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In Vitro Studies Investigating the Interactions between Degarelix, a Decapeptide Gonadotropin-Releasing Hormone Blocker, and Cytochrome P450

Anders Sonesson and Birgitte Buur Rasmussen

Department of Bioanalysis, Ferring Pharmaceuticals A/S, Copenhagen, Denmark

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Abstract: The decapeptide degarelix is a novel competitive gonadotropin-releasing hormone receptor antagonist that has been approved for the treatment of advanced prostate cancer by the FDA and the EU authorities. In this study, the interaction of degarelix with human cytochrome P450 (CYP450) enzymes was investigated *in vitro*. Inhibition of CYP450 was performed in human liver microsomes using documented marker substrates for the CYP450 isozymes CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP2E1. The inhibitory effects on selected P450 enzyme activities were investigated with degarelix concentrations representing the range of 2–200 times of expected clinical concentrations. No inhibition of any isozyme-catalysed biotransformations studied was detected. Induction of CYP450 enzyme activity by degarelix was investigated using primary human hepatocytes. Cryopreserved plateable hepatocytes and fresh hepatocytes in culture were treated for two-three consecutive days with degarelix at concentrations of 0.1, 1.0 and 10 μM. The cultured hepatocytes were also treated with three prototypical CYP450 inducers: omeprazole, phenobarbital and rifampin as positive controls for CYP450 enzyme induction. No induction of the activity of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 isozymes was observed. Degarelix appears to be a poor substrate of the CYP450 enzyme system, and the *in vitro* results indicate that the interaction between CYP450 and degarelix is low. These results indicate that degarelix is unlikely to cause any clinically significant drug–drug interactions *in vivo*.

Cytochrome P450 (CYP450) is the most important enzyme system involved in the metabolism of endogenous molecules. Besides their role in the metabolism of endogenous molecules, CYP450 enzymes are also the most important enzymes involved in biotransformation of xenobiotics [1]. The metabolic routes of elimination of drugs through the CYP450 family of enzymes can be inhibited or induced by concomitant drug treatment. Drug–drug inhibition interactions can cause increased exposure of a parent drug or metabolites leading to toxic effects [2]. A compound causing an increased activity or biosynthesis of one or more enzymes through induction can also have serious consequences. The drug itself or a co-administered drug will be cleared more quickly causing its pharmacological effect to be reduced that may lead to therapeutic failure of the drug [3]. Therefore, it is an important part of drug development to perform drug–drug interaction studies to investigate whether an investigational agent is likely to significantly affect the metabolic elimination of drugs already on the market and likely in medical practice to be taken concomitantly [4]. According to guidelines from the American Food and Drug Administration (FDA), even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic drug–drug interactions should be explored even for an investigational compound that is not eliminated significantly by metabolism [5].

Degarelix (fig. 1) is a decapeptide that shows a high affinity/selectivity to hGnRH receptors. When administered by subcutaneous injection, the peptide forms a gel depot at the site of injection [6]. Degarelix is slowly released from the depot into the blood stream and results in plasma concentrations of 5–50 nM [7]. The decapeptide has a molecular weight of 1632 containing seven unnatural amino acids, D-2-naphthylalanine, D-4-chlorophenylalanine, D-3-pyridylalanine, 4-aminophenylalanine(L-hydroorotyl)[5], 4-aminophenyl-alanine(carbamoyl)[6], N(epsilon)-isopropyllysine and D-alanine (fig. 1). *In vitro* stability studies performed in liver microsomes and hepatocytes from animals have shown degarelix to be a very poor substrate to cytochrome P450 enzymes. *In vivo* non-clinical metabolism studies of degarelix have shown that degarelix is subject to proteolysis by endopeptidases and unchanged degarelix and metabolites are fully excreted via the hepatic and urinary pathways. Systemic exposure to any metabolic products appears to be very low [8]. Degarelix has been approved for the treatment of advanced prostate cancer by the FDA and the EU authorities and can now be prescribed under the name Firmagon® [9].

The study was designed to evaluate the ability of degarelix to inhibit *in vitro* the CYP450 enzymes in human liver microsomes with the aim of ascertaining the lack of potential of

Author for correspondence: Anders Sonesson, Department of Bioanalysis, Ferring Pharmaceuticals A/S, Kay Fiskers Plads 11, DE-2300 Copenhagen S, Denmark (fax +4528176850, e-mail anders.sonesson@ferring.com).

