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Original Research Article

Small Molecule Angiotensin II Type 2 Receptor (AT_2R) Antagonists as Novel Analgesics for Neuropathic Pain: Comparative Pharmacokinetics, Radioligand Binding, and Efficacy in Rats

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Abstract

Objective. Neuropathic pain is an area of unmet clinical need. The objective of this study was to define the pharmacokinetics, oral bioavailability, and efficacy in rats of small molecule antagonists of the angiotensin II type 2 receptor (AT_2R) for the relief of neuropathic pain.

Design and Methods. Adult male Sprague-Dawley (SD) rats received single intravenous (1–10 mg/kg) or oral (5–10 mg/kg) bolus doses of EMA200, EMA300, EMA400 or EMA401 (*S*-enantiomer of EMA400). Blood samples were collected immediately pre-dose and at specified times over a 12- to 24-hour post-dosing period. Liquid chromatography tandem mass spectrometry was used to measure plasma drug concentrations. Efficacy was assessed in adult male SD rats with a unilateral chronic constriction injury (CCI) of the sciatic nerve.

Results. After intravenous administration in rats, mean (±standard error of the mean) plasma clearance for EMA200, EMA300, EMA400, and EMA401 was 9.3, 6.1, 0.7, and 1.1 L/hour/kg, respectively. After oral dosing, the dose-normalized systemic exposures of EMA400 and EMA401 were 20- to 30-fold and 50- to 60-fold higher than that for EMA300 and EMA200, respectively. The oral bioavailability of EMA400 and EMA401 was similar at ~30%, whereas it was only 5.9% and 7.1% for EMA200 and EMA300, respectively. In CCI rats, single intraperitoneal bolus doses of EMA200, EMA300, and EMA400 evoked dose-dependent pain relief. The pain relief potency rank order in CCI rats was EMA400 > EMA300 > EMA200 in agreement with the dose-normalized systemic exposure rank order in SD rats.

Conclusion. The small molecule AT_2R antagonist, EMA401, is in clinical development as a novel analgesic for the relief of neuropathic pain.

Key Words. Pharmacokinetics; Oral Bioavailability; Angiotensin II Type 2 Receptor (AT_2R) Antagonist; Neuropathic Pain; Analgesia; Radioligand Binding

Introduction

Neuropathic pain is often intractable and represents a major unmet medical need [1]. Hence, over the past two decades, there has been a large global research effort directed at improving our collective understanding of the pathobiology of neuropathic pain [2]. New insights on

novel neuropathic pain targets have been generated [2] that have informed drug discovery programs aimed at producing a new generation of small molecule analgesics with suitable pharmacokinetics, oral bioavailability, potency, and efficacy for improved relief of neuropathic pain [3].

Recent work using molecular biological methods shows that the angiotensin II type 2 receptor (AT_2R) is expressed abundantly in the brain and viscera of adult rodents, whereas there were low AT_2R expression levels in the fetus with the exception of the skin [4]. Although these data appear to contradict earlier reports of abundant AT_2R expression in the fetal rat with low levels in the adult, re-examination of the original fetal rat autoradiographical images shows that the radioligand binding appears to be largely confined to the skin [4].

In vitro studies show that angiotensin II, the major endogenous effector of the renin-angiotensin system, induces neuronal excitability [5] in cultured cells of neuronal origin [6–8] as well as in cultured adult rat dorsal root ganglion (DRG) neurons [9] and that these effects are blocked by the selective AT_2R antagonist, PD-123,319 [5–9]. As hyperexcitability of DRG neurons is a key mechanism contributing to neuropathic pain, our laboratory is investigating the utility of small molecule AT_2R antagonists with >1,000-fold selectivity over the angiotensin II type 1 receptor (AT_1R) as potential novel analgesics for alleviation of neuropathic pain.

In drug discovery, information from rodent studies on the pharmacokinetics and oral bioavailability of new molecules is essential for guiding lead optimization and for informing selection of drug candidates for progression into preclinical drug development [10]. Additionally, data from rodent pharmacokinetic studies in conjunction with that from efficacy studies undertaken in relevant rodent models of the human disease are essential for estimating target plasma concentrations for subsequent early-phase clinical trials of the drug candidate in patients. As the pharmacokinetics and oral bioavailability of test compounds in rodents are derived from plasma concentration vs time curve data, sensitive and selective bioanalytical methods are required for quantification of the analytes of interest in small volumes of rodent plasma.

