Received: 6 May 2020 | Revised: 13 May 2020 | Accepted: 14 May 2020

DOI: 10.1002/prp2.619

ORIGINAL ARTICLE

The endothelin receptor antagonist macitentan for the treatment of pulmonary arterial hypertension: A cross-species comparison of its cytochrome P450 induction pattern

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Abstract

The dual endothelin receptor antagonist macitentan was approved in 2013 for the treatment of pulmonary arterial hypertension. Macitentan is an inducer of cytochrome P450 expression in vivo in animal species but not in man. In rat and dog, changes in P450 expression manifest as autoinduction upon repeat dosing. The induction pattern, however, significantly differed between both species, and between male and female rats. While macitentan exposure steadily declined with dose in the dog, P450 induction was saturable in the rat reaching levels of 40%-60% and 60%-80% at steady-state in male and female animals, respectively. The nature and number of P450 enzymes involved in macitentan clearance were identified as a major reason for the observed species differences. In the dog, macitentan was metabolized by a single P450 enzyme, that is, Cyp3a12, whereas several members of the Cyp2c and Cyp3a families were involved in the rat. Macitentan selectively upregulated Cyp3a expression in rat, whereas the expression of the Cyp2c enzymes involved in macitentan metabolism remained mostly unchanged, eventually leading to a higher contribution of Cyp3a upon induction. Macitentan also induced CYP3A4 expression in human hepatocytes via initial activation of the human pregnane X receptor. No such induction was evident in humans at the therapeutic macitentan dose of 10 mg as shown in a clinical drug-drug interaction study with the CYP3A4 substrate sildenafil.

KEYWORDS

endothelin receptor antagonist, macitentan, P450 induction, species differences

Abbreviations: [S], substrate concentration; AUC, area under the concentration vs time curve; BCA, bicinchoninic acid; BLAST, basic local alignment search tool; CL, clearance; CL_{int}, intrinsic clearance; C_{max}, peak plasma concentration; ct, cycle threshold; Cyp, cytochrome P450 gene; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; g, gravitation constant; HPLC, high-performance liquid chromatography; ID, internal diameter; K_m, Michaelis-Menten constant; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MEM, minimal essential medium; P450, cytochrome P450 enzyme; PAH, pulmonary arterial hypertension; PCR, polymerase chain reaction; PEG, polyethylene glycol; PXR, pregnane X receptor; Sf9, spodoptera frugiperda 9 (army fall worm); $T_{1/2}$, apparent terminal half-life; T_{max}, time to reach peak plasma concentration; V_{max}, maximal rate (of enzyme reaction); Vss, volume of distribution at steady-state.

1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease, ultimately leading to right heart failure and death.[1] Several drug classes have shown benefits in the treatment of PAH, among which endothelin receptor antagonists,[2-5] phosphodiesterase-5 inhibitors,[1,6] epoprostenol,[7] and prostanoid analogues including the recently approved selexipag[8-10] are the most prominent. The endothelin receptor antagonist bosentan (Tracleer®) was the first oral treatment option for PAH approved in 2001, followed by ambrisentan (Letairis®/Volibris®) in 2007 and macitentan (Opsumit®) in 2013. As demonstrated in the pivotal phase III SERAPHIN trial (Study with an Endothelin Receptor Antagonist in Pulmonary arterial Hypertension to Improve cliNical outcome), macitentan alone or in combination with phosphodiesterase-5 inhibitors or prostanoid receptor agonists reduces the risk of a composite endpoint of morbidity and mortality in PAH patients.[5]

Macitentan was developed as a follow-up drug of bosentan with the aim to find a second-generation dual endothelin receptor antagonist with an improved liver safety profile, a drug-drug interaction profile devoid of cytochrome P450 induction, and a once-a-day dosing regimen. Bosentan treatment was associated with transient increases in liver transaminases and plasma bile salts as a result of intrahepatic cholestasis. Mechanistic work suggested that bosentan initially triggers a disruption of bile salt homeostasis through inhibition of the bile salt pump BSEP[11] eventually leading to bile salt accumulation in liver cells up to cytotoxic levels. The increases in liver transaminases in man are thus believed to result from the secondary bile salt toxicity in hepatocytes. Moreover, bosentan was a moderate CYP2C9 and CYP3A4 inducer in man[12]). Macitentan was optimized with respect to endothelin receptor binding,[13-15] improved pharmacokinetics allowing for once-a-day dosing,[16] a superior liver safety profile,[17] and is devoid of P450 induction at the therapeutic dose of 10 mg.[18] Like bosentan, macitentan in vitro is a P450 inducer and an inhibitor of BSEP. The improved safety profile over bosentan was achieved by modifying its hepatic disposition profile. While bosentan is an OATP substrate and consequently accumulates in liver cells, macitentan is more lipophilic and less acidic. As a result of these changes in physicochemical properties, it is not an OATP substrate and partitions into liver by passive diffusion.[17] Local drug concentrations are thus limited by the extensive binding of macitentan to plasma proteins to levels too low to inhibit bile salt transport[19] or to induce P450 enzymes.[18]

During its nonclinical safety assessment, macitentan has been given to rats, mice and dogs at doses far above those in clinical trials for periods up to 2 years. Decreased macitentan exposure has been consistently observed in all animal species after repeat treatment though the pattern of these time-dependent changes varied between species. In contrast, no such changes were observed in clinical trials.[19] This report describes the mechanistic work and biochemical processes underlying these changes in macitentan exposure in the rat and dog, together with an assessment of the P450 induction potential in humans on the level of mRNA expression changes in human hepatocytes and pregame X receptor (PXR) activation.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Two batches of macitentan were used in this study: batch no. 600083/Batch 1 was used for the two 4-week toxicity studies in the rat and dog and its pharmacokinetic characterization in both species, whereas batch 178193/batch 13 was used for the 8-day enzyme induction study in the rat. Both batches were manufactured at Lonza AG (Visp, Switzerland) with purities in excess of 98%. Metabolite M6 (batch LJC136-929.6) and the tetra-deuterated analogues of macitentan and M6 were synthesized in the Medicinal Chemistry department of Actelion Pharmaceuticals Ltd (Allschwil, Switzerland). ^{14}C-Labeled macitentan (lot nos. 031215 and 030514) was obtained from American Radiolabeled Chemicals Inc (St. Louis, MO, USA) with a specific activity of 50 mCi/mmol as a stock solution in acetonitrile, with chemical and radiochemical purities in excess of 97%. General purpose chemicals and solvents were of highest commercially available quality and obtained from Sigma-Aldrich or Fluka (Buchs, Switzerland). Liquid scintillation cocktails, Irga Safe Plus and Flow Safe 2, were from Perkin Elmer (Zürich, Switzerland) and Berthold Technologies (Regensdorf, Switzerland).

2.2 | Animal experiments

Male and female Sprague-Dawley rats, used in the single-and multiple dose pharmacokinetic experiments, with body weights ranging from 200 to 250 g were supplied by RCC Ltd, Biotechnology and Animal Breeding Division (Füllinsdorf, Switzerland). The animals were acclimatized to the experimental unit for at least 7 days before use in the study. During this period, animals were carefully monitored to ensure good health and suitability for inclusion in the study. All animals were housed under the same conditions in accordance with the US National Institute of Health guidelines and as approved by the cantonal veterinary office (license nos. 169 and 222). During pretrial holding periods, rats were housed in groups of up to five in Makrolon type-3 cages with wire mesh tops and standardized softwood bedding. Standard laboratory diet of known composition (Provimi Kliba, Kaiseraugst, Switzerland) and domestic mains tap water were available ad libitum.

Pharmacokinetic experiments in the dog were performed at RCC Ltd, Itingen, Switzerland under study number 850715 in accordance with the Swiss animal protection law and license no. 12. Three male Beagle dogs with body weights from 8.6 to 10.2 kg and an age of 16 months at the start of treatment were obtained from RCC Ltd Laboratory Animal Services (Füllinsdorf, Switzerland). Dogs were identified by individual kennel number and ear tattoo and underwent routine veterinary inspection prior to and during the study. They were

group-housed during the study and only separated for feeding and monitoring of clinical signs. Except for a period of fasting the night before and until 4 hour after dose administration, animals were given a daily allowance of 350 g of a standard laboratory diet of known formulation (Provimi Kliba 3353 dog maintenance diet, Provimi Kliba AG, Kaiseraugst, Switzerland) and had free access to tap water.