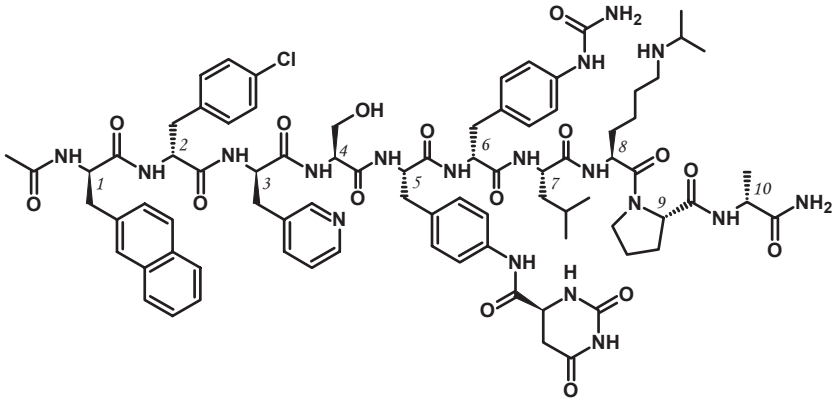

Fig. 1. Structure of degarelix. Peptide sequence: Ac-D-2Nal-D-4Cpa-D-3Pal-Ser-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys-Pro-D-Ala-NH_2. Ac, acetyl; 2Nal, 2-naphthylalanine; 4Cpa, 4-chlorophenylalanine; 3Pal, 3-pyridylalanine; 4Aph, 4-aminophenylalanine; Hor, hydroorotyl; Cbm, carbamoyl; Leu, leucine; ILys, N(epsilon)-isopropyllysine; Pro, proline; Ala, alanine.

degarelix to inhibit the metabolism of concomitantly administered drugs.

Further, the effects on the expression of the CYP450 enzymes were investigated by treating cultured human hepatocytes with degarelix. Cultured hepatocytes have proven to be a reliable test system for evaluation of the inductive effects of new chemical entities [10]. Thus, the possible interaction of degarelix with CYP450 enzymes was investigated, and the results are presented in this report.

Materials and Methods

Chemicals. Magnesium chloride, sulfaphenazole and troleandomycin were obtained from Fluka (Buchs, Switzerland). Acetic acid, 4-acetamidophenol, calcium chloride dehydrate, chlorzoxazone, diclofenac sodium salt, disulfiram, EDTA, 6α-hydroxypaclitaxel, 6β-hydroxytestosterone, ketoconazole, Krebs–Henseleit Buffer, miconazole, NADPH, omeprazole, paclitaxel, PBS, phenacetin, quinidine, sodium bicarbonate, salicylamide, sulfaphenazole, testosterone, TOX7 kit and tranylcypromine were purchased from Sigma-Aldrich (St Louis, MO, USA). (+/−)-Bufuralol hydrochloride salt, (+/−)-hydroxybufuralol maleate salt, furafylline, S-mephenytoin and 4-hydroxymephenytoin were obtained from Salford Ultrafine Chemicals (Manchester, UK). DMSO (Seccosolv), glacial acetic acid (pro analysi), acetonitrile (isocratic grade for liquid chromatography), hydrochloric acid (pro analysi), methanol (gradient grade for liquid chromatography), potassium dihydrogen phosphate (pro analysi) and potassium hydroxide (pro analysi) were obtained from Merck (Darmstadt, Germany). Montelukast sodium salt was obtained from Larodan Fine Chemicals (Limhamn, Sweden). Efavirenz was purchased from US Pharmacopeia (Rockville, MD, USA), and 8-hydroxyefavirenz was purchased from Toronto Research Chemical Inc. (North York, ON, Canada).

Degarelix was produced by Polypeptide Laboratories (Torrance, CA, USA). *InVitro*GRO HT medium, *InVitro*GRO CP medium, *InVitro*GRO HI medium, Torpedo antibiotica and collagen-coated 24-well plates were purchased from In Vitro Technologies (Baltimore, MA, USA).

Liver tissue. Pooled liver microsomes from male human beings containing 20 mg protein/ml from two suppliers were used. One batch was purchased from In Vitro Technologies (pool of 15), and one batch was acquired from XenoTech, LLC (pool of 50 individuals) (Lenexa, KS, USA). The liver microsome preparations were stored at −80°C.

Cryopreserved human plateable hepatocytes were purchased from In Vitro Technologies. Three batches were used from two male donors (lot no. ZCA and DJV) and one female donor (lot no LOF). Experiments with fresh human hepatocyte cultures were performed at a Xenotech, LLC; three liver batches were used.

Solutions prepared. Bufuralol HCl salt, degarelix, diclofenac HCl salt and NADPH or NADPH-generating system were dissolved in buffer 100 mM, potassium phosphate (pH 7.4) containing 1 mM EDTA and 3 mM magnesium chloride. Disulfiram, efavirenz, furafylline, miconazole, quinidine and phenacetin were dissolved in methanol. Chlorozoxazone, ketoconazole, sulfaphenazole, testosterone were dissolved in acetonitrile. Omeprazole, orphenadrine and paclitaxel were dissolved in DMSO.

CYP450 inhibition studies. The ability of degarelix to selectively inhibit enzyme activity was investigated against CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 activity. All assays were performed at our laboratory except the CYP2B6 assay that was performed at a Xenotech LLC. In-house incubation was carried out in duplicates in glass test tubes and in a shaking water bath at 37°C. The incubation buffer used was 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 3 mM magnesium chloride. Liver microsome preparations were diluted in the phosphate buffer. The marker substrate used and concentration of marker substrate and standard inhibitor used for each isozyme are listed in table 1. Control samples representing solvent added without degarelix or control inhibitor were included in each assay.

