Metabolite Profiles of Degarelix, a New GnRH Antagonist, in Rat, Dog and Monkey

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Running title: Metabolite profiles of degarelix in rat, dog and monkey

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Abstract: Number of words 224

Introduction: Number of words 423

Discussion: Number of words 1057

Number of text pages: 22

Number of Tables: 2

Number of Figures: 5

Number of references: 20

ABBREVIATIONS: FSH, follicle-stimulating hormone, hGnRH, human gonadotropin-releasing hormone, LC, liquid chromatography; LH, luteinising hormone, MS, mass spectrometry; MS/MS, tandem mass spectrometry; CYP450, cytochrome P450; RAD, radiochemical detection; SPE, solid phase extraction; TIC, total ion current, Tof, time of flight.
Abstract

Degarelix is a novel competitive gonadotropin-releasing hormone receptor blocker (antagonist). In this study the non-clinical metabolism and excretion of degarelix was investigated in Sprague-Dawley rat, Beagle dog and Cynomolgus monkey. Degarelix was found to be stable when incubated in microsomes and cryopreserved hepatocytes from animal liver tissue. ADME studies in male rat, dog and monkey showed that after a subcutaneous dose of tritium labelled degarelix the peptide was rapidly absorbed with $C_{\text{max}}$ in plasma of 1-2 h. The predominant route of excretion was via the kidneys and the bile. In both rat and dog, the majority of the degarelix dose was eliminated within 48 h via urine and feces in equal amounts (40-50% in each matrix), whereas in monkey the major route of excretion was faecal (50%) and 22% renal. In plasma and urine samples from all three species mainly intact degarelix was detected. In bile and feces samples from rats and dogs, the same truncated peptides of the parent decapeptide were detected. The major metabolites identified represented the N-terminal tetrapeptide, the pentapeptide and the heptapeptide. From the animal studies it could be concluded that degarelix is subject to common peptidic degradation in the liver/bile, and is fully excreted via metabolic and biliary (as metabolites and parent compound) and urinary (mainly as parent compound) pathways. Systemic exposure to metabolic products seems to be low.
Introduction

Degarelix (Fig. 1) is a decapeptide that shows a high affinity/selectivity to hGnRH receptors (Jiang et al., 2001). GnRH blockers bind to GnRH receptors in the pituitary gland without any subsequent receptor activation, which results in a prompt suppression of LH (and FSH) secretion from this gland, which in turn results in decreased production of testosterone from the Leydig cells of the testes (Chengalvala et al., 2003; Ortmann and Diedrich, 1999). The suppression of testosterone occurs almost immediately after administration of the peptide drug, and continues as long as degarelix is available at the receptors (de Pineaux et al., 2001). The efficacy and duration of action of a single subcutaneous injection of degarelix at 2 mg/kg to the intact male rat was compared to that of surgical castration (Broqua et al., 2002, Princivalle et al., 2007). Degarelix suppresses testosterone to castration levels for a total of 42 days and thereafter plasma testosterone levels began to increase gradually and returned to baseline levels at day 77 (Broqua et al., 2002). In nude mice and rat grafted with androgen-dependent human prostate tumour, degarelix injected at 2 mg/kg subcutaneously every 2 weeks produced complete suppression of tumour growth (de Pineaux et al., 2001). Based on the successful non-clinical findings, degarelix has entered and undergone successful clinical development, which resulted in approval for the treatment of advanced prostate cancer by both FDA and EMA, and can now be prescribed under the name Firmagon® (Boccon-Gibod et al., 2009, Pommerville and de Boer, 2010).

The present study was aimed at elucidating the hepatic metabolism and excretion profile of this small peptide hormone analogue with profound biologic activity on the
pituitary gland and testicular function. Due to the presence of several non-natural amino acids in the molecule (D-2Nal, D-4Cpa, D-3Pal, 4Aph(L-Hor), D-4Aph(Cbm), ILys and D-Ala, Fig. 1) degarelix was suspected not to follow the classical route of peptide hydrolysis, which made these explorations of particular interest. The metabolite profile of degarelix in plasma and excreta was compared with reported results of other LH-RH antagonists (Chan et al., 1991, Schwahn et al., 2000). Furthermore, it was also of interest to explore whether the compound is substrate for the CYP450 enzyme in hepatocytes with direct implications for the risk of drug-drug interactions.

This report summarises studies conducted to investigate in vivo metabolism of degarelix in the rat, dog and monkey species as well as in vitro stability studies in liver microsomes and cryopreserved hepatocytes from the same animal species as a part of the preclinical development performed in compliance with regulatory demands.

**Materials and Methods**

**Radiolabeled Test Material.** Tritium-labeled degarelix ([3H]-degarelix, Fig. 1) was prepared by a modification of the method used for synthesis of tritiated version of structurally related GnRH antagonist in (Parnes and Shelton, 1986). Briefly, 2-N-acetylamino-3-(2-naphtyl)acrylic acid was stereospecifically tritiated to N-acetyl-D-[2,3-3H]-2-naphtylalanine with the homogeneous catalyst (S,S)-BPPM-Rh⁺ in methanol. After evaporation of the solvent the crude product was coupled to the unprotected (2-10) degarelix fragment D-4Cpa-D-3Pal-Ser-4Aph(L-Hor)-D-
4Aph(Cbm)-Leu-ILys-Pro-D-Ala-NH₂ with PyBOP/diisopropylethylamine in DMF in 2 h at room temperature. During this coupling step approximately 20% racemisation of the N-Acetyl-D-naphthylalanine occurred, but the resulting [Ac-2Nal₁]-degarelix epimer could easily be separated during the final purification. No byproduct from coupling to the secondary nitrogen of Isopropyllysine⁸ was found, possibly due to steric hindrance. Tritiated degarelix was obtained by reversed phase LC (C₁₈) purification using triethylamine phosphate pH 2.3/acetonitrile buffer followed by counterion exchange for acetate using the same column. The radiosynthesis was performed at Moravek Biochemicals Inc (Brea, California, USA) to afford [Ac-D-[2,3-³H]2Nal₁]degarelix with specific radioactivity of 40 Ci/mmol and radiopurity >95%. The radiolabeled peptide was stored in solution in ethanol-water 1:1 at -40 °C, conditions under which degarelix was found to be chemically stable for at least 4 months.