2.3 | Single-dose pharmacokinetics in rat and dog

Two days prior to drug dosing, rats were anesthetized with a mixture of ketamin (90 mg/kg) and intraperitoneal xylazine 2% (10 mg/kg), and a catheter was implanted under aseptic conditions into the jugular vein to allow for serial blood sampling. After recovery from general anesthesia, animals were housed individually with free access to water and food during the recovery period and the entire duration of the experiment. Pharmacokinetic experiments were started after 2 days of recovery. For intravenous dosing, macitentan was formulated as 55:45 (v/v) mixture of 50 mmol/L TRIS-HCl buffer (pH 8.3) and PEG400. For oral experiments, macitentan was given as an aqueous suspension in 7.5% modified gelatin. Serial blood samples of 0.25 mL each were taken over a period of 32 hour and collected in vials fortified with EDTA. Plasma was generated by centrifugation and stored at −20°C pending analysis.

Experiments in the Beagle dog were performed using three male animals in a cross-over design with a wash-out period of 7 days between dosing events. For intravenous administration, macitentan was formulated as solution in 1:1:2 (v/v/v) mixture of PEG400, N-methylpyrrolidone and 50 mmol/L TRIS-HCl buffer (pH 8.3). For oral purposes, macitentan was given as an aqueous suspension in 7.5% succinylated gelatin (B. Braun, Melsungen, Germany). Serial blood samples of 2 mL each were taken over a period of 32 hour and transferred into vials fortified with EDTA as anticoagulant. Plasma was generated by centrifugation and stored at −20°C pending analysis.

2.4 | Multiple-dose pharmacokinetics in rat and dog

Pharmacokinetic data after repeat dosing in both species were generated as part of the toxicokinetic monitoring program of the respective 4-week toxicity studies. Macitentan was formulated in modified gelatine and given once daily to rats at doses of 0 (vehicle control), 50, 150, 450, and 1500 mg/kg, and to dogs at 0, 5, 50, and 500 mg/kg. Six blood samples over a 24 hour interval were collected into EDTA-containing vials from all study dogs, whereas blood samples in the rat study were taken from a satellite group allocated to toxicokinetic evaluation. Plasma was generated by centrifugation and stored at −20°C pending analysis. In the mechanistic follow-up study, macitentan was given to male Sprague-Dawley rats (n = 4) at daily doses of 0, 5, 15, and 50 mg/kg for a period of 8 days as a suspension in modified gelatine to mimic the conditions of the 4-week study. Blood samples were taken on day 1 and day 8 at 1, 2, 3, 4, 5, 8, and 24 hours postdosing, whereas liver samples for mRNA analysis were taken on day 8.

2.5 | Bioanalytical method for macitentan in plasma

Plasma samples (50-100 µL) were fortified with two volume equivalents of a 1:1 (v/v) mixture of acetonitrile and ethanol containing the tetra-deuterated internal standard. After centrifugation at 2000*g* for 20 minutes, supernatants were diluted with a 1:9 (v/v) mixture of acetonitrile and water. Lower and upper limits of quantification in plasma were 1 and 1000 ng/mL, respectively. Macitentan was quantified using liquid chromatography coupled to mass spectrometry on an TSQ Quantum triple stage quadrupole mass spectrometer (Thermo Finnigan, San Jose, United States) in selected reaction monitoring mode using a mass transition of 588.8 to 201.0. Chromatographic separation was achieved on a Waters XTerra C18 column (3.5 µm, 2.1 × 5 mm ID) at room temperature with a flow rate of 0.4 mL/min and a linear gradient from 40%-90% mobile phase B with a run time of 4 minutes. Mobile phases consisted of 0.1% (v/v) aqueous formic acid (phase A) and acetonitrile containing 0.1% formic acid (phase B). The parameters of the mass detector were set as follows: capillary voltage 4 kV, ion source gas 60 psi, auxiliary ion source gas 20 psi, collision gas 1 mTorr and transfer capillary temperature 350°C, with a scan time of 200 ms.

2.6 | Pharmacokinetic analysis

Pharmacokinetic parameters were estimated using the WinNonlin software package (Professional Version 5.0, Pharsight Corporation, Mountain View, United States). The following parameters were calculated: C_{max} (peak plasma concentration), T_{max} (time to reach peak plasma concentration), $T_{1/2}$ (apparent terminal half-life, calculated as $T_{1/2} = \ln(2)/\lambda_z$); $AUC_{0\text{-last}}$ (area under the plasma concentration vs time curve up to the last measurable concentration, calculated by the log-linear trapezoidal rule); $AUC_{0\text{-inf}}$ (area under the plasma concentration vs time curve extrapolated to infinity, calculated as $AUC_{0\text{-inf}} = AUC_{0\text{-last}} + C_{last}/\lambda_z$ where λ_z (terminal rate constant, lambda z) is the negative of the slope of a ln-linear regression of the unweighted data considering the last concentrations vs time points above the lower limit of quantification). $AUC_{0\text{-inf}}$ was accepted if the $AUC_{\%extp}$, that is, the percentage of area extrapolated to infinity, did not exceed 20%, otherwise $AUC_{0\text{-last}}$ was reported. Values below the lower limit of quantification were set to "zero" before the first measurable concentration, and to "missing" after the last measurable concentration.

2.7 | Origin of rat and dog liver tissue for P450 induction studies

Rat livers from male and female animals (n = 5-6) were obtained from the satellite groups of the respective 4-week toxicity studies and

the 8-day mechanistic follow-up study (n = 4, males only). Dog liver samples (n = 2-3) were obtained from the main group animals of the 4-week toxicity study in the Beagle dog. Whole livers (rat) or aliquots (dog) were extracted after sacrifice and stored at −80°C.

2.8 | Taqman analysis of P450 expression in rat and dog liver

50-100 µg aliquots from each individual rat and dog liver were used for total RNA extraction with a Qiagen RNeasy Midi kit. DNase (Qiagen, Venlo, The Netherlands) was added during RNA extraction to avoid contamination by genomic DNA. Complementary DNA (cDNA) was synthesized using 500 ng or 300 ng of total RNA as template for rat and dog, respectively, with the Taqman reverse transcription reagents kit from Applied Biosystems (Rotkreuz, Switzerland) and MultiscribeTM reverse transcriptase at 1.25 IU/µL, random hexamers at 2.5 µmol/L, and RNAase inhibitor at 0.4 IU/µL. PCR was performed with the Taqman PCR Mastermix from Applied Biosystems. The mix contained AmpErase® uracil N-glycosylase in order to prevent re-amplification of PCR carry-over products. Primers and probes for rat Cyp2b2, Cyp2c6, Cyp2c11, Cyp2c12, Cyp2c13, Cyp3a1, Cyp3a2, Cyp3a9, and dog Cyp3a12 were designed using the Primer Express® software (Applied Biosystem) and BLAST searches were performed to assess for correct annealing. The primers and probes of all P450 enzymes and eukaryotic 18S rRNA as endogenous control were obtained from Applied Biosystems and Microsynth AG (Balgach, Switzerland). Transcript levels were quantified with an ABI Prism 7000 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. Relative transcript levels were determined using the comparative cycle threshold (Ct) method ($\Delta\Delta C_T$). The amount of target, normalized to an endogenous control (18S) and relative to a calibrator (controls from vehicle-treated animals) is given by $2^{-\Delta\Delta CT}$. The normalization of each individual transcript to 18S was used to estimate basal mRNA expression levels, whereas the additional normalization to the corresponding vehicle-treated transcript yielded the fold increases in mRNA.

2.9 | Preparation and characterization of liver microsomes

Pooled liver tissues of 1.5-2 g per dose group and species were transferred into ice-cold 50 mmol/L TRIS-HCl buffer (pH = 7.4) isotonized with 250 mmol/L sucrose, and homogenized with a tissue grinder (Potter S, Vitaris, Baar, Switzerland). Homogenates were initially centrifuged at 9000 g and 4°C for 20 minutes. The supernatants were collected and centrifuged again at 100 000 g and 4°C for 30 minutes (Sorvall Pro80, rotor T-865, ThermoFischer Scientific). The microsomal pellet was suspended in 50 mL of a 100 mmol/L pyrophosphate buffer (pH 7.25) containing 1 mmol/L EDTA, centrifuged again at 100 000 g and 4°C for 30 minutes. The resulting pellet was re-suspended in 100 mmol/L sodium phosphate buffer, aliquoted into 0.15 mL portions and stored at −80°C. Total microsomal protein was quantified using the BCA protein assay kit from Pierce (Perbio Science, Lausanne, Switzerland) on a FLUOstar OPTIMA (BMG Labtech GmbH, Offenburg, Germany) using bovine serum albumin as standard. Total P450 concentrations were determined on a PerkinElmer UV/VIS spectrophotometer (lambda 35, Perkin Elmer, Schwerzenbach, Switzerland) using the carbon monoxide assay with an extinction coefficient of 91 mmol/L^{-1} cm^{-120}).