For enzyme activities of CYP1A2, CYP2C19, CYP2D6 and CYP2E1, the incubation experiments were performed with a liver microsome protein concentration of 4 mg/ml in a final volume of 0.25 ml. The concentrations of added degarelix were 0 (control sample), 4.2, 42, 420 and 4200 nM. The incubations were performed by first adding degarelix solution (10 µl) or inhibitor solution (2 µl) after which phosphate buffer was added to a total volume of 98 µl in each test vial. Freshly prepared NADPH-generating system (2.5 mM NADP, 12.5 mM glucose-6-phosphate and 40 U/ml glucose-6-phosphate dehydrogenase) solution (100 µl) was added, and the tubes were mixed. Pooled human liver microsomes (50 µl) were then added to each tube. The tubes were capped, gently mixed and thereafter placed in a water bath for pre-incubation for 5 min. After pre-incubation, marker substrate (2 µl) was added to each tube, and the tubes were gently mixed and thereafter incubated for 45 min. After 45 min., the tubes were placed on ice and proteins were precipitated by the addition of 0.25 ml of acetonitrile/glacial acetic acid 96/4 (v/v) followed by vortexing. The tubes were then centrifuged (4000 × *g* for 20 min. at 8°C) to pellet the proteins, and the supernatant from

Table 1.

Assay conditions including marker substrates, concentrations, metabolites, multiple reaction monitoring (MRM) transitions, selected ion monitoring (SIM) transitions and inhibitor used for CYP450 interaction studies in liver microsomes.

Isozyme	Marker substrate	Concentration (μM)	Metabolite	MRM	SIM	Inhibitor
CYP1A2	Phenacetin [11]	200	Acetaminophen	n.u.	152.2	Furafylline [12]
CYP2B6	Efavirenz [13]	3	8-hydroxyefavirenz	330 → 286	n.u.	Orphenadrine [14]
CYP2C8	Paclitaxel [15]	12.2	6α-hydroxypaclitaxel	870 → 286	n.u.	Montelucast [16]
CYP2C9	Diclofenac [17]	11	4′-Hydroxydiclofenac	310 → 266	n.u.	Sulfaphenazole [17]
CYP2C19	Omeprazole [18]	200	5′-hydroxyomeprazole	n.u.	384	Ketoconazole [19]
CYP2D6	Bufuralol [20]	200	1′-hydroxybufuralol	n.u.	278	Quinidine [20]
CYP3A4	Testosterone [21]	200	6β-hydroxytestosterone	n.u.	305	Miconazole [22]
CYP2E1	Chlorozoxazone [23]	200	6-hydroxychlorzoxazone	n.u.	184	Disulfiram [24,25]

n.u., not used.

each tube was transferred to 1 ml liquid chromatography (LC) autosampler vials (Waters, Milford, MD, USA).

For measurement of CYP3A4 activity, the incubation conditions and procedure were as described above with the exception that the incubation time was 10 min. Also, after the incubation with the tubes placed on ice, testosterone and metabolites were extracted from the incubation mixture by adding 3 ml of methylenchloride to each tube followed by vortexing. The tubes were then centrifuged ($4000 \times g$ for 20 min. at 8°C) after which the lower organic phase was transferred to new test tubes and the organic extracts were taken to dryness in a SpeedVac vacuum-centrifuge (Savant Instruments Inc., Farmingdale, NY, USA). The dried down samples were redissolved in 0.4 ml of water/methanol/acetonitrile 64/35/1 (v/v/v) and transferred to LC autosampler vials (Waters).

The CYP2B6 activity assay was performed at Xenotech LLC. The protein concentration for the assay was 0.1 mg/ml in a final volume of 0.2 ml. The incubation buffer was 50 mM, pH 7.4, potassium phosphate buffer containing 1 mM EDTA and 3 mM magnesium chloride. The concentrations of degarelix investigated were 0 (control samples), 10, 30, 100, 300, 1000, 3000 and 10,000 nM. All pipetting steps were performed using a Tecan liquid-handling system into 96-well plates. Degarelix was dissolved in methanol in this assay, and 2 μl of degarelix or orphenadrine was added in the assay. Reactions were initiated by the addition of an aliquot of an NADPH-generating system (1 mM NADP, 5 mM glucose-6-phosphate and 1 unit/ml of glucose-6-phosphate dehydrogenase). The incubation time was 5 min., and the proteins were precipitated by the addition of acetonitrile containing the internal standard [^{2}H]-8-hydroxyefavirenz. Precipitated protein was removed by centrifugation ($920 \times g$ for 10 min. at 10°C), and the supernatants were transferred to a new 96-well plate for analysis by LC with tandem mass spectrometry (MS/MS) detection.

The CYP2C8 activity assay was performed with a microsomal protein concentration of 0.25 mg/ml and a NADPH concentration of 2 mM. The concentrations of degarelix investigated were 0 (control samples), 100, 1000 and 10,000 nM. The incubation was started by tempering 320 μl buffer for 1 min. at 37°C, whereupon 2 μl of degarelix solution or montelukast solution was added. The test tubes were mixed and incubated for another minute. Then, NaDPH solution (80 μl) was added, and the tubes were mixed and incubated for another 5 min. The reaction was started with the addition of paclitaxel (2 μl). After 10 min. of incubation, 100 μl sample was quenched with 100 μL acetonitrile/glacial acetic acid 96/4 (v/v) in a polypropylene tube and put on ice for 30 min. The samples were then centrifuged ($13,000 \times g$) for 5 min., and the supernatants were diluted 1/1 (v/v) with Milli-Q water in an LC autosampler vial (Waters).

