. Ovariectomy of adult female rats causes reduction in female-dominant CYP3A9, which is reversed by treatment with estrogen (20). These factors result in well-defined gender differences in metabolism and pharmacokinetics of various CYP3A substrates in rats .

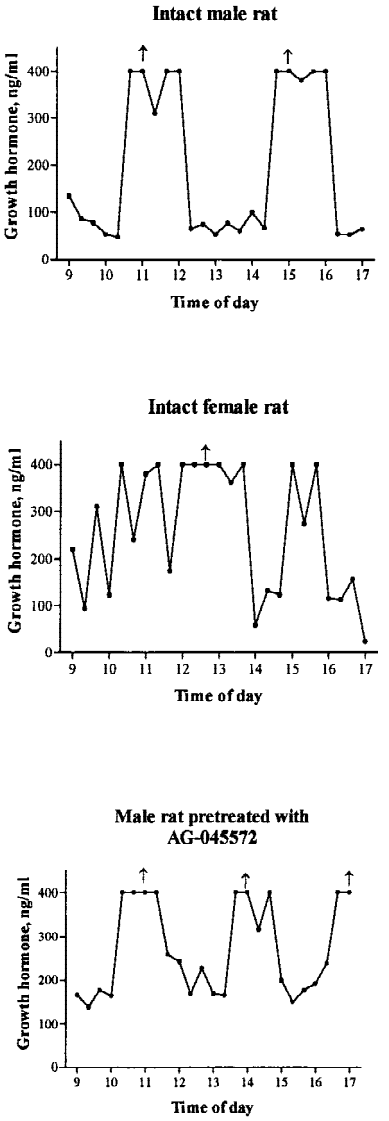

Fig. 5. Representative plasma growth hormone secretory profiles in male, female, and pretreated with AG-045572 male rats.

Our work showed that AG-045572 is metabolized primarily by CYP3A enzymes in both male and female rats. However, the rates of metabolism were significantly lower in female rats. It has been reported that although total hepatic mRNA content of CYP3A genes in female rats is only 2/3 of that in male, CYP3A-mediated activities (such as 6β-hydroxylation of testosterone) are 10-fold lower than in male rats (21). The existence of gender-specific (or gender-dominant) CYP3As is most likely responsible for the observed difference in CYP3A-dependent activities between male and female rats. In the case of metabolism of AG-045572 we observed different enzyme kinetic parameters in male and female rat liver microsomes, suggesting the contribution of different isozymes of CYP3A.

AG-045572 is a potent GnRH receptor antagonist with Ki of 6.0 and 3.8 nM at human and rat GnRH receptor, respectively (22). Four-day pretreatment of male rats with this compound effectively suppressed testosterone to castrate levels. This was followed by an increase in oral bioavailability of AG-045572 to that seen in surgically castrated male or intact

female rats. The observed suppression of testosterone was not accompanied by a change in pulsatile pattern of growth hormone secretion; however, baseline levels were slightly elevated. It has been published earlier that neonatal or prepubertal castration of male rats elevated baseline levels of growth hormone but did not change its sectretory pattern (23). In adult rats different hormones can act in concert as well as individually on expression of CYPs in various organs (6). Our data suggest that testosterone, rather than the secretory pattern of growth hormone is the primary factor responsible for the change in pharmacokinetics of AG-045572 after pretreatment. Chronic treatment of male rats with any GnRH receptor agent will likely cause a similar effect on hormone-dependent drug metabolizing enzymes.

Most researchers have observed sex-related differences in drug metabolism in rats and mice but not in humans (5). We also did not see statistically significant differences in the metabolism of AG-045572 between male and female human liver microsomes. The absence of clear gender differences in metabolism in humans is generally attributed to the large interindividual variability in CYP expression (24). This variability is most likely due to a substantial number of factors (age, nutritional status, various diseases, medications etc.) that can alter the hormonal levels and as a result expression of CYPs (25,26). Also, Rebbeck *et al.* (27) recently reported a mutation in the transcriptional regulatory element of CYP3A4 gene, which may be important for its expression. The authors showed that patients carrying this mutation had faster developing prostate cancer compared to patients without the mutation. Since CYP3A is involved in the metabolism of endogenous testosterone, factors affecting its expression may have an impact on hormone-dependent diseases such as prostate cancer.

In conclusion, AG-045572 is a potent GnRH receptor antagonist that effectively suppresses testosterone. At the same time, this compound is metabolized by CYP3A whose expression is testosterone-dependent. This results in the "feminization" of AG-045572 pharmacokinetics after multiple-dose pretreatment of male rats with the compound. The possibility that chronic treatment of humans with GnRH receptor antagonist will alter the expression of CYPs requires further investigation.

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