2.10 | Metabolic profiles with liver microsomes and recombinant P450 enzymes

In a reaction volume of 200 µL, 10 µmol/L ^{14}C-macitentan in 100 mmol/L phosphate buffer (pH 7.4) was incubated with 1 mg/mL of microsomal protein. After a preincubation period of 5 minutes at 37°C, the reaction was started by addition of 1 mmol/L NADPH and terminated after 0, 10, 20, 30, or 40 minutes by addition of 0.1 mL ice-cold methanol. After centrifugation, total radioactivity assessed by liquid scintillation counting using a Tricarb 2300TR liquid scintillation analyzer (Perkin Elmer, Zürich, Switzerland. Control experiments in the absence of NADPH or microsomal protein were run in parallel under otherwise identical conditions. All incubations were conducted in duplicate and the supernatants stored frozen at −20°C prior to analysis. The NADPH-regenerating system was prepared as a 10-fold concentrated stock solution and consisted of 11 mmol/L NADP, 100 mmol/L glucose-6-phosphate and 50 mmol/L magnesium chloride in 0.1 mol/L phosphate buffer (pH 7.4). 20 UI/mL of glucose-6-phosphate dehydrogenase was added to this mixture before addition to the incubation mixture. Recombinant rat and dog P450 enzymes expressed in baculovirus-infected Sf9 cells with supplemental cDNA-expressed P450 reductase and cytochrome b_5 were purchased from Becton Dickinson (Basel, Switzerland) with a P450 enzyme concentration of 1.0 nmol/mL. Incubations with rat Cyp2b1, Cyp2c6, Cyp2c11, Cyp2c12, Cyp3a1, Cyp3a2, and dog Cyp2c21 and Cyp3a12 were performed at 10 µmol/L ^{14}C-macitentan. In a 96-well format, 1.0 µL of the 1 mmol/L ^{14}C-stock solution in DMSO was added into 100 µL of 100 mmol/L phosphate buffer (pH 7.4) containing the recombinant P450 enzyme at a concentration of 200 pmol/mL. The reaction was initiated by the addition of 10 µL of the prewarmed NADPH-regenerating system and incubation was continued for up to 40 minutes at 37°C in an Eppendorf thermomixer rotating at 400 rpm. The reaction was terminated by addition of 50 µL of ice-cold acetonitrile. After sealing the 96-well plate with aluminum foil and centrifugation at 465*g* and 4°C for 20 minutes, a 100 µL aliquot was submitted to HPLC analysis.

2.11 | Enzyme kinetics of M6 with liver microsomes and recombinant P450 enzymes

Prior to the main experiment, initial rate conditions for M6 formation were individually determined for liver microsomes and recombinant P450 enzymes such that metabolite formation was in the range

of 10%-25% and linear with respect to protein concentration and incubation time. For the determination of enzyme kinetic parameters, macitentan was incubated in a concentration range from 1 to 200 µmol/L. In a 96-well format, a 1.0 µL-aliquot of the 100-fold concentrated macitentan stock solution in DMSO was added into 100 µL of 100 mmol/L phosphate buffer (pH 7.4) containing either the liver microsomes or recombinant P450 enzymes. The reaction was initiated by addition of 10 µL prewarmed NADPH-regenerating system and incubation continued for up to 30 minutes at 37°C in an Eppendorf thermomixer rotating at 400 rpm. The reaction was stopped by addition of 50 µL ice-cold acetonitrile. Samples were centrifuged at 46*g* and 4°C for 20 minutes, 10 µL aliquots were submitted to LC-MS/MS analysis of liquid chromatography with ^{14}C-radiodetection. Enzyme kinetic parameters were calculated by nonlinear regression with the Origin software package (version 7.5, Redacom AG, Nidau, Switzerland) using the following equation

$$v = \frac{v_{max} \cdot [S]}{K_m + [S]}$$

in which v is the rate of M6 formation, [S] is the substrate concentration, K_m is the Michaelis-Menten constant, and V_{max} is the maximal rate.

2.12 | Induction of P450 enzymes in human hepatocytes

Three batches of human hepatocytes were cultured as monolayers in a 24- or 48-well format to investigate CYP1A2, CYP2C9, and CYP3A4 induction. Hepatocytes were maintained in William's E medium with Glutamax I supplemented with penicillin/streptomycin, bovine insulin and hydrocortisone hemisuccinate. Hepatocytes, cell culture media and additives were obtained from Biopredic International (Rennes, France). Hepatocytes were maintained at 37°C in a humidified atmosphere with 5% CO_2 for at least 2 hour prior to incubation with macitentan at 0.1, 1, 3, and 10 µmol/L. Rifampicin and omeprazole were used as positive controls. Solutions of all drugs were prepared by diluting the DMSO stock solutions with cell culture medium by a factor of 1000. Incubations were carried out for 48-89 hour and medium was replaced every 24 hour with medium containing fresh drug or vehicle. Nifedipine, diclofenac and phenacetin were used as P450 marker substrates to quantify CYP3A4, CYP2C9 and CYP1A2 activity. At the end of the incubation period, hepatocytes were washed with MEM (Oxoid AG Basel, Switzerland) and incubated for 10 minutes at 37°C. Medium with the P450 markers was prepared by dilution of an aqueous 7.5% $NaHCO_3$ solution (w/v) with MEM to a final concentration of 2.2 g/L. The P450 marker stock solutions in DMSO were diluted with MEM by a factor of 200 to obtain nifedipine, diclofenac and phenacetin solutions at concentrations of 200, 50, and 200 µmol/L, respectively. 400 µL (24-well format) or 250 µL (48-well format) were distributed per well and hepatocytes were incubated for 2 hour (nifedipine), 6 hour (diclofenac), or 17 hour (phenacetin) at 37°C in a humidified atmosphere with 5% CO_2. At the end of the incubation period, supernatants were collected, immediately frozen, and stored at −20°C pending LC-MS/MS analysis. Hepatocytes were lysed for 5 minutes at room temperature using RLT® lysis buffer from Qiagen AG (Hombrechtikon, Switzerland), cell lysates transferred into Eppendorf tubes, homogenized by vortexing, and aliquots taken for protein quantification. Total protein was quantified in a 96-well plate format using the BCA™ protein assay kit from Pierce (Perbio Science Switzerland, Lausanne, Switzerland) on a FLUOstar OPTIMA and bovine serum albumin as standard. The remaining hepatocyte lysates were used for total RNA extraction with a Qiagen Rneasy micro kit. DNase (recombinant human desoxyribonuclease purchased from Qiagen) treatment was applied during the RNA extraction process to avoid contamination by genomic DNA. cDNA was synthesized using 100 ng or 50 ng of total RNA as template with the Taqman reverse transcription reagents kit from Applied Biosystems and Multiscribe™ reverse transcriptase at 1.25 IU/µL, random hexamers at 2.5 µmol/L, and RNAase inhibitor at 0.4 IU/µL. PCR was performed with the Taqman PCR Mastermix. P450 primers and probes were designed using the Primer Express® software and BLAST searches were performed to assess for correct annealing. The primers and probes for CYP3A4, CYP2C9 and 18S quantification were obtained from Applied Biosystems and Microsynth AG. CYP3A4 and CYP2C9 transcript levels were quantified with an ABI Prism 7000 sequence detection system using the comparative cycle threshold method ($\Delta\Delta C_T$ method, cf. above). Statistical analysis and curve fitting for EC_{50} determinations were performed using the GraphPad Prism software (version 4, GraphPad Software Inc, United States). For the construction of the concentration-response curve, a three-parameter equation (sigmoid) was used (Y = Bottom + (Top-Bottom)/(1 + $10^{((\log EC50-X)}$), in which X equals the logarithm of concentration, and Y equals the response.

2.13 | Activation of the human pregnane X receptor

Green monkey kidney CV-1 cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere in DMEM high glucose medium supplemented with 10% fetal bovine serum and 50 IU/mL penicillin/streptomycin. Three days prior to the experiment, cells were expanded in DMEM/F12 with 10% charcoal-treated FBS and 50 IU/mL penicillin/streptomycin, thereafter plated into 96-well dishes at a density of 25 000 cells per well and grown overnight. For transfection, 0.1 ng of expression vector for pCDNA3-hPXR, 20 ng of the luciferase reporter CYP3A4pGL3, 60 ng of an expression vector for βgalactosidase (pSVβ Gal) used for the determination of the transfection efficiency and carrier plasmid was added to a total of 100 ng DNA per well. Cells were transiently transfected using 1 µL of LipofectAMINE in 50 µL of OptiMemI per well. This mixture was then added dropwise to the DNA solution previously prepared in 50 µL of OptiMemI per well. The mixture was incubated at room temperature for 45 minutes to allow for the DNA/LipofecAMINE complexes to form, and then distributed into the wells (100 µL/well). The cells were incubated for 24 hour in order to allow for protein

synthesis. All media, chemicals and serum for CV-1 cell culture and transfection were obtained from Invitrogen AG (Basel, Switzerland).