**Chemicals and Reagents.** The development compound code of degarelix was FE 200486; all metabolites are named according to the usual conventions for modified peptides (IUPAC-IUB joint commission, 1983) using FE 200486 as basis. Degarelix (FE 200486) was synthesised by Polypeptide Laboratories (Torrance, California, USA). The unlabeled reference compounds FE 200486(1-4)-OH, FE 200486(1-6)-OH, FE 200486(1-7)-OH, FE 200486(1-9)-OH, FE 200486(1-10)-OH, FE 200486(4-10) and FE 200486(7-10) were synthesised by Polypeptide Laboratories (Wolfenbüttel, Germany). Internal standard [Ile³(²H₁₀)]-barusiban was prepared at Ferring Research Institute Inc. (San Diego, California, USA). Reagents and solvents were of analytical grade and were obtained from commercial sources.
Liver Tissue. Pooled liver microsomes from three male species: Beagle dogs (pool of 4), Cynomolgus monkey (pool of 6) and Sprague-Dawley rats (pool of 200) were purchased from XenoTech (Kansas, USA) through Tebu-Bio (Le Perray-en-Yvelines, France). The liver microsome preparations were stored at -80°C until use.

Cryopreserved preparations of male rat Sprague-Dawley hepatocytes (#M00005/MSE) and male Beagle dog (#M00205/VWL) hepatocytes were purchased from In Vitro Technologies (Baltimore, Maryland, USA) and stored in liquid nitrogen until use.

In Vitro Studies

Microsomal incubation conditions. Degarelix (10 µM) was incubated in a 1.5 ml solution containing magnesium chloride (3 mM) and EDTA (1 mM) in potassium phosphate buffer (0.1 M, pH 7.4). The liver microsomes protein concentration was 3 mg/ml. The reaction was started by adding NADPH (2 mM). Control samples containing no NADPH were also included in the study. All incubations were performed at 37°C in a shaking water bath. Samples, aliquots of 100 µl, were removed after 0, 5, 10, 20, 40 and 60 min and added to polypropylene tubes containing 100 µl acetonitrile/glacial acetic acid 96/4 (v/v). The precipitated microsomal solutions were vortexed and put in ice for at least 30 min. Internal standard, [Ile\(^3\)\(^{2H_{10}}\)]-barusiban was added to a concentration of 952 nM. The test tubes were centrifuged and the supernatant was diluted with an equal volume of water in an LC auto-sampler vial. The samples were analysed for degarelix substance stability and screening of metabolites using LC-MS.
Stability studies with cryopreserved hepatocytes. The stability of degarelix in hepatocytes was studied using cryopreserved preparations from male Sprague-Dawley rats and male Beagle dog origin. Cryopreserved hepatocytes were thawed and prepared according to a protocol from In Vitro Technologies. The thawed cell suspensions were counted and diluted to 2 million viable cells/ml in a Krebs Henseleit buffer (pH 7.4) containing 2.2 g/l sodium hydrogen carbonate, 22.6 mM HEPES and 1 mM calcium chloride. The metabolic activity of the hepatocyte suspensions were investigated by degradation studies of test substrates naloxone (glucuronic acid activity) (Wahlström et al., 1989), propranolol (CYP2D6 activity) (Masubuchi et al., 1994) and verapamil (CYP3A4 activity) (Kroemer et al., 1993). These activity control samples were analysed by LC-MS/MS.

Substance stability studies were performed at concentrations of 40 µM [³H]-degarelix. Hepatocyte suspensions were tempered at 37˚C for 5 min prior to adding the test substrate. Samples were removed after 0, 10, 30, 60 and 120 min of incubation. Aliquots of 100 µl were withdrawn and terminated in 100 µl acetonitrile/glacial acetic acid 96/4 (v/v) in eppendorf polypropylene tubes. After centrifugation, 100 µl of supernatant was diluted with 100 µl milli-Q water in an LC autosampler vial. The samples were stored at 8˚C until analysis by LC-RAD and LC-MS.

In Vivo Samples.

Animals and treatments. All animal studies were performed at contract laboratory facilities. The animal experiments were performed according to ethical guidelines of animal experimentation and all animal work was carried out in compliance with U.K.
or Dutch legislation and was approved by a local ethical review process as appropriate. Sprague-Dawley rats, Beagle dogs and Cynomolgus monkeys were used. All samples collected were frozen within 1 hour after collection and stored at -80°C until the time of analysis. For metabolite screening analysis the biological samples were shipped on dry ice to Ferring Pharmaceuticals lab facility and stored at -80°C until analysis except the samples from rat study R1 (see below) where the sample analyses were performed at the contract research organisation. All mass balance determinations were performed at the contract research organisations.

Rats. Three studies in rats were performed. The first study (R1) was an ADME study and comprised one group (A) dosed intravenously and two groups (B and C) dosed subcutaneously, respectively. Both male and female rats were used. All rats (bodyweight 200-400 g) were dosed with 30 µg [3H]-degarelix free base peptide/kg (~300 µCi/kg). Feces, heparin plasma and urine samples from groups A and B were collected up to 240 h after dosing. Bile sampling from cannulated rats (group C) as well as urine and feces were collected for up to 48 h. All samples in this study were analysed by LC-RAD at the contract research organisation.