In the CYP2C9 activity assay, the incubation was performed with a microsomal protein concentration and a NADPH concentration as in the CYP2C8 assay. The inhibitory effect of degarelix was tested at five different concentrations: 1, 10, 100, 1000 and 10,000 nM. The incubations were started by tempering 240 μl microsome suspension (0.31 mg/ml buffer) for 1 min. at 37°C water bath. Degarelix solution (3 μl) was added, or sulfaphenazole solution (1.5 μl) was added. The solution was tempered for another minute, whereupon NADPH solution (60 μl) was added followed by a 5-min. pre-incubation. The reaction was started with the addition of the diclofenac solution (3 μl). After 10 min. of incubation, 100 μl sample was quenched with 100 μl acetonitrile/glacial acetic acid 96/4 (v/v) in a polypropylene tube and put on ice for 30 min. The samples were then centrifuged ($13,000 \times g$) for 5 min., and the supernatants were diluted 1/1 (v/v) with Milli-Q water in an LC autosampler vial (Waters).

Study of the induction potential of degarelix on CYP450 enzymes using cryopreserved plateable human hepatocytes. The induction potential of degarelix on CYP1A2, CYP2C9 and CYP3A activity was investigated in cryopreserved plateable human hepatocytes. Rifampin at a concentration of 25 μM was used as positive control for induction of CYP3A4 and CYP2C9, and omeprazole at a concentration of 50 μM was used as a positive control for induction of CYP1A2. Degarelix was tested at the concentrations of 0.1, 1 and 10 μM in hepatocytes from three different donors. Probe substrates were phenacetin (CYP1A2), diclofenac (CYP2C9) and testosterone (CYP3A4). All substances were dissolved in DMSO and diluted with *InVitro*GRO HI medium keeping the final concentration of DMSO at 0.1% (v/v).

The cryopreserved cells were thawed according to a protocol supplied by In Vitro Technologies. The cells were counted using the Trypan blue exclusion method, and the cell suspension was diluted to 600,000 cells/ml.

The experiments were performed in 24-well plates during 5 days. First, 0.5 ml of the hepatocyte suspension was added to each well for cell attachment. The plates were incubated at 37°C, 5% CO_2 in a saturating humidity chamber. After 3–4 hr, the plates were examined for cell attachment. Any remaining unattached cells were removed, and the wells were filled with 0.5 ml *InVitro*GRO CP medium and placed in the incubator. The media were changed after 24 hr. On day 3, the wells were refilled with 0.5 ml *InVitro*GRO CP medium containing the degarelix substance, vehicle control and positive controls, and the media were changed after 24 hr. On day 5, after 48 hr of exposure of the inducers, the cells were washed and the wells refilled with 0.5 ml of CYP-specific standard substrate diluted in Krebs–Henseleit buffer. The plates were returned to the incubation chamber, and samples for measurement of 4′-hydroxydiclofenac were removed after 2 hr of incubation. Samples for measurement of 6β-hydroxytestosterone were removed after 3 hr of incubation, and samples for measurement of acetaminophen were removed after 4 hr. Sample volume removes was 50 μl at each time-point, and the samples were mixed with 50 μl acetonitrile/glacial acetic acid 96/4 (v/v) to precipitate the proteins. After centrifugation, the supernatant was diluted 1/1 (v/v) with water in a LC autosampler vial (Waters), and the diluted sample was analysed directly by LC–MS/MS analysis.

Study of the induction potential of degarelix on CYP450 enzymes using freshly cultured human hepatocytes. The induction potential of degarelix on CYP2B6, CYP2C8 and CYP2C19 activity was

investigated in freshly cultured human hepatocytes at Xenotech LLC. Phenobarbital at a concentration of 750 μM was used as a positive control for induction of CYP2B6, and rifampin at a concentration of 10 μM was used as positive control for induction of CYP2C8 and CYP2C19.

Fresh hepatocytes were isolated and cultured according to Quistorff *et al.* [26]. The hepatocytes were seeded on 60-mm Permanox culture dishes (Fisher Scientific, Pittsburgh, PA, USA), approximatively 1.1–1.5 × 10^6 viable cells/ml and 3 ml per dish, and thereafter coated with collagen (PureCol) and placed in a humidified culture chamber (37 ± 1°C, at 95% relative humidity, 95/5% air/CO_2). After an attachment period of 2–3 hr, media and dead or unattached cells were removed by aspiration, and the media were replaced with Modified Eagle's medium Dr. Chee's modification (MCM) containing ITS+ (6.13 μg/ml transferrin and 6.13 μg/ml selenous acid), linoleic acid (5.25 μg/ml), BSA (1.23 mg/ml), penicillin (49 U/ml), streptomycin (49 μg/ml), dexamethasone (0.098 μM) and Matrigel (250 μg/ml). Cultures were allowed to adapt to the culture environment for three days, during which the medium was replaced daily with supplemented MCM (without Matrigel) [27,28]. After the adaption period, the hepatocyte cells were examined under a light microscope and determined to be morphologically normal and suitable for treatment with the test article.

The hepatocyte cultures were treated daily for three consecutive days with supplemented MCM (each culture dish was treated with approximately 3 ml) containing 0.1% (v/v) DMSO (vehicle, negative control), one of the three concentrations of degarelix (0.1, 1 or 10 μM) or one of the known CYP enzyme inducers, phenobaribital and rifampin. Approximately 24 hr after final treatment, microsomal samples were prepared based on the method described by Madan *et al.* [29]. The resulting microsomal pellets were resuspended in 250 mM sucrose and stored at −80 ± 5°C.