After 24 hour incubation, cells were treated with macitentan, rifampicin or DMSO vehicle using DMEM/F12 cell culture medium supplemented with 10% charcoal-treated, delipidated fetal bovine serum (Autogen Bioclear UK Ltd, Calne, United Kingdom) and 50 IU/mL penicillin/streptomycin. Macitentan and rifampicin were used at 0.03, 0.1, 0.3, 1, 3, 10, and 30 µmol/L by diluting the respective DMSO stock solutions with culture medium by a factor of 1000. Triplicate 100 µL-aliquots of medium containing either drug or vehicle were distributed per well. After 24 hours, cell extracts were prepared by removing the medium, addition of 100 µL passive lysis buffer (Promega, Catalys AG, Wallisellen, Switzerland) to each well and incubation for 20 minutes at room temperature. Beta-galactosidase activity was determined by adding 180 µL of chlorophenol red-1β-galactopyranoside solution to 20 µL of cell lysates. After 60-min incubation at 37°C, absorption at 540 nm was measured. Luciferase activity was quantified using the luciferase assay kit from Promega and data were normalized against β-galactosidase values to compensate for varying transfection efficiencies. Luciferase activity for treated cell extracts was then standardized against DMSO-treated control cells and expressed as x-fold activation. Luciferase and βgalactosidase measurements were carried out on a FLUOstar OPTIMA (BMG Labtech GmbH, Offenburg, Germany).

3 | RESULTS

The pharmacokinetic properties of macitentan in the rat and dog have been determined after single dose intravenous and oral administration. Pharmacokinetic parameters for both species are summarized in Tables 1 and Table 2. In the male rat, macitentan had plasma clearances (CL) of 6.5-8.2 mL/min·kg and volumes of distribution at steady-state (V_{ss}) of 1.2-1.6 L/kg, with no obvious difference between doses. Whereas plasma clearance in female rats was about three to fourfold lower, the V_{ss} of 1.4 L/kg indicated no sex difference in general tissue distribution. Apparent terminal half-lives were 1.9-3.8 hour in male rats, and 8.9 hour in female animals. In the dog, macitentan plasma CL was 5.2-5.7 mL/min·kg and V_{ss} was 1.1-1.2 L/kg, resulting in apparent terminal half-lives of 3.9 hour to 4.3 hour. As in the rat, CL and V_{ss} were independent of dose. Oral absorption was slow in rats and peak plasma concentrations (C_{max}) were only reached after 6-16 hour postdose. Bioavailability in male animals at 3 mg/kg was 53%. At 30 mg/kg, $AUC_{0\text{-inf}}$ and C_{max} increased in a more than dose-proportional manner, that is, by 30-fold and 19-fold over an only 10-fold increase in dose, likely due to saturation of clearance processes. Oral bioavailability was not calculated for 30 mg/kg as matching intravenous data were not available. In female rats, the higher $AUC_{0\text{-inf}}$ likely resulted from lower CL rather than sex differences in oral absorption. Oral bioavailability was comparable to males at 62%. Oral pharmacokinetic data at higher doses were generated as part of the toxicokinetic monitoring program of the 4-week toxicity study in the rat, in which macitentan $AUC_{0\text{-24h}}$ was determined on days 1 and 28 of treatment at daily doses of 50, 150, 450, and 1500 mg/kg (Table 3). At these doses, $AUC_{0\text{-24h}}$ increased in a less than dose-proportional manner, that is a 4.6-fold increase in $AUC_{0\text{-24h}}$ with a 30-fold increase in dose, likely as a consequence of macitentan's low aqueous solubility.[21] $AUC_{0\text{-24h}}$ in female rats exceeded those in males by about threefold. Macitentan pharmacokinetics after oral dosing to the dog differed from those in the rat. Oral absorption was fast and C_{max} was reached within 2 hour postdose (Table 2). C_{max} and $AUC_{0\text{-inf}}$ increased in proportion to dose and oral bioavailability at these doses was 74%-86%. Pharmacokinetic data at doses up to 500 mg/kg were again available from the toxicokinetic monitoring of the 4-week toxicity study (Table 3). $AUC_{0\text{-24h}}$ increased about dose-proportionally up to 50 mg/kg but increased by only 5.1-fold from 50 to 500 mg/kg.

Repeat dose pharmacokinetic data in the rat and dog were initially obtained from the day 28 data of two 4-week toxicity studies. $AUC_{0\text{-24h}}$ declined in both species after 4 weeks of macitentan treatment, but with notable differences between rat and dog (Table 3). In the rat, day 28/day 1 ratios of $AUC_{0\text{-24h}}$ were 0.4-0.6 in males and 0.6-0.8 in females with no obvious dose dependence in the magnitude of decline in either sex. In contrast, $AUC_{0\text{-24h}}$ after 4 weeks of treatment decreased dose-proportionally in the dog, by 10%-20% at 5 mg/kg to 80%-94% at 500 mg/kg. To better

TABLE 1 Pharmacokinetics of macitentan in rats and dogs after single intravenous dosing[a]

Species	Dose (mg/kg)	Sex	$AUC_{0\text{-inf}}$ (µg h/mL)	CL (mL/min·kg)	V_{ss} (L/kg)	$T_{1/2}$ (h)
Rat	0.1	M	0.27 (0.16-0.33)	6.5 (5.0-10)	1.6 (1.1-2.2)	3.8 (1.7-5.8)
	0.3	M	0.61 (0.56-0.66)	8.2 (7.5-9.0)	1.2 (1.1-1.6)	1.9 (1.7-2.3)
	0.3	F	2.99 (1.47-4.95)	2.0 (1.0-3.4)	1.4 (1.0-2.0)	8.9 (6.6-12)
Dog	0.1	M	0.32 (0.29-0.34)	5.2 (5.0-5.7)	1.2 (0.9-1.5)	3.9 (3.0-4.4)
	0.3	M	0.91 (0.71-1.04)	5.7 (4.8-7.0)	1.1 (0.9-1.3)	4.3 (3.3-5.2)

[a]Data are geometric means and range of n = 5 for rat and n = 3 for dog.

TABLE 2 Pharmacokinetics of macitentan in rats and dogs after single oral dosing[a]

Species	Dose (mg/kg)	sex	$AUC_{0\text{-inf}}$ (μg h/mL)	C_{max} (μg/mL)	T_{max} (h)	F^b (%)
Rat	3	M	3.24 (1.04-6.20)	0.38 (0.08-0.73)	6 (6-8)	53
	3	F	18.4 (11.0-28.6)	0.85 (0.63-1.23)	8 (8-8)	62
	30	M	97.4 (82.5-139)	7.30 (5.55-8.96)	16 (8-24)	n.c.[c]
Dog	1	M	2.37 (1.59-2.94)	0.41 (0.19-0.52)	2 (2-3)	74 (47-89)
	10	M	27.5 (20.1-32.1)	4.59 (2.18-7.24)	2 (1-3)	86 (60-104)

[a]All data are geometric means and range of n = 4-6 for rat, and n = 3 for dog, median and range is given for T_{max}.

[b]Oral bioavailability was calculated using intravenous AUC data at 0.3 mg/kg for rat, and 0.1 mg/kg for dog.

[c]n.c., not calculated due to lack of intravenous data with matching AUC, formal calculation of F using 0.3 mg/kg intravenous data yields F of 160%.

TABLE 3 Macitentan plasma exposures in rat and dog repeat oral dosing

Species	Dose (mg/kg)	$AUC_{0\text{-}24\,h}$ (day 1, μg/mL.h)		$AUC_{0\text{-}24\,h}$ (day 8/28, μg/mL.h)		AUC ratio (day 8/28-day 1)	
		Males	Females	Males	Females	Males	Females
Rat[a]	50	213	726	114	490	0.5	0.7
	150	382	1160	200	776	0.5	0.7
	450	595	1590	327	1280	0.6	0.8
	1500	979	2500	365	1590	0.4	0.6
Rat[b]	3	3.09	–	3.05	–	1.0	–
	15	20.3	–	12.3	–	0.6	–
	50	26.8	–	16.8	–	0.6	–
Dog[a]	5	17.5	11.6	14.7	10.5	0.8	0.9
	50	134	94.8	39.1	46.5	0.3	0.5
	500	692	504	44.9	84.4	0.06	0.2

Note: Mechanistic follow-up study in male Sprague-Dawley rats.