The second rat study (R2) was a biliary excretion study. Three male rats under anaesthesia, had a flexible plastic cannula inserted in the common bile duct to allow complete collection of bile. A second cannula was inserted into the stomach in order to be able to infuse sodium taurocholate in saline solution during the study as a replacement for lost bile salts. The animals were administrated a single subcutaneous dose of [3H]-degarelix (26 µg/kg bodyweight with non-radiolabelled degarelix added to a total dose of 30 µg/kg bodyweight) and placed in metabolism cages. Bile, urine
and feces samples were collected during 48 h after dosing. The collected samples were analysed by LC-RAD and LC-MS.

The third rat study (R3) was performed in order to study the enterohepatic circulation of degarelix and its metabolites. Three pairs of male rats were surgically prepared under anaesthesia. Bile duct cannulation was surgically established, so that bile was directly and quantitatively transferred from the bile duct of the first ('donor') rat to the duodenum of the second ('recipient') rat. A further cannulation of the bile duct of the 'recipient' rat allowed the completion collection of bile produced in the 'recipient' animal. A single subcutaneous dose of $[^3]H$-degarelix (26 µg/kg bodyweight with non-radiolabelled degarelix added to a total dose of 30 µg/kg bodyweight, ~300 µCi/kg) was administrated to the ‘donor’ rats and during the following 48 h, urine and feces were collected from both ‘donor’ and ‘recipient’ rats and bile was collected from the ‘recipient’ rats.

**ADME study in dogs.** Male and female dogs (body weight 10 – 15 kg) were treated with either a single subcutaneous (group A) or intravenous dose (group B) of $[^3]H$-degarelix at 3 µg/kg bodyweight. Feces, heparin plasma and urine samples from groups A and B were collected up to 240 h after dosing.

In addition, two male dogs had a surgically inserted catheter placed in the gall bladder via the bile duct for bile collection. Prior to surgery the dogs were fasted for at least 24 h and were allowed a recovery period of 3 days before dosing. The dogs were administrated a subcutaneous injection of 1 mg non-radiolabelled degarelix/ kg body
weight (injection volume 0.2 ml/kg) and heparin plasma and bile samples were collected up to 24 h post-dose for metabolite screening analysis.

**ADME study in monkeys.** Four male Cynomolgus monkeys (4.2 – 7.5 kg) were dosed for a disposition of radioactivity study. The animals were administrated a single subcutaneous dose of 8.2 µg/kg (200 µCi/kg) [3H]-degarelix. Urine and feces samples were collected quantitatively from each animal after dosing until the time of sacrifice. Blood samples were collected at the time of the sacrifice of the individual animal (6, 24, 48 and 240 h) into tubes containing EDTA as anticoagulant.

**Analysis of total radioactivity**
Plasma, blood, urine, cage washings, homogenates of feces and tissue and carcass digests were combusted in oxygen using Packard Model 307 automatic sample oxidise (Meriden, Connecticut, USA). The products of combustion were absorbed into 15 ml Monophase (Canberra Packard Ltd) for radioactivity measurement. Radioactivity was measured by liquid scintillation spectrometry using a Wallac 1409 automatic scintillation counters (Wallac Oy, Turku, Finland). Radioactivity in hepatocyte uptake studies was measured using a Tricarb model 1600TR liquid scintillation analyzer (Packard).

**Analysis of plasma samples for metabolites**
Plasma samples were thawed and then centrifuged at 3,000g for 20 min at 8°C.
Plasma, 0.5-1.0 ml, was transferred to a 6 ml glass vial and precipitated by adding 2 equivalents of acetonitrile/glacial acetic acid 96/4 (v/v). Internal standard, 50 µl 4 µg/ml [Ile3(2H10)]-barusiban, was added to each tube. The tubes were vortexed and
thereafter centrifuged at 3,000g for 20 min at 8°C. The supernatants were transferred to new 6 ml glass vials and evaporated to dryness under vacuum in a SpeedVac (Farmingdale, New York, USA) at 40°C. The sample residues were re-dissolved in 0.2 ml water/acetonitrile 64/36 (v/v) containing 0.05% (v/v) trifluoroacetic acid and the supernatants were transferred to LC autosampler vials. The samples were analysed by LC-RAD and LC-MS.

Analysis of urine samples for metabolites

Urine samples were purified and concentrated by SPE using Isolute (Hengoed, Mid-Glamorgan, UK) C\textsubscript{18} columns. The SPE column was first conditioned with one column volume of methanol followed by equal volume of acetonitrile and finally with 5 mM ammonium acetate buffer (pH 4.6). The urine sample, with 20 ng/ml IS ([Ile\textsuperscript{3}(\textsuperscript{2H\textsubscript{10}})]-barusiban) added, was applied and the container and resin were washed with a column volume of 5 mM ammonium acetate buffer (pH 4.6). Retained material on the SPE C\textsubscript{18} column was eluted using 3 ml acetonitrile/water 9/1 (v/v) containing 5 mM ammonium acetate (pH 4.6). The acetonitrile/water 9/1 eluate was evaporated to dryness under reduced pressure using a SpeedVac. Each sample residue was re-dissolved in 0.2 ml water/acetonitrile 64/36 (v/v) containing 0.05% (v/v) trifluoroacetic acid and transferred to a total recovery vial (Waters, Milford, Massachusetts, USA). The samples were analysed by LC-RAD and LC-MS. Recoveries of degarelix and synthesized standards FE 200486(1-4)-OH, (1-6)-OH, (1-7)-OH, (1-9)-OH and (1-10)-OH, FE 200486(4-10) and (7-10) and ([Ile\textsuperscript{3}(\textsuperscript{2H\textsubscript{10}})]]-barusiban in the SPE method for spiked urine samples at concentrations of 10 and 100 ng/ml were >80% for all peptides tested.
Analysis of bile samples for metabolites

Bile samples were thawed at room temperature. For direct analysis of the bile, 100 µl bile from each sample was transferred to auto sampler vials for analysis by LC-RAD and LC-MS. Larger volumes of bile was also purified and concentrated by SPE prior to analysis according to the SPE procedure described for urine above. Recoveries in the SPE method for spiked bile samples at concentrations of 10 and 100 ng/ml were > 80% for all peptides tested.