The protein concentration in each microsomal sample was determined with a BCA protein assay kit (Pierce Chemical Company, Rockford, IL, USA). The microsomal incubations were performed at 37°C in 0.2 ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4) with 3 mM $MgCl_2$ and 1 mM EDTA. The reactions were initiated by the addition of a NADPH-generating system (1 mM NADP, 5 mM glucose-6-phosphate and 1 unit/ml of glucose-6-phosphate dehydrogenase). Tecan robotics was used for liquid handling, and the incubations were performed in 96-well plates.

A bupropion hydroxylase assay was used for measurement of CYP2B6 activity [30]. The concentration of bupropion was 500 μM in the incubation mixture, and the microsomal protein concentration was 0.04 mg/ml. The incubation was performed for 30 min. before terminating it by the addition of acetonitrile containing [$^2H_6$]-hydroxybupropion.

CYP2C8 activity was measured using an amodiaquine *N*-dealkylase assay [31]. The starting incubation mixture contained a 20 μM of amodiaquine and 0.02 mg/ml microsomal protein. The reaction was terminated after 10 min. of incubation by the addition of acetonitrile containing [$^2H_5$]-*N* desethylamodiaquine.

To measure CYP2C19 activity, an S-mephenytoin 4′-hydroxylase assay was used [32]. The starting concentration of S-mephenytoin was 400 μM, and the protein concentration in the incubation mixture was 0.1 mg/ml. The reaction was terminated after 30 min. of incubation by the addition of acetonitrile containing [$^2H_3$]-4′-hydroxymephenytoin. Precipitated protein was removed by centrifugation (920 × *g* for 10 min. at 5°C), and the supernatant fractions were analysed by LC–MS/MS.

LC–MS and LC–MS/MS analysis. CYP1A2 and CYP2D6 inhibition assays. Acetaminophen and 1′-hydroxybufuralol were analysed by LC–MS using a Waters 2690 LC with a 996 diode-array detector connected to a Micromass (Manchester, UK) Q-Tof MS instrument using an electrospray interface operating in positive ion polarity mode. Table 1 lists the selected ion monitoring (SIM) transitions used for MS quantification. The LC column used was a Symmetry C_{18} column (150 × 2.1 mm, 5 μm; Waters) equipped with a Symmetry Shield C_{18} guard column (12.5 × 2.1 mm, 5 μm; Waters). The column flow rate was 0.25 ml/min., and the column temperature was 40°C. The samples were analysed using a gradient elution with a starting composition of 91/9 (v/v) water/methanol containing 5 mM ammonium acetate and 0.2% (v/v) formic acid. The mobile-phase gradient was first increased linear to 13.5% methanol during 5 min., and then, the organic content was increased more rapidly to 64.5% during 10 min. The mobile-phase composition was subsequently kept isocratic for an additional 5 min. before returning to the starting composition with a linear decrease in organic content during 3 min. after which the column was equilibrated for 5 min. before injecting the next sample.

CYP2C19 inhibition assay. 5′-hydroxyomeprazole was analysed with the same instrument and conditions as described above with exception of the LC column used, which was an Zorbax SB-C_{18} (150 × 2.1 mm, 5 μm; Agilent, Santa Clarita, CA, USA) with an Eclipse C_8 pre-column (12.5 × 2.1 mm, 5 μm; Agilent). The starting composition of the mobile phase was 91% water and 9% acetonitrile; the concentration of acetonitrile was increased linear to 64% during 15 min. and then kept isocratic at this composition for 2 min. before returning to the starting composition with a linear decrease during 3 min. The column was equilibrated with the starting composition for 10 min. before injecting the next sample.

CYP2E1 inhibition assay. 6′-hydroxychlorzoxazone was analysed with the same instrument and conditions as described for the CYP1A2 and CYP2D6 assays with the exception that an APCI interface was used on the MS working in negative ion polarity mode. The mobile phase and gradient used were also different. The starting composition was 91/9 (v/v) water/methanol containing 25 mM ammonium acetate and 0.2% (v/v) formic acid. The organic content was increased to 64.5% during 16 min. The mobile-phase composition was then kept isocratic for an additional 3 min. before returning to the starting composition with a linear decrease in organic content during 3 min., and then the column was equilibrated for 5 min. before injecting the next sample.

CYP3A4 inhibition assay. 6β-hydroxytestosterone was analysed with the same instrument and conditions as described for the CYP1A2 and CYP2D6 assays with the exceptions of the column, mobile phase and gradient used. The LC column used was an Zorbax SB-C_{18} (150 × 2.1 mm, 5 μm; Agilent) with an Eclipse C_8 pre-column (12.5 × 2.1 mm, 5 μm; Agilent). The starting composition of the mobile phase was water/methanol/acetonitrile 64/35/1 (v/v/v) containing 0.2% (v/v) formic acid. This composition was held isocratic for 10 min. before the linear gradient was started, and after 20 min., the composition was water/methanol/acetonitrile 43.5/55/1.5 (v/v/v) containing 0.2% (v/v) formic acid, and then after an additional 5 min., the composition was water/methanol/acetonitrile 18/80/2 (v/v/v) containing 0.2% (v/v) formic acid. The final composition was held at isocratic conditions from 10 min., and then the mobile-phase composition changed linear during 5 min. to the starting composition. The column was equilibrated with the starting composition for 6 min. before injecting the next sample.