[a]Data from the 4-week toxicity studies in the Sprague-Dawley rat and Beagle dog.

[b]Data from an 8-day.

understand the apparent lack of a dose dependence in the rat, a second study with 8 days of treatment was performed in male rats at lower doses of 3, 15, and 50 mg/kg (Table 3). In this later study, AUC at 3 mg/kg on day 1 was comparable to the outcome of the PK experiment at the same dose (Table 2). At 50 mg/kg, however, $AUC_{0\text{-}24h}$ was only 26.8 μg·h/mL (day 1), that is, only 13% of the $AUC_{0\text{-}24h}$ (day 1) in the 4-week study. Differences in particle size distribution were identified between both macitentan batches and likely explain the varying plasma exposures. No change in $AUC_{0\text{-}24h}$ was noted after 8 days treatment at 3 mg/kg, whereas $AUC_{0\text{-}24h}$ was reduced to 60% of day 1 levels at 15 and 50 mg/kg. The above species differences in macitentan autoinduction prompted further work with the aim to understand the biochemical basis of these changes, the nature of metabolizing enzymes involved and their regulation upon macitentan treatment, in particular in light of the absence of such changes in clinical use.[18]

For the identification of the rat and dog P450 enzymes involved in metabolism, ^{14}C-macitentan was incubated with liver microsomes and a panel of recombinant P450 enzymes commercially available at the time of study conduct. Experiments were performed at 10 μmol/L macitentan and metabolic profiles recorded by HPLC coupled to radiodetection. The results are summarized in Table 4. Liver microsomes catalyzed three metabolic reactions, that is, oxidative depropylation to M6, aliphatic hydroxylation to yield M7, and hydrolysis to the aminopyrimidine M3 (Figure 1). M6 formation was by far the most prominent pathway, whereas M3 and M7 were minor components. With exception of Cyp2b1, all recombinant rat P450s yielded M6, whereas M7 was only formed by Cyp2c11. Dog

Cyp3a12 yielded mostly M6, but no M7 and only small amounts of M3. Neither M6 nor M7 were observed with Cyp2c21. M3 was detected in all experiments in the presence but also the absence of enzyme or NADPH, indicating that hydrolysis to M3 was not dependent on specific enzyme catalysis.

Enzyme kinetic parameters, K_m and V_{max}, were determined for M6 for those recombinant rat and dog P450 enzymes showing the largest turnover, that is, rat Cyp2c6, Cyp2c11, Cyp2c12, Cyp3a1, Cyp3a2, and dog Cyp3a12. Intrinsic clearance CL_{int}, the ratio of V_{max}/K_m, was calculated as a measure of catalytic efficiency (Table 5). Among the rat Cyp2c enzymes, M6 formation is most efficiently catalyzed by Cyp2c11 with a CL_{int} of $0.28 \cdot 10^{-6}$ $min^{-1} \cdot pmol\ P450^{-1}$.

TABLE 4 Metabolic profiles with liver microsomes and recombinant rat and dog P450 enzymes[a]

Species/P450 enzyme	M3	M6	M7	macitentan
Rat liver microsomes	5.5	53	4.8	37
Rat Cyp2b1	5.8	n.d.[b]	n.d.	94
Rat Cyp2c6	3.8	4.4	n.d.	93
Rat Cyp2c11	1.3	32	14	52
Rat Cyp2c12	6.1	4.3	n.d.	89
Rat Cyp3a1	4.6	85	n.d.	10
Rat Cyp3a2	5.6	80	n.d.	14
Dog liver microsomes	2.9	30	1.9	64
Dog Cyp2c21	5.3	n.d.	n.d.	94
Dog Cyp3a12	2.8	75	n.d.	22
No NADPH	0.8	n.d.	n.d.	99
No microsomes	3.2	n.d.	n.d.	97

[a]Results represent percent radioactivity in the chromatogram.
[b]n.d., not detected.

CL_{int} of Cyp2c6 and Cyp2c12 was about two orders of magnitude lower and their contribution to total intrinsic clearance therefore considered negligible. K_m for rat Cyp3a1 and Cyp3a2 were significantly lower than for the Cyp2c enzymes. Based on CL_{int}, both were by far the most important rat P450 enzymes involved in M6 formation. Dog Cyp3a12 displayed K_m and V_{max} values of 4.3 ± 1.0 µM and 6.1 ± 0.3 pmol/(min·pmol P450), respectively, indicating little species differences in the affinity of macitentan for the different cyp3a enzymes. The relative abundance of Cyp2b2, Cyp2c and Cyp3a mRNA was determined in liver samples of vehicle-treated rats from the 4-week toxicity study, using the housekeeping gene S18 as a reference. (Table 6). Results were in good agreement with literature[22-24] and were used to estimate the contribution of each P450 enzyme to total CL_{int} in both sexes (Table 5).

K_m and V_{max} together with total P450 concentrations were also determined from liver microsomes of the 4-week toxicity study in the rat (Table 7). Total P450 concentrations were 0.87 nmol/mg in untreated male rats. In treated males, P450 concentrations were in a narrow range of 1.33-1.45 nmol/mg, without an obvious dependence on dose. The K_m for M6 formation was 4.9 ± 0.8 µmol/L in liver microsomes of control males, and 7.4 ± 2.6 µmol/L at 50 mg/kg. At all higher doses, K_m was in a narrow range from 1.2 to 3.3 µmol/L. Data for V_{max} followed the same trend. Total P450 concentration was 0.71 nmol/mg in microsomes of untreated animals and increased irrespective of dose by about 50% in all treated females. The K_m derived from a Michaelis-Menten plot was 20 ± 4 µmol/L in untreated female livers which sharply dropped to 1.7-3.8 µmol/L in treated females. In contrast, V_{max} steadily increased with dose from 124 to 478 pmol/(min·mg). For the vehicle and 1500 mg/kg doses, K_m and V_{max} were independently determined using Eadie-Hofstee plots (Table 7 and Figure 2). For both sexes at 1500 mg/kg and for untreated males, estimates were in good agreement with those derived from the Michaelis-Menten plots. The Eadie-Hofstee plot

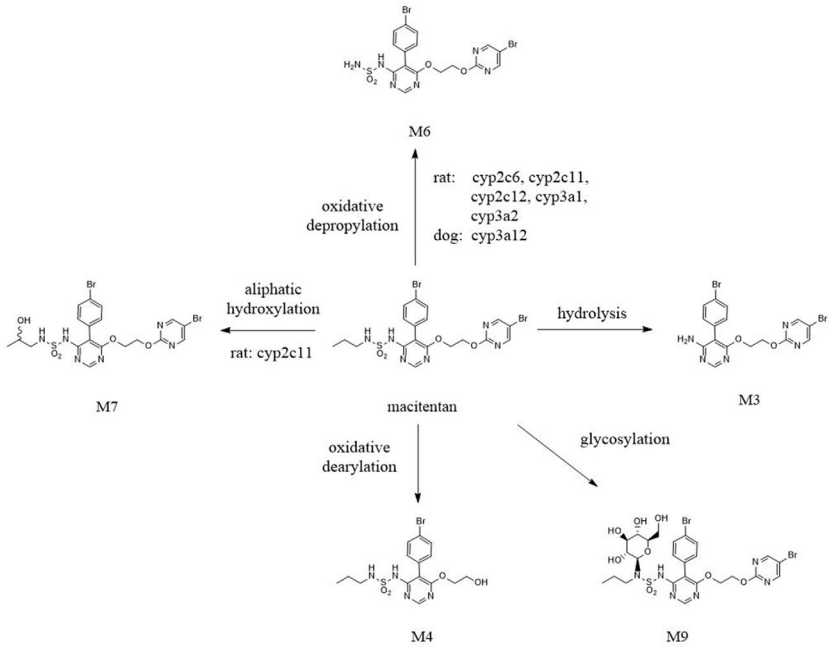

FIGURE 1 Primary metabolism of macitentan in rat and dog

TABLE 5 Enzyme kinetics of metabolite M6 with recombinant rat and dog P450 enzymes

Species	P450	V_{max} (pmol/min·pmol P450)	K_m (µmol/L)	CL_{int} (V_{max}/K_m) (10^{-6} min^{-1}·pmol $P450^{-1}$)	Contribution to total CL_{int}[a]	
					Males (%)	Females (%)
Rat	Cyp2c6	0.15 ± 0.02	18 ± 6	0.008	0.1	9
	Cyp2c11	12 ± 1	43 ± 6	0.28	20	—[b]
	Cyp2c12	0.34 ± 0.03	139 ± 18	0.002	< 0.1	19
	Cyp3a1	6.4 ± 0.7	2.8 ± 1.8	2.28	13	73
	Cyp3a2	4.4 ± 0.3	2.4 ± 0.8	1.83	67	—[b]
Dog	Cyp3a12	6.1 ± 0.3	4.3 ± 1.0	1.42	100	100

[a]Estimated using the relative abundance data from Table 6.