Analysis of feces samples for metabolites

Thawed feces samples were transferred to pre-weighed Kimax tubes (100 x 16 mm), about 0.9-2.3 g material. The feces material was homogenised in 1 ml of 1.5% (v/v) aqueous formic acid using a Covaris (Woburn, Massachusetts, USA) acoustic mixing system. Internal standard, 50 µl of 4 µg/ml [Ile3(2H10)]-barusiban in 5 mM ammonium acetate (pH 4.6) was added and 5 ml of water/acetonitrile 6/4 (v/v) was added to each sample tube. The tubes were extracted for 30 min using a Heidolph (Schwabach, Germany) multi Reax at 90% of max setting. The Kimax tubes were centrifuged at 3,000 g for 30 min at 8°C. The supernatant from each tube was evaporated under vacuum in a SpeedVac at 40°C. The sample residue in the first test tube were redissolved in 0.5 ml water/acetonitrile 64/36 (v/v) containing 0.05% (v/v) trifluoroacetic acid and transferred to a LC autosampler vial. The feces extract samples were analysed by LC-RAD and LC-MS. Recoveries in the extraction method after spiking homogenate of blank rat feces at concentrations of 50 and 500 ng/ml were ~50% for all peptides tested, the test peptides used were the same as those used for investigating the SPE recovery for the bile and urine samples.
Metabolite profiling using radiochemical detection

Rat bile, feces and urine from the rat study R1 were analysed using a LC-RAD system consisted of a Spectra Series P200 binary gradient pump (Thermo Separations, Fremon, California, USA) and a Canberra Packard A515 Radiometer. The LC analysis was achieved using a Hypersil (Thermo Fisher Scientific) BDS phenyl column (5 µm, 250 x 4.6 mm) eluted at 1 ml/min with 70% eluent A (20 mM aqueous phosphate buffer, pH 6.9 with 0.3% triethylamine) and 30% eluent B (20 mM phosphate buffer in acetonitrile with 0.3% triethylamine) for 10 min followed by a linear gradient to 34% B in 1 minute, and a isocratic elution (66% A/ 34% B) for additional 34 min. The column temperature was 40°C. The RAD had a liquid scintillation flow cell with a volume of 0.5 ml and the scintillation flow was 3 ml/min FloScint A (Perkin-Elmer, Shelton, Connecticut, USA).

LC-RAD of bile, urine and feces samples from the rat studies R2 and R3 were performed using an Agilent (Santa Clara, California, USA) 1100 LC system connected to a Packard (Perkin Elmer) Flow Scintillation Analyser. The column used was an Agilent HP Zorbax SB-C18, 5 µm (150 x 2.1 mm) with guard column SB-C8, 5 µm (12.5 x 2.1 mm). The LC flow-rate was 0.25 ml/min. The samples were analysed using a gradient system starting at 9% (v/v) acetonitrile and 91% aqueous 5 mM ammonium acetate (pH 4.6) buffer to 56% acetonitrile during 50 min. The RAD flow-cell volume was 0.5 ml and the scintillation flow was 1 ml/min Ultima Flo Gold scintillation cocktail (Perkin Elmer).

LC-RAD of monkey plasma and urine samples were performed by using a Waters 2695 Alliance system connected to an IN/US Systems (Tampa, Florida, USA) β-RAM
model 3. FlowLogic 1:1 scintillation cocktail (AIM Research Inc, Hockessin, Delaware, USA) was delivered by an ARC (AIM Research Inc) StopFlow Model B unit. The column used was an YMC (Kyoto, Japan) basic C18, 5 µm (150 x 2.1mm) lot 3096 with guard column C18, 5 µm (10 x 2.1mm). The LC flow-rate was 0.22 ml/min. The gradient used was as above for the Agilent system. Agent/Eluate ratio was 2.0, thus, 0.44 ml/min scintillation cocktail was mixed with the LC column eluant. Samples were analysed in nonStopFlow mode.

**Metabolite profiling and metabolite identification by mass spectrometry detection.**

Plasma, bile, urine and feces samples and liver microsome and hepatocyte samples were analysed for metabolite identification using LC-MS. The LC instrument was a Waters 2690 Alliance system and the MS system a Micromass (Manchester, UK) Q-Tof 1.0 upgraded to a 3.6 GHz TDC. The column used was an YMC basic C18, 5 µm (150 x 2.1 mm) with guard column C18, 5 µm (10 x 2.1mm). The LC flow-rate was 0.22 ml/min. The samples were analysed using a gradient starting at 5% acetonitrile isocratic during 5 min and then a linear increase to 60% acetonitrile during 50 min. The mobile phase was acidified with 0.05% (v/v) trifluoroacetic acid. The MS interface used was an electrospray ionization interface operating in positive ionization mode.

The LC retention times of the synthesised standard truncated peptides were known as well as their MS spectra and product ion spectra. In addition, all MS data files were screened for m/z values of possible truncated peptide ions as FE 200486(1-3)-OH, FE 200486(1-5)-OH and FE 200486(1-8)-OH, and FE 200486(6-10) as well as
oxidation products of degarelix and oxidation products of truncated metabolites of degarelix. Possible presence of glucuronyl derivative of degarelix was also investigated. Also, TIC data from post-dose samples were compared with the TIC data from pre-dose samples for any unique ions present in the post-dose samples.