CYP2B6 inhibition assay. 8-hydroxyefavirenz was analysed by LC–MS/MS using a system of Shimadzu (Kyoto, Japan) LC pumps and autosampler and an Applied Biosystems/MDS Sciex (Foster City, CA, USA) API3000. An Atlantis C_{18} (100 × 2.1 mm, 5 μm; Waters) column was used equipped with a Security Guard C_8 pre-column (4.0 × 2.0 mm; Phenomenex, Torrance, CA, USA). The electrospray ionization was performed with negative potential, and the multiple reaction monitoring (MRM) transition is described in table 1.

CYP2C8 inhibition assay. The formation of the metabolite 6α-hydroxypaclitaxel was measured by UPLC–MS/MS with the MRM transition listed in table 1. An ACQUITY UPLC system (Waters) interfaced to a Quattro micro instrument (Waters) was used

equipped with an electrospray interface operating in positive potential mode. The column used was an ACQUITY UPLC BEH Shield RP18 (2.1 × 50 mm, 1.7 μm; Waters) with a frit (0.2 μm, 2.1 mm; Waters) placed prior to the column. The mobile-phase flow rate was 0.4 ml/min., and the column temperature was 60°C. The samples were analysed using a gradient. The starting mobile-phase concentration was 10 mM ammonium bicarbonate (pH 10)/acetonitrile 8/2 (v/v) for 0.15 min., and then the concentration was increased linearly to 95/5 (v/v) during 1.15 min. The mobile-phase composition remained at 10 mM ammonium bicarbonate (pH 10)/acetonitrile 8/2 (v/v) for an additional 0.5 min. before returning to 8/2 (v/v) to equilibrate the column for 0.6 min.

CYP2C9 inhibition assay. The formation of the metabolite 4′-hydroxydiclofenac was determined by LC–MS/MS MRM detection of negative ions; the MRM transition measured is listed in table 1. The LC–MS/MS system was a Waters Alliance 2690 LC system connected to a Micromass Quattro Ultima using an electrospray interface operating in negative potential mode. The column was an XTerra RP18 (2.1 × 50 mm, 3.5 μm; Waters) equipped with a C_{18} pre-column (2.1 × 10 mm, 3.5 μm; Waters). The LC flow rate was 0.3 ml/min., and the column temperature was 40°C. The starting mobile-phase condition was water/acetonitrile 93/7 (v/v) with 0.5% (v/v) glacial acetic acid; after 0.5 min., a linear gradient started, and after 4 min., the composition was water/acetonitrile 1/9 (v/v) with 0.5% (v/v) glacial acetic acid. This composition remained isocratic for 1.5 min. and returned thereafter to the starting composition during 0.5 min. followed by conditioning of the column for 3 min. before the next sample was injected.

Conditions used in inducer investigation. The CYP450 marker substance metabolites measured for monitoring CYP450 induction in the hepatocyte preparations are listed in table 2. For the CYP1A2, CYP2C9 and CYP3A4 assays, the instrument used was a 2690 Alliance LC (Waters) connected to a Quattro Ultima (Micromass) MS/MS instrument using an electrospray ionization interface. The LC column used was an Zorbax MS C_{18} (50 × 2.1 mm, 3 μm; Agilent), the column temperature was 40°C, and the column flow rate was 0.3 ml/min. Starting composition of the LC gradient used was water/acetonitrile 93/7 (v/v) containing 0.5% (v/v) glacial acetic acid, and after a 0.5-min. isocratic period, the organic content increased linearly during 4 min. to a final composition of methanol/water 9/1 containing 0.5% (v/v) glacial acetic acid. The high organic composition was kept isocratic for 1.5 min. before a linear decrease to the starting composition during 0.5 min. The column was equilibrated for 5 min. prior to the injection of the next sample.

For measurement of hydroxybupropion and N-desethylamodiaquine, an API2000 (Applied Biosystems/MDS Sciex) MS/MS instrument was used whereas for 4′-hydroxymephenytoin analysis, an API3000 (Applied Biosystems/MDS Sciex) was used. The MS/MS instruments were used with Shimadzu LC pumps and autosampler systems. An Atlantis C_{18} column (100 × 2.1 mm, 5 μm; Waters) with a connection guard column C_8 (4.0 × 2.0 mm, Phenomenex) was used for all three assays.

Table 2.
Analytical conditions including metabolites, multiple reaction monitoring (MRM) transitions and polarity of detected ions for CYP450 assays used in CYP450 induction studies in human hepatocytes and liver microsomes prepared from human hepatocytes.

Isozyme	Metabolite	MRM	ESI polarity
CYP1A2	Acetaminophen	152 → 110	Positive
CYP2B6	Hydroxybupropion	256 → 119	Positive
CYP2C8	N-desethylamodiaquine	328 → 283	Positive
CYP2C9	4′-hydroxydiclofenac	310 → 266	Negative
CYP2C19	4′-hydroxymephenytoin	233 → 190	Negative
CYP3A4	6β-hydroxytestosterone	305 → 269	Positive

ESI, electrospray ionization.