[b]Male-specific P450.

TABLE 6 mRNA levels of selected P450 genes in liver samples of vehicle-treated male and female Sprague-Dawley rats[a]

Gene	Male (rel. to S18.1000)	Male (% of total)	Female (rel. to S18.1000)	Female (% of total)
Cyp2b2	0.64 ± 0.53	1.6	0.02 ± 0.01	0.03
Cyp2c6	3.54 ± 0.65	8.7	5.84 ± 2.38	10
Cyp2c11	19.7 ± 1.7	48	n.s.[b]	—
Cyp2c12	2.58 ± 1.10	6.3	51.2 ± 18.3	88
Cyp2c13	3.02 ± 0.37	7.4	n.s.	—
Cyp3a1	1.46 ± 0.27	3.6	0.21 ± 0.24	0.3
Cyp3a2	9.67 ± 2.90	24	n.d.[c]	—
Cyp3a9	0.09 ± 0.05	0.2	0.78 ± 0.49	1.3

[a]Relative expression levels (×1000) normalized to the S18 housekeeping gene; data are means and SD of n = 5-6.

[b]n.s., not studied.

[c]n.d.: not measurable.

TABLE 7 Enzyme kinetic parameters of M6 with rat liver microsomes of the 4-week toxicity study

Sex	Dose (mg/kg)	P450 (nmol/mg)	K_m (µmol/L)	V_{max} (pmol/min·mg)
Male	0	0.87	4.9 ± 0.8[a]	332 ± 14[a]
			4.7 ± 0.8[b]	328 ± 23[b]
	50	1.35	7.4 ± 2.6	709 ± 67
	150	1.41	3.3 ± 1.0	543 ± 33
	450	1.33	1.2 ± 0.8	405 ± 36
	1500	1.45	2.4 ± 0.7	522 ± 29
			2.2 ± 0.2	516 ± 15
Female	0	0.71	20 ± 4[a]	124 ± 9
			K_m1: 26 ± 2[b]	V_{max}1: 132 ± 5[b]
			K_m2: 3.4 ± 1.7[b]	V_{max}2: 53 ± 12[b]
	50	1.05	3.5 ± 1.5	166 ± 16
	150	1.15	1.7 ± 0.8	195 ± 15
	450	1.18	2.1 ± 0.6	345 ± 18
	1500	0.90	3.8 ± 1.0	478 ± 27
			3.0 ± 0.5	458 ± 25

[a]Determined by nonlinear regression (Michaelis-Menten plot).

[b]Determined from an Eadie-Hofstee plot.

for untreated females was, however, clearly biphasic. Differential analysis yielded K_m values of 26 ± 2 µmol/L and 3.4 ± 1.7 µmol/L, whereas the corresponding V_{max} were 132 ± 5 pmol/(min·mg) and 53 ± 12 pmol/(min·mg).

In parallel, changes in mRNA expression relative to the vehicle-treated controls have been determined for all rat P450 enzymes involved in M6 formation, that is, Cyp2c6, Cyp2c11, Cyp2c12, Cyp3a1, and Cyp3a2 (Table 8). In male rats, Cyp2c6, Cyp3a1, and Cyp3a2 were upregulated in a generally dose-dependent manner by 6.5- to 10-fold at the highest macitentan dose of 1500 mg/kg (Figure 3). Expression of Cyp2c11 responded only weakly to macitentan treatment, and mRNA levels were only increased by about 50% vs control at 450 mg/kg and 1500 mg/kg. No induction was observed for the male-specific Cyp2c13 and for Cyp2c12 in either sex. In female rats, Cyp2c6 mRNA increased up to 150 mg/kg by about fourfold over vehicle and appeared to plateau at higher doses. Upregulation of Cyp3a1 mRNA increased dose-dependently up to 106-fold at 1500 mg/kg. Reproducible amplification of Cyp3a2 mRNA was not possible with female liver samples, as basal expression levels were too low. mRNA levels were also determined in the mechanistic follow-up study and generally confirmed the observations from the 4-week study.

Enzyme kinetic data from dog liver microsomes revealed a much simpler picture (Table 9). Total P450 content increased steadily with dose in both sexes. V_{max} and Cyp3a12 mRNA levels increased in parallel to total P450 concentrations. In male dogs, a 3.3-fold increase in V_{max} over vehicle control was observed for the 500 mg/kg dose, which was paralleled by an 18.6-fold increase in Cyp3a12 mRNA expression (Figure 3). The increases in female dogs were lower, that is, 1.8-fold for V_{max} and 12.7-fold for Cyp3a12. K_m ranged from 4.5-10 µmol/L and was largely independent from dose and sex.

Induction of human CYP1A2, CYP2C9, and CYP3A4 was investigated on mRNA and P450 enzyme activity level using human

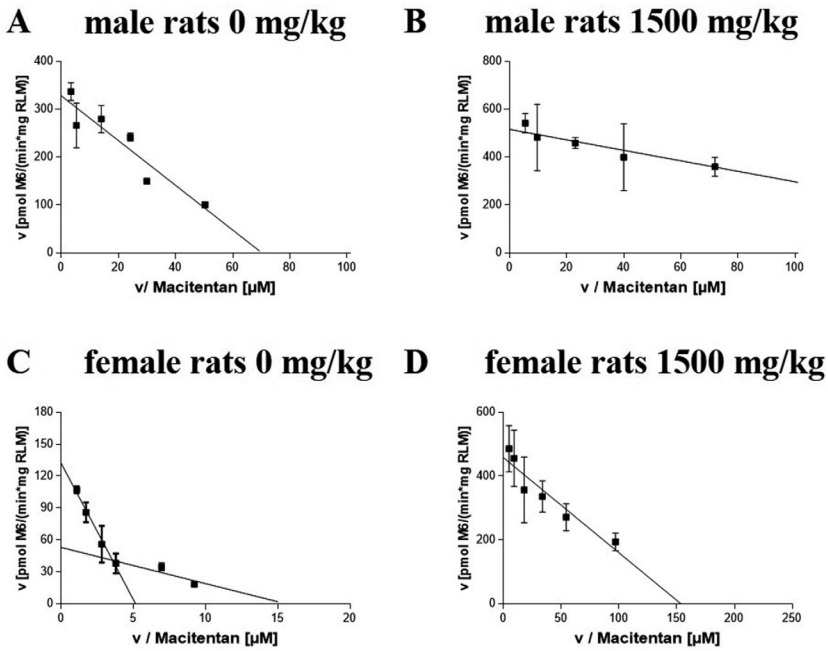

FIGURE 2 Eadie-Hofstee plots of M6 formation with rat and dog liver microsomes of the control and 1500 mg/kg dose groups

TABLE 8 Upregulation of selected rat P450 mRNAs in liver samples from the 4-week toxicity study and the mechanistic follow-up study[a]

Species	Sex	Dose (mg/kg)	Cyp2c6	Cyp2c11	Cyp3a1	Cyp3a2
Rat	Male	0	1.00 ± 0.18	1.00 ± 0.09	1.00 ± 0.19	1.00 ± 0.30
		50	6.21 ± 1.02	n.d.[b]	2.17 ± 0.37	3.65 ± 1.03
		150	4.36 ± 1.96	n.d.	3.51 ± 1.36	4.53 ± 1.31
		450	9.53 ± 1.26	1.48 ± 0.48	4.87 ± 0.51	5.21 ± 1.33
		1500	9.20 ± 3.29	1.43 ± 0.55	9.96 ± 4.18	6.50 ± 2.40
Rat	Male	0	1.00 ± 0.67	1.00 ± 0.72	1.00 ± 0.68	1.00 ± 0.37
		5	1.92 ± 0.45	1.55 ± 0.22	1.66 ± 0.75	1.46 ± 0.18
		15	1.55 ± 1.21	1.65 ± 1.35	2.53 ± 2.02	2.37 ± 1.43
		50	3.08 ± 2.45	2.50 ± 0.85	6.04 ± 3.00	4.04 ± 0.98
Rat	Female	0	1.00 ± 0.41	—[c]	1.00 ± 1.16	—[d]
		50	2.78 ± 0.95	—	12.1 ± 4.4	—
		150	4.21 ± 0.51	—	38.3 ± 10.7	—
		450	4.35 ± 1.54	—	55.3 ± 13.1	—
		1500	3.29 ± 0.77	—	106 ± 34	—