Advanced Chemical Development ACD/Labs (Toronto, Canada) software MS Manager was used for comparing data sets in order to detect unique differences.

Product ion spectra of detected metabolite ions were recorded. The collision energy used was optimised for each metabolite.

Results

Radiolabeling of degarelix. Tritium (two atoms per peptide molecule) was chosen as a radiolabel due to higher specific radioactivity than the alternative $^{14}\text{C}$. The label was placed on alpha and beta carbon atoms of D-2-naphthylalanine (D2Nal) in position 1 of the peptide ($[^{3}\text{H}]$-degarelix, Fig. 1), where tritium would not be expected to exchange with hydrogen under physiological conditions. The choice of D2Nal as tritiation site was dictated by ease of synthesis. At the same time loss of label via enzymatic cleavage in this part of the molecule is highly unlikely due to D configuration of the amino acid bearing the label as well as two consecutive amino acids following it.

Stability in liver microsomes. No degradation of degarelix was detected when the substrate was incubated in liver microsomes from dog and monkey. A tendency to minor decrease of degarelix peak area was seen when incubating the peptide in liver microsomes from rats. However, no metabolites of degarelix could be detected in the samples representing 60 min incubation in buffered rat liver microsome mixture.
Stability in hepatocytes. No degradation of degarelix was detected when [³H]-degarelix was incubated in cryopreserved hepatocytes from male rat and male dog for up-to 120 min at 37°C. The metabolic activity of the cryopreserved hepatocytes as evaluated using three test substrates, all hepatocyte batches used expressed metabolic activities such as CYP3A4 and CYP2D6 mediated metabolism as well as glucuronic acid conjugation activity.

Recovery of radioactivity in rat. After a subcutaneous dose of 30 μg/kg (study R1), [³H]-degarelix radioactivity appeared to be rapidly absorbed with a remainder of 10–14% of the radioactivity at the injection site after 10 days. Maximal plasma concentration was achieved within 1-2 h. The predominant routes of excretion were via the kidneys (50%) and the bile (40-50%). The majority of the radioactivity was excreted within 48 h.

Metabolite profiling in rats. In the first study (R1), the samples were only analysed by LC-RAD, the identity of the peaks detected were assigned by retention time comparison with standards but the identities were not confirmed by LC-MS analysis. In plasma, most of radioactivity detected was the parent compound [³H]-degarelix representing >94% of total radioactivity measured in female rats and >87% in male rats respectively. One metabolite assumed to represent FE 200486(1-9)-OH detected in the plasma samples representing about 6% of the peak integrated radioactivity in female rats whereas in male rats about 13% was detected (Table 1). In urine, the major part of excreted radioactivity was the parent compound for both genders. Low levels of 6 metabolites were also detected. In bile and feces a clear difference in
metabolic profile was detected depending on the gender. In bile the same five metabolites were detected in both genders but in female rats the majority of the excreted radioactivity was the parent compound and the two metabolites assumed to represent FE 200486(1-4)-OH and FE 200486(1-6)-OH present in almost equal amounts. In bile collected from male rats, the amount of metabolite FE 200486(1-6)-OH was almost twice as large as the parent compound. In feces the same six metabolites were detected in samples from female rats and male rats. The major part of excreted radioactivity in feces from male rats was metabolite FE 200486(1-4)-OH whereas in female rats equal amounts of FE 200486(1-4)-OH and FE 200486(1-10)-OH were the largest metabolites detected.

In the second rat study (R2), bile, feces and urine samples were analysed by LC-RAD and LC-MS. In urine, the majority of the radioactivity (94%) detected was the parent compound [3H]-degarelix (see Fig. 2). In addition seven smaller metabolites were detected representing <5% of total radioactivity detected. In bile four metabolites were identified; the metabolites were identified by LC-MS/MS to represent the truncated peptides of degarelix: FE 200486(1-4)-OH, FE 200486(1-6)-OH, FE 200486(1-7)-OH and FE 200486(1-9)-OH. The product ion spectra data of metabolites detected as well as the product ion spectrum of degarelix (FE 200486) are summarised in Table 2. In addition the product ion spectrum of degarelix is shown in Fig 3. Besides the b-ions and y’-ions present in the product ion spectrum of degarelix, the product ions m/z 121.1, 154.1 and 170.1 represent immonium ions of amino acids 3Pal3, 4Cpa2 and Nal1, respectively. The origin of product ion m/z 171.2 is related to amino acid Ilys8.
A triethylamine phosphate buffer containing mobile phases was used for LC-RAD analysis in rat study R1 which enabled base line separation of the metabolites.

However, in order to use a MS as a detector, ammonium acetate was the buffer salt used in the mobile phases for LC-RAD and LC-MS analysis of the samples from rat study R2. Using this mobile phase mixture the metabolites FE 200486(1-4)-OH and FE 200486(1-6)-OH unfortunately co-eluted and so did the metabolites FE 200486(1-7)-OH and FE 200486(1-9)-OH (see Fig. 4). The peak representing the tetrapeptide and the hexapeptide was 46%, the peak representing the heptapeptide and the nonapeptide was 31% and the peak representing $[^3]$H-degarelix was 22% of total radioactivity detected, respectively, in the 0-6 h bile samples which is close to the results found in study R1 for male rats (Table 1). Feces samples were investigated to confirm the completeness of the surgical procedure of bile-duct cannulation. No metabolites of $[^3]$H-degarelix or the parent compound were detected in the feces extracts by LC-RAD or by LC-MS.

**Enterohepatic circulation of degarelix in rat (study R3).** Following a single subcutaneous dose of 30 μg/kg, $[^3]$H-degarelix radioactivity was almost entirely absorbed from the dose site and about one half of the dose excreted in bile. Only about 1% of this biliary excreted fraction was reabsorbed from the gastrointestinal tract, and thus, enterohepatic recirculation of $[^3]$H-degarelix and/or its metabolites plays virtually no part in the disposition of the drug in the rat.