Results

Inhibition of CYP450 activities.
The rate of inhibition was calculated using peak area data of biotransformed metabolite by the formula:

$$\%\,\text{Inhibition} = (100 - [(\text{Test inhibitor sample after final time of incubation}) - (\text{Test inhibitor sample after 0 min. of incubation})]/[(\text{Control sample after final time of incubation}) - (\text{Control sample after 0 min. of incubation})]) \times 100$$

Inhibition of P450 enzyme activity.
No inhibitory effects of degarelix were observed on relevant CYP450 marker enzyme activities in buffered human liver microsome suspensions at concentrations investigated as summarized in table 3. Inhibition of CYP1A2, CYP2C19, CYP2D6, CYP3A4 and CYP2E1 was investigated with a maximum degarelix concentration of 4.2 μM whereas inhibition of CYP2B6, CYP2C8 and CYP2C9 was investigated with a maximum degarelix concentration of 10 μM.

Selective inhibitors of the different P450-catalysed biotransformations studied were included in the experiments to check the validity of the assays performed. All inhibitors included in the study inhibited the isozyme-catalysed biotransformation with ≥50% at the concentrations used. Degarelix appeared to increase CYP2C9 activity slightly; however, the effect did not seem to be dose dependent (table 3). This was also observed in a previous study (data not shown).

Induction of CYP450 enzyme activity.
No induction of the activity of CYP450 enzymes CYP3A4, CYP1A2 and CYP2C9 after dosing with degarelix at the concentration levels of 0.1, 1 and 10 μM in human cryopreserved hepatocytes was observed, as summarized in table 4. At 10 μM, degarelix decreased the enzyme activity of CYP1A2 and CYP2C8 by 33% and 35%, respectively, and with minor effects on CYP3A4. Control substrate rifampin induced CYP2C9 activity 2–3 times and CYP3A4 activity 7–11 times, whereas omeprazole induced CYP1A2 activity 11–19 times in the cryopreserved hepatocyte preparations used.

Treatment of fresh cultured human hepatocytes with degarelix concentrations at the concentration levels of 0.1 and 1 caused no increased activity in the prepared liver microsomes of CYP2B6, CYP2C8 and CYP2C19 (table 5). Treatment with a degarelix concentration of 10 μM caused a decrease in CYP2B6, CYP2C8 and 2C19 activity (38%, 41% and 22% compared to vehicle control, respectively). The typical CYP2B6 inducer phenobarbital increased the CYP2B6 activity 3–19 times whereas rifampin increased CYP2C8 activity 2–4 times and CYP2C19 activity 2–5 times.

Table 3.
Effect of degarelix on various CYP450 activities in human liver microsomes.

Isoenzyme	Reaction	Test article	% Inhibition	Concentration (μM)
CYP1A2	Phenacetin	Furafylline	79	10
	O-deethylation	Degarelix	−4	4.2
CYP2B6	Efavirenz	Orphenadrine	75	750
	8-hydroxylation	Degarelix	6	10
CYP2C8	Paclitaxel	Montelucast	50	0.35
	6α-hydroxylation	Degarelix	−20	10
CYP2C9	Diclofenac	Sulfaphenazol	74	1.2
	4′-hydroxylation	Degarelix	−23	0.001
		Degarelix	−10	0.010
		Degarelix	−2	0.10
		Degarelix	2	1.0
		Degarelix	−11	10
CYP2C19	Omeprazole	Ketoconazole	80	10
	5′-hydroxylase	Degarelix	7	4.2
CYP2D6	Bufuralol	Quinidine	69	10
	1′-hydroxylase	Degarelix	3	4.2
CYP2E1	Chlorzoxazone-	Disulfiram	74	10
	6-hydroxylase	Degarelix	9	4.2
CYP3A4	Testosterone	Miconazole	97	100
	6β-hydroxylase	Degarelix	2	4.2

Table 4.
Induction potential of degarelix on activities of CYP2A1, CYP2C9 and CYP3A4 in three different lots of cryopreserved human hepatocytes.

	Fold induction			
	Degarelix (μM)			Rifampin (μM)
CYP3A4	0.1	1	10	25
Donor 1	0.9	1.2	0.8	6.6
Donor 2	1.1	1.1	0.7	11.5
Donor 3	0.9	1.1	0.7	7.9
CYP1A2	Degarelix (μM)			Omeprazole (μM)
	0.1	1	10	50
Donor 1	0.8	1.0	0.9	19.0
Donor 2	1.5	1.3	0.3	18.8
Donor 3	1.2	1.1	0.8	10.9
CYP2C9	Degarelix (μM)			Rifampin (μM)
	0.1	1	10	25
Donor 1	1.0	1.1	1.0	2.2
Donor 2	1.0	1.1	1.1	3.3
Donor 3	1.0	1.0	1.1	1.8

Table 5.
The effects of treating cultured fresh human hepatocytes with degarelix or prototypical inducers on activities of CYP2B6, CYP2C8 and CYP2C19.