Here, we report on the comparative pharmacokinetics and oral bioavailability of three AT_2R blockers, viz. EMA200 (also known as PD-123,319), EMA300 (also known as PD-121,981) EMA400 (also known as PD-126,055), and its *S*-enantiomer (EMA401) administered by single intravenous (i.v.) and oral bolus doses to groups of adult male Sprague-Dawley (SD) rats. Additionally, we assessed the analgesic efficacy of single intraperitoneal (i.p.) bolus doses of EMA200, EMA300, and EMA400 in rats with a chronic constriction injury (CCI) of the sciatic nerve, a widely utilized rat model of neuropathic pain.

Together, our data show for the first time that selective, small molecule AT_2R antagonists produce dose-dependent relief of hindpaw hypersensitivity in the CCI rat model of neuropathic pain. The antineuropathic potency rank order was EMA400 > EMA300 > EMA200, which mirrored their rank order for dose-normalized systemic exposure in the adult male SD rat.

Materials and Methods

Drugs and Reagents

Test Items

The selective AT_2R antagonists (Figure 1), EMA200, EMA300 (as the sodium salt), EMA400, and EMA401 (*S*-enantiomer of EMA400 as the sodium salt) were used herein. EMA200 administered to rats in the pharmacokinetic study was synthesized by Dr Craig Williams, School of Chemistry and Molecular Biosciences (The University of Queensland). PD123,319 was purchased from Sigma Aldrich (Sydney, Australia) and was used for the efficacy study in rats and for generating the calibration standards for the Liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for EMA200. EMA300, EMA400, EMA401, and EMA401-d7 were synthesized by Glycosyn[trl] (Lower Hutt, New Zealand) and supplied by Spinifex

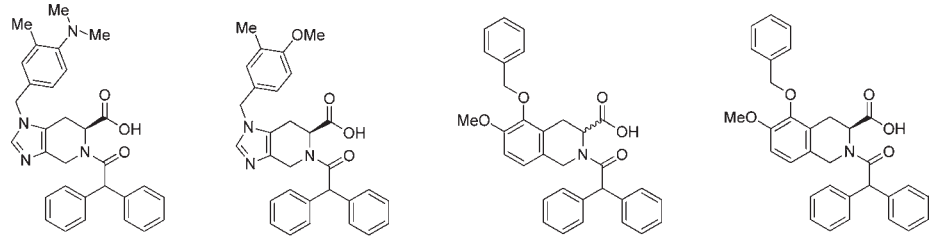

Figure 1 Chemical structures of the small molecule angiotensin II type 2 receptor antagonists assessed herein, viz. EMA200 (also referred to as PD-123,319), its structural analog, EMA300 (also referred to as PD-121,981), EMA400 (also referred to as PD-126,055), and EMA401 ([S]-enantiomer of EMA400).

Pharmaceuticals Pty Ltd. (Melbourne, Australia). Losartan was from Dent Global (Lower Hutt, New Zealand). EMA300 is a structural analog of EMA200 (Figure 1). EMA400/EMA401 is a member of the tetrahydroisoquinoline class of AT_2R antagonists.

Materials

Sodium benzylpenicillin vials (600 mg) were from CSL Ltd (Melbourne, Australia). Isoflurane (Forthane®) was from Abbott Australasia (Sydney, Australia), while medical grade CO_2 and O_2 were from BOC Gases Ltd. (Brisbane, Australia). Heparinized saline (50 IU/mL) and saline ampoules were from Astra Pharmaceuticals Pty Ltd. (Sydney, Australia). Polyethylene tubing was from Dural Plastics and Engineering Pty Ltd. (Sydney, Australia), and silk sutures (Dysilk Black Braided Siliconized Silk) were from Dynek Pty Ltd. (Hendon, Australia). Minivials (Eppendorf™) were from Disposable Products (Brisbane, Australia).

Reagents

Analytical reagent (AR) grade formic acid and hydrochloric acid were from The University of Queensland's Biological and Chemical Sciences Store (Brisbane, Australia). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were either from The University of Queensland's Biological and Chemical Sciences Store or from Burdick & Jackson (Muskegon, MI, USA). Citric acid monohydrate United States Pharmacopoeia (USP) and glacial acetic acid were from EM Science (Gibbstown, NJ, USA). 2-butanol, 1-methylpiperidine, and methadone were from Sigma Aldrich (St Louis, MO, USA). Hexane and HPLC grade water were from Burdick & Jackson.