**Recovery of radioactivity in dogs.** Beagle dogs were dosed with a single subcutaneous dose of 3 μg/kg $[^3]$H-degarelix. The radiolabelled compound reached a maximum in plasma after 2 h. The radioactivity was excreted rapidly with the
majority excreted within 48 h. The routes of excretion were via the kidneys (40-42%) and the bile (50-52%).

**Metabolite profiling in dogs.** In plasma, only the parent compound degarelix was detected in post dose plasma samples, no metabolites of degarelix were detected. In bile samples, intact degarelix and five metabolites of degarelix were detected in post-dose bile samples. The identity of the four metabolites FE 200486(1-4)-OH, FE 200486(1-6)-OH, FE 200486(1-7)-OH and FE 200486(1-9)-OH were confirmed by retention time comparison and MS/MS product ion spectra data with synthesised compounds. The fifth metabolite was identified to be FE 200486(1-5)-OH based on the product ion spectrum recorded (Table 2). The heptapeptide, the largest metabolite detected, constituted 47% of total integrated peak area whereas the other peak detected were smaller, the pentapeptide (26%), the hexapeptide (10%), the nonapeptide (6%), the tetrapeptide (4%) and degarelix (7%), respectively.

**Recovery of radioactivity in monkey.** Four male monkeys were dosed with a single subcutaneous dose of 8.2 µg/kg [³H]-degarelix. The major route of excretion was fecal, with 50% of the radioactivity recovered at 240 h. Excretion by the fecal route was slow with only 1% excreted at 6 h post-dose. This rose to 30% at 48 h post-dose, and 50% at 240 h post-dose. Urinary excretion was lower, but more rapid. At 6 h post dose 10% of the radioactivity was excreted, rising to 20% at 240 h post-dose. The mean overall recovery of radioactivity from the time of dosing up to the time of sacrifice, including excreta, tissues and remaining carcass, was 83.3 ± 5.9% of dose.
Metabolite profiling in monkeys In plasma samples, only the parent compound was detected. No metabolites of \([^{3}\text{H}]\)-degarelix were detected in the post-dose plasma samples. Also, the majority of the radioactivity detected, 92%, in the urine samples collected during the first 24 h after dosing was the parent compound. At least five low abundant metabolites of \([^{3}\text{H}]\)-degarelix were detected in the urine samples. The major metabolite peak detected (5% of total radioactivity detected) was identified to be a co-elution of the metabolites FE 200486(1-9)-OH and the FE 200486(1-10)-OH by retention time comparison with synthesised standards.

Discussion

This is the first detailed report on the non-clinical testing of degarelix investigating the metabolic and excretion profile of the compound in numerous animal species combining both \textit{in vitro} and \textit{in vivo} approaches.

Based on previous experiences with peptide drug candidates containing less than 10 amino acids and that the degarelix molecule contains 7 non-natural amino acids, the peptide was subjected to stability studies in liver microsomes and hepatocytes as performed for a small molecule drug. However, degarelix was found to be a very poor substrate to any degrading enzymes, \textit{e.g.} CYP450 enzymes (Mabic et al., 1999), present in the buffered liver microsome suspensions or in hepatocyte suspensions. The minor loss of degarelix in the rat microsomes could not be explained and was not further pursued. Similar results have been reported for cetrorelix, a luteinizing hormone-releasing hormone receptor antagonist developed by ASTA Medica AG which has several structural similarities to degarelix. In incubations with liver
fractions from rat and dog, no degradation of cetrorelix was detected (Schwahn et al., 2000).

The ADME studies of degarelix performed in rat, dog and monkey showed that degarelix and metabolites were fully excreted via the biliary and urinary pathways in almost equal amounts. The total recovery of administrated radioactivity was >90% in rat and dog. The overall recovery of radioactivity was somewhat lower than expected in the monkey, however, it was considered likely that incomplete collection of excreta from the primates accounted for most of the radioactivity not recovered.

Other peptide based GnRH blockers have been reported to be mainly excreted by the biliary route in nonclinical studies. In an ADME study of cetrorelix in rats and dog (Schwahn et al., 2000) it was shown that rats excreted 70% of radiolabeled drug via feces and 24% into urine whereas in dogs equal amounts were excreted into feces and urine. In bile collected from bile-duct cannulated female rats 70% of the radioactive dose of cetrorelix administrated was recovered. A disposition study of RS-26306, a luteinizing hormone-releasing hormone antagonist developed by Syntex Research, showed that 16% and 55-69% of the radioactive dose was recovered in urine and feces, respectively, in rat and monkey (Chan et al., 1991).

Enterohepatic circulation was found to play no part in the disposition of degarelix and/or metabolites of degarelix in the rat. The same result has been reported for cetrorelix in the rat (Schwahn et al., 2000).
The *in vivo* metabolism of degarelix was similar in all species investigated. In plasma, mainly intact degarelix was detected. Low levels of circulating metabolites were detected in plasma from rats, whereas no circulating metabolites could be detected in plasma from dogs and monkey. Also in urine, mainly intact degarelix (>90%) was detected. Only low levels of truncated peptide metabolites were detected in the urine samples from animals. In bile and feces samples, mainly degradation products of degarelix, *i.e.* truncated peptides, were detected. In both rat and dog, the tetrapeptide, the pentapeptide and the heptapeptide metabolites of degarelix were found to represent the major part of the radioactivity excreted in faces.