[a]No induction observed on Cyp2C12 and Cyp2c13, data not included in table.
[b]n.d.: not determined as induction of 450 and 1500 mg/kg was minimal.
[c]Not analyzed, Cyp2c11 is male-specific.
[d]Not analyzed as basal expression in female rats was too low.

hepatocytes at macitentan concentrations up to 10 µmol/L. Omeprazole and rifampicin were used as positive controls. Results for CYP3A4 are shown in Figure 4, panel A (mRNA) and panel B (enzyme activity). Macitentan showed a concentration-dependent increase in CYP3A4 mRNA by about 11-fold vs DMSO control at 10 µmol/L, whereas the increase in CYP3A4 enzyme activity reached fivefold. No change in CYP1A2 and CYP2C9 mRNA expression was observed. The activation of PXR was determined in a reporter-gene assay expressed in green monkey kidney (CV-1) cells at concentrations up to 30 µmol/L, again using rifampicin as positive control. Results are presented in Figure 4, panel C, demonstrating a concentration-dependent activation of the PXR receptor with an EC_{50} of 1.2 µmol/L.

4 | DISCUSSION

The endothelin receptor antagonist macitentan, approved for the treatment of pulmonary hypertension, has shown significant species

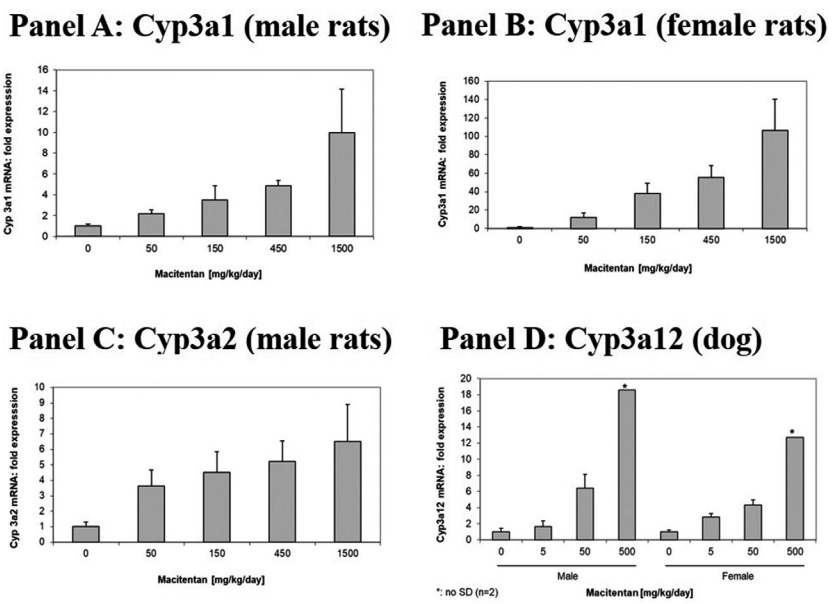

FIGURE 3 Up-regulation of Cyp3a mRNA in rat and dog livers after daily dosing with macitentan for 4 weeks

TABLE 9 Enzyme kinetic parameters of M6 and upregulation of Cyp3a12 mRNA in liver samples from the 4-week toxicity study in the dog

Sex	Dose (mg/kg)	P450 (nmol/mg)	K_m (μmol/L)	V_{max} (pmol/min·mg)	Cyp3a12
Male	0	0.45	6.7 ± 1.2	304 ± 14	1.00 ± 0.45
	5	0.50	8.6 ± 0.9	517 ± 15	1.63 ± 0.72
	50	0.73	10 ± 1.7	998 ± 51	6.45 ± 1.71
	500	1.00	5.3 ± 1.0	1011 ± 46	18.6[b]
Female	0	0.55	4.5 ± 1.0	682 ± 33	1.00 ± 0.23
	5	0.63	6.2 ± 0.5	1028 ± 23	2.84 ± 0.44
	50	0.87	7.2 ± 1.0	1730 ± 67	4.34 ± 0.61
	500	0.84	6.7 ± 2.2	1250 ± 107	12.7[a]

[a]n = 2 only.

differences in its induction pattern of P450 enzymes. Macitentan is not a P450 inducer at efficacious doses of 10 mg in man as shown in an interaction study with the CYP3A4 substrate sildenafil[18] and the lack of autoinduction upon repeated treatment.[19] In rat and dog, the main animal species used in the nonclinical safety assessment, macitentan exhibited time-dependent changes in its pharmacokinetic behavior. While the inductive effects increased with increasing doses in the dog, the decline in drug exposure in the rat reached a plateau already at relatively low doses. Moreover, sex differences were observed in the magnitude of decline in the rat. The purpose of this investigation was to better understand the biomolecular basis of these differences in the induction pattern of macitentan in animals. Additional work was done to assess the P450 induction potential in humans by investigating changes in cytochrome P450 mRNA expression in human hepatocytes and activation of the PXR receptor.

Macitentan is a low CL drug in the rat and dog. CL were 6.5-8.2 mL/min·kg and 5.2-5.7 mL/min·kg in the male rat and dog, respectively, translating into 15%-30% of the respective liver blood flows considering the blood/plasma ratios of 0.64 and 0.54 in rat and dog (data not shown). CL in female rats only reached 25% of the value in male animals as a result of differences in metabolic competence. Macitentan partitions well into tissues as judged by its volume of distribution at steady-state of 1-2 L/kg, which was independent from dose, species, and sex. Terminal half-lives, as a composite endpoint of volume and CL, were 1.9-4.3 hour in male rats and dogs, and 8.9 hour in female rats. Oral absorption in the rat was slow with a T_{max} of 6-16 hour, likely as a consequence of its low aqueous solubility[21]). Plasma exposure was about six times higher in female rats, mostly reflecting the sex differences in CL, whereas there was no such difference in oral bioavailability. Oral absorption was faster in the dog with T_{max} of 2 hour. Oral bioavailability reached 74%-86%, indicating complete absorption from the gastro-intestinal tract and a limited first-pass effect.

The metabolism of macitentan in the rat and dog has been recently published in detail.[25] In brief, macitentan is metabolized along five primary pathways (Figure 1), that is, 1) oxidative depropylation to M6 via initial hydroxylation at the α carbon atom of the propyl chain, 2) aliphatic hydroxylation at the β carbon atom to M7, 3) cleavage of the ethylene glycol linker to yield M4 with loss of the 5-bromopyrimidine moiety, 4) conjugation with glucose to yield M9, most likely at one of the nitrogen atoms of the sulfamide moiety, and 5) hydrolysis of the sulfamide moiety to yield M3. M3

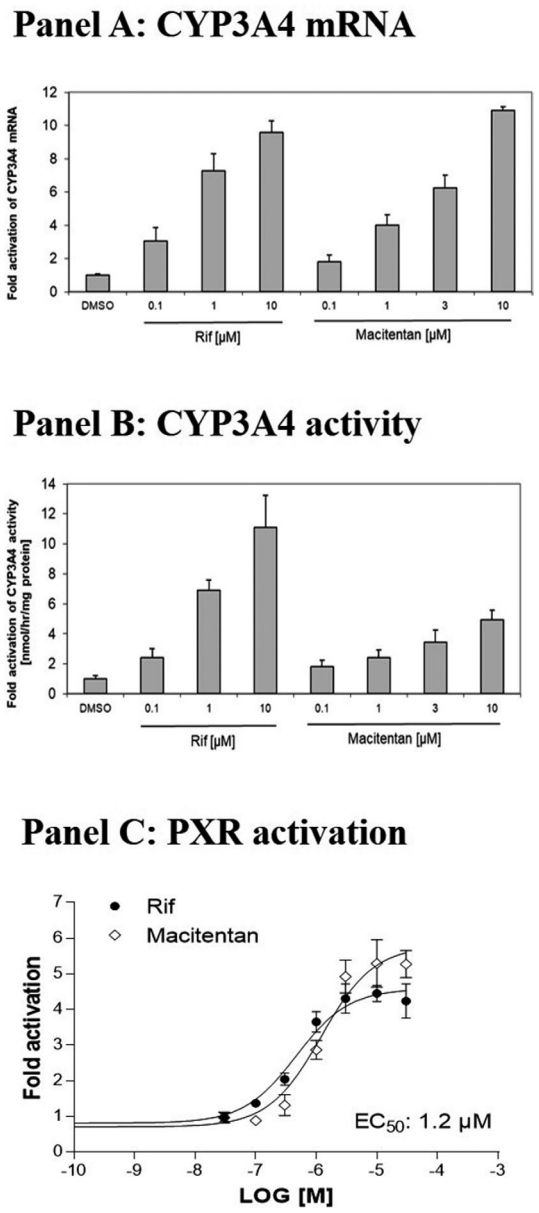

FIGURE 4 The effect of macitentan on CYP3A4 mRNA expression and enzyme activity in human hepatocytes and activation of human PXR

and M6 then undergo secondary metabolism including oxidation reactions and conjugation with glucose or glucuronic acid. With exception of the conjugation to glucose, all metabolic pathways were also present in man.[26] M6 and M7 are formed by P450 enzymes, whereas the enzymes involved in M3, M4, and M9 formation remain unknown. M6 formation was the major metabolic pathway in animals[25] and man[26] and the only major metabolite circulating in plasma of all species.