	Fold induction			
	Degarelix (μM)			Phenobarbital (μM)
CYP2B6	0.1	1	10	750
Donor 1	0.9	1.0	0.6	2.6
Donor 2	1.0	1.0	0.7	19
Donor 3	0.9	0.9	0.5	8.1
CYP2C8	Degarelix (μM)			Rifampin (μM)
	0.1	10	10	10
Donor 1	1.0	1.0	0.5	2.0
Donor 2	1.1	1.0	0.8	2.4
Donor 3	1.0	1.0	0.5	3.6
CYP2C19	Degarelix (μM)			Rifampin (μM)
	0.1	1	10	10
Donor 1	0.9	1.1	0.7	3.6
Donor 2	1.0	1.0	0.9	5.3
Donor 3	1.0	1.0	0.7	1.9

Discussion

The duration and/or intensity of action of numerous drugs and other xenobiotics are determined by their rate of metabolism (biotransformation) by several CYP enzymes that are localized primarily in the liver endoplasmic reticulum (microsomes) [33]. If a compound is metabolized by a certain CYP450 enzyme, it could inhibit the metabolism of the other drugs metabolized by the same enzyme and, conversely, these same drugs will inhibit the metabolism of that compound. The degree of inhibition will be determined by the concentration of the given compound and of other drugs and their affinity (K_m or K_i) for binding to the CYP450 enzyme that metabolizes them [34]. Our previous experience with peptide drugs in development is that smaller size peptides, tetrapeptide and heptapeptide are biotransformed by CYP450 enzymes, and inhibition interaction with CYP450 enzymes has been detected. However, degarelix has been shown to be stable in liver microsome preparations, and no biotransformation has been detected [8].

Degarelix is likely to be co-administered with other drugs as it is prescribed to patients with advanced prostate cancer. According to the guideline by the FDA on drug interaction studies [5], metabolic drug–drug interactions should be explored even for an investigational compound that is not eliminated significantly by metabolism. To our experience, it is important that all eight CYP enzymes listed by the FDA are investigated for possible drug–drug interactions with a new chemical entity (NCE) and that the data are included in the new drug application file to be submitted to regulatory authorities.

The inhibitory effects of degarelix on selected P450 enzyme activities were investigated with degarelix concentrations representing the range of 2–200 times of expected clinical concentrations. The findings that degarelix does not inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP2E1 metabolism *in vitro* make it highly unlikely that degarelix will cause clinically significant inhibition of the CYP450 enzymes *in vivo* and thus eliminate the need for clinical inhibition-based drug–drug interaction studies of degarelix and concomitant drugs eliminated by these enzymatic pathways.

The observed increase in CYP2C9 activity in the inhibition assay is not a sign of an enzyme induction process, as this would require protein synthesis. Human liver microsomes are a static system without prerequisites for the protein synthesis processes, such as RNA/DNA transcription, and thus, enzyme induction cannot occur in this matrix. Activation of the CYP2C9 enzyme *in vitro* has been described earlier [35,36] and is believed to be a direct effect of a drug on the active site of the enzyme. Notably, the *in vitro* activation demonstrated for the CYP2C9 enzyme has been shown to have no clinical impact [37]. Also, the induction studies performed in cultured hepatocytes showed no increase in CYP2C9 activity by degarelix.

Primary cultures of human hepatocytes, both freshly isolated human hepatocytes and plateable cryopreserved human hepatocytes, have proven to be a reliable *in vitro* model for evaluating NCEs as inducers of CYP450 [10]. The induction potential of a drug is measured by quantifying the enzyme activity of primary cultures following treatments including the NCE, a positive control inducer drug and vehicle-treated hepatocytes (negative control).

The possible inductive effect of degarelix was compared relative to three mechanistically distinct and clinically relevant CYP inducers, namely omeprazole (an Ah receptor activator and CYP1A2 inducer), phenobarbital (a CAR activator and CYP2B6 inducer) and rifampin (a PXR agonist and inducer of CYP2C8, CYP2C9, CYP2C19 and CYP3A4) [38–42]. The study design, the test system and the selection and concentration of prototypical inducers and probe substrates were based on recommendations by the draft guidance by the FDA [5].

Degarelix showed no tendency to induce the activity of CYP1A2, CYP2C9 and CYP3A4 in human cryopreserved hepatocytes and no tendency to induce CYP2B6, CYP2C8 and CYP2C19 in fresh human hepatocytes. At 10 μM, degarelix decreased the enzymatic activity of all tested enzymes by 22–41%. Because of the fact that a general suppression was seen on all CYPs analysed at 10 μM degarelix, and because of the fact that it is inconsistent with the results seen in the inhibition studies, where no inhibition (all tested CYPs) or time-dependent inhibition (CYP2B6) was seen, the decrease is believed to result from direct biochemical cytotoxicity towards the exposed hepatocytes at this very high supraclinical concentration of degarelix, and thereby resulting in a reduction in the activity of all investigated CYP enzymes.

Because the therapeutic concentration of degarelix is in the range of 5–50 nM, it is highly unlikely that the decrease in enzyme activity or the direct cytotoxic effect observed at 10 μM (200–2000 times higher than the clinical relevant concentrations) of the test article would be of any clinical relevance.

In summary, degarelix is a poor substrate of the P450 enzyme, and the *in vitro* results presented here indicate that there is no interaction between CYP450 and degarelix. It is highly unlikely that degarelix will cause any clinically relevant drug–drug interactions in human beings.

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