Angiotensin II Receptor Binding Assays

Cloned Rat AT_1 and AT_2 Receptors

Human embryonic kidney (HEK) cells were transfected with rat AT_1R complementary DNA (cDNA) (HEK-AT_1) alone or rat AT_2R cDNA (HEK-AT_2) alone using Lipofectamine™ 2000 as per the manufacturer's instructions. Selection of HEK cells stably expressing the receptors of interest was achieved by addition of G418 (500 μg/mL) to the growth medium. HEK cells stably expressing either the AT_1R or the AT_2R (HEK-AT_1 and HEK-AT_2, respectively) were maintained at 37°C in a 5% humidified CO_2 incubator in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and G418 (500 μg/mL).

Membranes were prepared from each of stably transfected HEK-AT_1 and HEK-AT_2 cells grown in 10-cm tissue culture plates to approximately 80% confluence. Briefly, cells were washed with binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$; pH 7.4; 3 × 5 mL aliquots), dislodged with a cell scraper in 1.5-mL binding buffer and dispersed by sonication (5 × 1 second bursts at 60 W) using a Vibracell Ultrasonic Processor on ice, and then centrifuged at 2,000 × *g* for 10 minutes at 4°C. After discarding the pellet, the supernatant was centrifuged at 44,800 × *g* for 30 minutes at 4°C, and the pellet was resuspended in binding buffer supplemented with the protease inhibitors, leupeptin at 5 μg/mL, and phenylmethylsulphonyl fluoride at 1 mM, as well as the trypsin inhibitor, aprotinin at 0.3 TIU.[1] Protein concentrations were determined using the Bradford procedure (Bio-Rad, Sydney, NSW, Australia). Binding affinities for the ligands of interest were determined using competitive displacement binding assays.

Radioligand binding assays were performed using membranes prepared from each of HEK-AT_1 and HEK-AT_2 cells (40 μg protein), and incubated with [^{3}H]-angiotensin II (40 nM) and varying concentrations (0.1 nM to 1.0 μM for the AT_2R assay and 10 nM to 100 μM for the AT_1R assay) of the unlabelled ligands of interest, viz. EMA200, EMA300, EMA400, EMA401, EMA402, and angiotensin II. Nonspecific binding was determined in the presence of unlabelled angiotensin II at 1.0 μM. Following incubation for 1 hour at 4°C, radioligand binding was terminated by rapid filtration through Whatman™ GF/B filters (Sigma Aldrich, Sydney, NSW, Australia) that had been presoaked for 1 hour at 4°C in filtration buffer (1% polyethyleneimine, 0.1% bovine serum albumin, Tris-HCl buffer 50 mM, pH 7.4), using a Brandell™ cell harvester (Perkin Elmer, Dewers Grove, IL, USA). After washing three times with ice-cold Tris-HCl buffer (50 mM, pH 7.4), individual filters containing the bound [^{3}H]-angiotensin II were placed into separate vials containing scintillation fluid (3 mL) for 16 hours prior to counting in a Packard™ liquid scintillation counter (Tricarb 2700 TR; Gaithersberg, MD, USA).

Cloned Human AT_1 and AT_2 Receptors

Chinese hamster ovary (CHO) cells or Hela cells expressing either the cloned human AT_1R or AT_2R, respectively, were generated according to published methods [11,12]. These experiments were conducted according to standard protocols at Cerep (Redmond, WA, USA) [13]. Briefly, radioligand binding assays were performed using CHO-AT_1 or CHO-AT_2 cells, and incubated with the radioligands, [^{125}I][Sar^1,Ile^8]-angiotensin II, or [^{125}I]CGP 42112A at 0.05 nM for the AT_1R and AT_2R, respectively. The concentration ranges for the unlabelled ligands of interest, viz. EMA401, EMA402, and saralasin were 0.01 nM to 100.0 μM for the AT_2R assay and 0.01 pM to 1 μM for the AT_1R assay. Nonspecific binding was determined in the presence of unlabelled angiotensin II at 10 and 1.0 μM for the AT_1R and AT_2R assays, respectively. Following incubation for 1 hour at 37°C for the AT_1R assay and 3 hours at 37°C for the AT_2R assay, radioligand binding was terminated, and bound radioligand was determined using liquid scintillation counting [13].