Similar metabolite profiles have been reported for rats and dogs receiving cetorelix or RS-26306 (Schwahn et al., 2000, Chan et al., 1991). For both compounds plasma and urine samples were found to mainly contain intact parent and in bile and feces samples the four main metabolites were truncated peptides of the parent decapeptide. The metabolites detected were (1-4), (1-6), (1-7) and (1-9) for cetorelix (Schwahn et al., 2000) and the (1-4), (1-6) and (1-7) form of RS-26306, (Chan et al., 1991), *i.e.* the same size truncated peptides of degarelix found in bile from *in vivo* rat and dog studies in this study.

The *in vivo* excreta results reported in this study show that degarelix is mainly excreted unchanged via the urine and is subject to extensive sequential peptidic degradation during its elimination via the hepato-biliary pathway. The results are in line with data for other compounds of similar size and structure as degarelix (Schwahn et al., 2000, Chan et al., 1991) and in line with the fact that compounds of this size (molecular weight 1632) are more likely to be eliminated by the hepato-
bilary pathway (Sahi, 2005). Peptidases that play an important role in the metabolism of several other peptide drugs (Lin, 2009; Malm-Erjefält et al., 2010) do not degrade degarelix in the blood circulation.

Seven of the ten amino acids in degarelix are unnatural, in spite of this, proteolytic fragments from cleavage at most positions were found with two notable exceptions. Proteolysis of the very unnatural all D and lipophilic (1-3) partial sequence does not appear to have occurred. In addition, no products of oxidative metabolism of the (1-4) fragment were observed suggesting that it may not be a substrate of the CYP450 enzymes. The fragment by cleavage between ILys^8 and Pro^9 was not observed either, suggesting that none of the proteases that came into contact with degarelix were able to productively bind the partial sequence around these amino acids. Interestingly, the (1-5) and (1-6) fragments were found in high proportion in bile, urine, or feces of several species, which would require proteases capable of accommodating in their S1 pocket either a bulky side chain (Aph(Hydroorotyl)^5) or a D amino acid (D-Aph(Cbm)^5). As all mass spectrometry data collected were screened for the ions representing all theoretically cleavages of the peptide backbone, we most likely can rule out that other proteolytic cleavages should have occurred to a great extent as they were not detected by LC-MS. No other metabolites of degarelix than the truncated peptide metabolites reported above were detected when the LC-MS data was evaluated using ACD labs compare MS data set software function.

The metabolite FE 200486(1-10)-OH was only detected in feces but not in bile of rats. The metabolite might have been formed by two possible mechanisms: (1) proteases present in the duodenum and jejunum of the rat or (2) bacteria in the large intestine.
(cecum/colon) of the rats (Langguth et al., 1994). However, incubations of degarelix with rat jejunal juices or with colonic contents/feces have not been performed to confirm if any these mechanisms is responsible for the formation of FE 200486(1-10)-OH.

As outlined in the summary figure (Fig. 5) degarelix is subject to proteolysis by endopeptidases, and unchanged degarelix and metabolites are fully excreted via the hepatic and urinary pathway. Systemic exposure to any metabolic products seems to be low.

Acknowledgments

The authors would like to thank Claudio Schteingart for valuable comments to this manuscript.

Authorship Contributions

*Participated in research design:* Sonesson A, and Buur Rasmussen B.

*Conducted experiments:* Sonesson A

*Contributed new reagents or analytical tool:* Stalewski J

*Performed data analysis:* Sonesson A, and Buur Rasmussen B

*Wrote or contributed to the writing of the manuscript:* Sonesson A, Buur Rasmussen B, Koechling W, Stalewski J, and Tankó LB

*Conduct of animal trials:* Koechling W

*Clinical interpretation of data:* Tankó LB
References


Legends to Figures.

FIG. 1. Structure of degarelix (FE 200486) with \[^3\text{H}\] indicating the positions of the tritium radiolabeled FE 200486. Peptide sequence: Ac-D-2Nal-D-4Cpa-D-3Pal-Ser-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys-Pro-D-Ala-NH$_2$

Abbreviations: Ac = Acetyl, 2Nal = 2-Naphtylalanine, 4Cpa = 4-Chlorophenylalanine, 3Pal = 3-Pyridylalanine, Ser = Serine 4Aph = 4-Aminophenylalanine, Hor = Hydroorotyl, Cbm = Carbamoyl, Leu = Leucine, ILys = N(epsilon)-Isopropyllysine, Pro = Proline, Ala = Alanine.

FIG. 2. Radio chromatogram from LC-RAD analysis of urine collected between 0-6 h post-dose from a male rat administrated with a subcutaneous dose of 30 µg/kg [\(^3\text{H}\)]-degarelix

FIG. 3. Product ion spectrum of degarelix (below) with fragmentation pattern indicated (above).

FIG. 4. Radio chromatogram from LC-RAD analysis of bile sampled between 0-1 h post-dose from a male rat administrated with a subcutaneous dose of 30 µg/kg [\(^3\text{H}\)]-degarelix.

FIG. 5. Outline of the \textit{in vivo} metabolism of degarelix based on animal data.

Abbreviations: b = bile, f = feces, p = plasma, u = urine, matrix.
TABLE 1

Metabolite pattern in plasma, urine, bile and feces after s.c. administration of 30 µg [3H]-degarelix/kg in rats (rat study R1).