In line with these previous reports, metabolite M6 was also by far the major product with rat and dog liver microsomes accompanied by only small amounts of M7 and M3 (Table 4). The identification of metabolizing enzymes and determination of enzyme kinetic parameters was therefore focussing on M6. Whereas only a single P450 enzyme, that is, Cyp3a12, appeared to be involved in M6 formation in the dog, several P450 enzymes catalyzed M6 formation in the rat. Based on CL_{int}, catalytic efficiency was highest for the two Cyp3a enzymes, whereas CL_{int} was significantly lower for the Cyp2c enzymes (Table 5). The contribution of each P450 enzyme to total CL_{int} was estimated considering the relative abundance of each P450 enzyme in vehicle-treated liver microsomes (Table 6). In noninduced male rats, M6 was mostly catalyzed by Cyp3a2 (67%), followed by Cyp2c11 (20%), Cyp3a1 (13%) and negligible contributions of Cyp2c6 and Cyp2c12 (both < 1%). In female rats, Cyp3a1 was the major P450 involved in M6 formation accounting for 73% of total CL_{int}. Cyp2c6 and Cyp2c12 were more important in female rats and together responsible for 28%.

Macitentan induced P450 enzyme expression in rat and dog, leading to autoinduction in both 4-week toxicity studies. Increases in total P450 content and V_{max} and the upregulation of several P450 enzymes were biochemical hallmarks of this induction. (Tables 7-9). Despite these similarities, there were notable species differences between rat and dog. While the inductive effect appeared to plateau in male and female rats, macitentan plasma AUC declined steadily with dose in the dog, down to 6%-20% of day 1 values at the highest dose of 500 mg/kg (Table 3).

The P450 induction pattern in rat resembled that of a typical phenobarbital-type inducer. While all Cyp2c genes but Cyp2c6 were refractory to induction, Cyp3a mRNAs were upregulated in a dose-dependent manner. In males, Cyp3a1 mRNA increased steadily with dose up to about 10-fold vs control at 1500 mg/kg, whereas most of the Cyp3a2 mRNA increase was already present at the lowest dose of 50 mg/kg. The dose dependence in Cyp3a2 upregulation was more evident at lower doses and started already at 5 mg/kg. As the contribution of Cyp2c6 to macitentan clearance was small, the increase in V_{max} in liver microsomes of the 4-week study is likely a reflection of the higher Cyp3a levels after induction, leading to an even larger shift toward Cyp3a enzymes. This is reflected in the unchanged K_m across doses, which was close to those of the recombinant Cyp3a1 and Cyp3a2 (Tables 5 and 7). In female rats, Cyp3a1 mRNA was upregulated in a dose-dependent manner up to 106-fold vs control at 1500 mg/kg. Basal levels of Cyp3a2 were too low to allow for amplification, as Cyp3a2 is a male-specific P450 enzyme.[27] Cyp2c6 was upregulated by three- to fourfold vs control but without an obvious dose response. The resulting shift in macitentan clearance toward Cyp3a1 upon induction was again evident in the liver microsomes from the 4-week toxicity study. While the composite K_m in the vehicle group was 20 μmol/L, it sharply dropped to 1.7-3.8 μmol/L in all macitentan-treated groups, that is, close to the K_m of recombinant Cyp3a1. The large increase in Cyp3a1 mRNA did not translate into functional protein as V_{max} increased by only fourfold at 1500 mg/kg, whereas total P450 protein was similar at all treated doses.

In contrast to the rat, the induction pattern was straightforward in the dog. Based on the enzyme kinetic data with liver microsomes and recombinant P450 enzymes, macitentan is a Cyp3a12 substrate which is upregulated in a dose-dependent manner upon macitentan treatment. V_{max} in liver microsomes increased with dose and in parallel to Cyp3a12 mRNA with no obvious difference between male and female animals.

Macitentan induced CYP3A4 mRNA and enzyme expression in human hepatocytes and activated the human PXR receptor in a concentration-dependent manner with an EC_{50} of 1.2 µmol/L (Figure 4). Although not experimentally proven, the same mechanism is likely operating in animals as the P450 induction pattern in rats strongly resembled that of phenobarbital, a known PXR activator in the rat.[28] In clinical trials, macitentan neither changed the pharmacokinetics of the sensitive CYP3A4 substrate sildenafil,[18] nor was there any autoinduction upon multiple dosing.[19] Macitentan plasma exposure ($AUC_{0\text{-}24h}$) at its therapeutic dose of 10 mg was 5.4 µg·h/mL, whereas first signs of autoinduction in the rat and dog were only evident at $AUC_{0\text{-}24h}$ of 20.3 µg·h/mL (15 mg/kg) and 17.5 µg·h/mL (5 mg/kg), respectively (Table 3). Macitentan has been shown to partition into liver by passive diffusion and is not a substrate of hepatic OATP transporters.[17,21] Unbound plasma concentrations are therefore a good approximation of free concentrations in the liver. Plasma protein binding in animals and man was in excess of 99%.[29] Unbound peak plasma concentrations in man and consequently free peak concentrations in the liver are therefore in the range of about 10-20 nmol/L, that is, significantly below the EC_{50} determined on PXR activation. The absence of P450 induction in man is thus best explained by too low unbound drug levels in the liver to translate into relevant upregulation of P450 enzymes. This is in contrast to bosentan, another endothelin receptor antagonist used for the treatment of pulmonary hypertension, which is classified as a moderate CYP3A4 inducer in clinical use.[30] Despite the significantly higher daily dose, that is, 250 mg vs 10 mg, its plasma concentrations are also in the range 1-2 µmol/L.[12] Also bosentan is a PXR activator and upregulates P450 expression in hepatocytes.[30] Both compounds, however, differ in their mechanism of hepatic disposition. While macitentan partitions into liver via passive diffusion, bosentan is an OATP substrate and has been shown to accumulate in the liver.[31,32]

In summary, macitentan is a P450 enzyme inducer in animals and man. The induction pattern, however, significantly differed between species. Total P450 content and enzyme activity steadily increased in the dog resulting in a dose-dependent reduction in macitentan exposure after repeated treatment. In contrast, the inductive effect was saturable in the rat leading to a plateau in the reduction of macitentan exposure at steady-state. The inductive effect was more pronounced in the male rat. The absence of P450 induction in clinical use is likely the consequence of unbound hepatic drug concentrations well below the EC_{50} for PXR activation.

ACKNOWLEDGEMENTS

The authors thank Aude Weigel, Sybille Fläschel, Gabriele Buczinsky, and Marie Philibert for their dedication and experimental support.

CONFLICT OF INTEREST

At the time, the experimental work described herein was done, all authors were employees of Actelion Pharmaceuticals Ltd, Allschwil, Switzerland. Actelion Pharmaceuticals Ltd was acquired by Johnson & Johnson in 2017. After a demerger, all authors are now employees of Idorsia Pharmaceuticals Ltd.

For the above reasons, the data presented in this publication belong to Johnson & Johnson. The authors are therefore not in the position to share them on a public platform.

AUTHOR CONTRIBUTIONS

Treiber, Delahaye, Seeland, and Gnerre participated in research design, performed data analysis, and wrote or contributed to the writing of the manuscript. Delahaye, Seeland, and Gnerre conducted experiments.

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How to cite this article: Treiber A, Delahaye S, Seeland S, Gnerre C. The endothelin receptor antagonist macitentan for the treatment of pulmonary arterial hypertension: A cross-species comparison of its cytochrome P450 induction pattern. *Pharmacol Res Perspect*. 2020;e00619. https://doi.org/10.1002/prp2.619