<table>
<thead>
<tr>
<th>Gender</th>
<th>Sample</th>
<th>Sampling period [h]</th>
<th>% of component in sample</th>
<th>[3H]-degarelix</th>
<th>FE 200486 (1-4)-OH</th>
<th>FE 200486 (1-6)-OH</th>
<th>FE 200486 (1-5)-OH</th>
<th>FE 200486 (1-9)-OH</th>
<th>FE 200486 (1-7)-OH</th>
<th>U*</th>
<th>U*</th>
<th>FE 200486 (1-10)-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Plasma</td>
<td>0-24</td>
<td></td>
<td>87.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Urine‡</td>
<td>0-48</td>
<td></td>
<td>96.4</td>
<td>0.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>0.32</td>
<td>0.17</td>
<td>0.53</td>
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<td>Bile‡</td>
<td>0-48</td>
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<td>17.2</td>
<td>25.9</td>
<td>30.5</td>
<td>1.17</td>
<td>9.98</td>
<td>13.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Feces‡</td>
<td>0-48</td>
<td></td>
<td>5.84</td>
<td>25.7</td>
<td>19.2</td>
<td>12.9</td>
<td>15.5</td>
<td>3.90</td>
<td>-</td>
<td>-</td>
<td>14.4</td>
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<td>Female</td>
<td>Plasma</td>
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<td>94.3</td>
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<td>-</td>
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<td>92.4</td>
<td>2.97</td>
<td>2.97</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
<td>0.17</td>
<td>0.12</td>
<td>0.64</td>
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<tr>
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<td>Bile</td>
<td>0-48</td>
<td></td>
<td>28.3</td>
<td>24.9</td>
<td>23.4</td>
<td>1.36</td>
<td>7.91</td>
<td>14.1</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>Feces‡</td>
<td>0-48</td>
<td></td>
<td>5.95</td>
<td>26.6</td>
<td>12.0</td>
<td>9.95</td>
<td>15.2</td>
<td>3.15</td>
<td>-</td>
<td>-</td>
<td>24.7</td>
</tr>
</tbody>
</table>

*Numbers represent mean values of 4 individual animals. ‡Radioactivity detected in void peak adds up the percentage to 100.
## TABLE 2

**Identifications of degarelix and metabolites of degarelix using product ion mass spectrometry data**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion (m/z)</th>
<th>Characteristic product ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degarelix</td>
<td>816.4</td>
<td>121.1, 154.1, 170.1, 171.2, 186.1 (y₂”), 240.1 (b₁), 356.3 (y₃”), 421.2 (b₂), 469.4 (y₄”), 569.2 (b₃), 606.3 (y₅”), 656.1 (b₄), 674.4 (y₆”), 696.9 (y₇”), 794.9 (y₈”), 807.9 (b₁₀), 958.4 (b₃), 976.6 (y₉’’), 1063.6 (y₇’’), 1163.6 (b₆), 1211.7 (y₈’’), 1276.5 (b₇), 1393.0 (y₉”’’), 1063.6 (y₇”’’), 1163.6 (b₆), 1211.7 (y₈”’’), 1276.5 (b₇)</td>
</tr>
<tr>
<td>FE 200486(1-10)-OH</td>
<td>816.9</td>
<td>121.1, 154.1, 170.1, 171.2, 187.1 (y₂”’’), 240.1 (b₁), 357.3 (y₃”’’), 421.2 (b₂), 470.4 (y₄”’’), 569.2 (b₃), 606.8 (y₅”), 656.1 (b₄), 675.4 (y₆”), 697.4 (y₇”), 795.4 (y₈”’’), 807.9 (b₁₀), 958.4 (b₃), 977.6 (y₉”’’), 1064.6 (y₇”’’), 1163.6 (b₆), 1212.7 (y₈”’’), 1276.5 (b₇)</td>
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<tr>
<td>FE 200486(1-9)-OH</td>
<td>781.4</td>
<td>121.1, 154.1, 170.1, 171.2, 240.1 (b₁), 286.3 (y₂”’’), 399.4 (y₃”’’), 421.2 (b₂), 569.2 (b₃), 604.6 (y₄”), 656.1 (b₄), 662.0 (y₅”), 760.1 (y₆”’’), 773.6 (b₆), 906.8 (y₇”), 958.4 (b₃), 993.8 (y₅”), 1141.9 (y₇”), 1163.6 (b₆), 1323.2 (y₈”’’), 1276.5 (b₇)</td>
</tr>
<tr>
<td>FE 200486(1-7)-OH</td>
<td>1294.5</td>
<td>121.1, 154.1, 240.1 (b₁), 337.2 (y₂”’’), 421.2 (b₂), 569.2 (b₃), 639.3 (y₃”’’), 656.1 (b₄), 604.6 (y₄”), 656.1 (b₄), 662.0 (y₅”), 760.1 (y₆”’’), 773.6 (b₆), 906.8 (y₇”), 958.4 (b₃), 993.8 (y₅”), 1141.9 (y₇”), 1163.6 (b₆), 1323.2 (y₈”’’), 1276.5 (b₇)</td>
</tr>
<tr>
<td>Compound</td>
<td>m/z</td>
<td>Assignments</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>FE 200486(1-6)-OH</td>
<td>1181.4</td>
<td>726.3 (y₄”), 874.4 (y₅”), 958.4 (b₅), 1055.4 (y₆”), 1163.6 (b₆), 1251.5 (y₇”), 1276.5 (b₇)</td>
</tr>
<tr>
<td>FE 200486(1-5)-OH</td>
<td>976.4</td>
<td>121.1, 154.1, 240.1 (b₁), 421.2 (b₂), 526.3 (y₂”), 569.2 (b₃), 613.2 (y₃”), 656.1 (b₄), 761.3 (y₄”), 942.3 (y₅”), 958.4 (b₅), 1139.4 (y₆”), 1163.6 (b₆)</td>
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<tr>
<td>FE 200486(1-4)-OH</td>
<td>674.3</td>
<td>121.1, 154.1, 240.1 (b₁), 321.2 (y₁”), 408.1 (y₂”), 421.2 (b₂), 556.2 (y₃”), 569.2 (b₃), 656.1 (b₄), 737.4 (y₄”), 934.3 (y₅”), 958.4 (b₅)</td>
</tr>
</tbody>
</table>

Figure 1
Figure 4