Ethics Statement

For the efficacy studies in CCI rats and the pharmacokinetic studies of EMA200 and EMA300 in rats described herein, ethics approval was obtained from the Animal Ethics Committee of The University of Queensland and experiments adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes

(7th edition, 2004). The pharmacokinetic and oral bioavailability study of EMA400 in rats was conducted at Cerep in accordance with the requirements of the Animal Ethics Committee at Cerep [14]. The in-life phase of the pharmacokinetic study of the *S*-enantiomer of EMA400 (EMA401) was undertaken at MPI Research, Inc. (Mattawan, MI, USA) [15]. It was conducted in accordance with the requirements of the Institutional Animal Care and Use Committee at MPI Research, Inc and was in compliance with the relevant Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals [14].

Animals

Efficacy

For the efficacy studies in the CCI rat model of neuropathic pain, adult male SD rats were purchased from The University of Queensland Biological Resources. Animals were housed in a temperature-controlled facility (21 ± 2°C) with a 12/12-hour light-dark cycle, and experimentation was conducted during the light phase. Standard rodent chow and water were available *ad libitum*.

Pharmacokinetics

For the pharmacokinetic studies of EMA200 and EMA300, adult male SD rats were purchased from the Herston Medical Research Centre at The University of Queensland (Brisbane, Australia). Rats were housed in a temperature-controlled environment (21 ± 2°C) on a 12/12-hour light/dark cycle with rodent chow and water available *ad libitum*. For the pharmacokinetic study of EMA400, adult male SD rats were purchased from Charles River Laboratories (Ann Arbor, MI, USA) by Cerep [14]. For the pharmacokinetic study of EMA401, adult male SD rats were purchased from Charles River Laboratories by MPI Research, Inc. [15].

CCI Rat Model of Neuropathic Pain

A unilateral CCI of the sciatic nerve was induced in rats according to a published method [16]. Briefly, rats were anesthetized with 3% isoflurane delivered in oxygen, and a CCI was induced by tying four loose ligatures that were ~1 mm apart around one sciatic nerve. Development of mechanical hypersensitivity in the ipsilateral (operated side) hindpaws was assessed using von Frey filaments (Stoelting, Wood Dale, IL, USA) to measure paw withdrawal thresholds (PWT values) presurgery and at 14 days post-CCI surgery in rats.

Assessment of Mechanical Allodynia in the Hindpaws

Specifically, von Frey filaments were used to determine the lowest mechanical threshold required to evoke a brisk paw withdrawal reflex of the rat hindpaw. Briefly, rats were transferred to wire mesh testing cages and allowed to acclimatize for 15–20 minutes prior to von Frey testing. Commencing with the 6-g filament, it was applied to the plantar surface of the hindpaw until the filament buckled slightly. Absence of a response after 3 seconds prompted use of the next filament of increasing force. Conversely, a hindpaw withdrawal response within 3 seconds prompted use of the next filament of decreasing force. A score of 20 g was given to rats that did not respond to any of the von Frey filaments in the range 2–20 g. Mechanical allodynia in the ipsilateral hindpaws of CCI rats was fully developed when von Frey PWTs were ≤6 g. The treatment goal is to increase von Frey PWTs in the ipsilateral hindpaws from ≤6 g to pre-CCI surgery values (~12 g), which represents complete alleviation of mechanical allodynia.

Efficacy Assessment: Test Item Administration in Rodents

CCI rats (300–350 g) with fully developed mechanical allodynia in the ipsilateral hindpaws received single bolus doses of one of EMA200, EMA300, EMA400, or vehicle by the i.p. route. von Frey PWTs were assessed in the hindpaws pre-dose and at the following post-dosing times, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, and 3 hours. Vehicle for dissolution of the test compounds comprised dimethyl sulfoxide (DMSO): sterile water for injection (90:10) for EMA200 and EMA400, and water for injection for EMA300 (sodium salt). CCI rats were administered single i.p. bolus doses of EMA200 at 1, 3, and 10 mg/kg (N = 3–11 per dose); EMA400 at 0.003, 0.01, and 0.03 mg/kg (N = 4 per dose); or vehicle (N = 8) according to a "washout" protocol such that each rat received up to three doses of EMA200, EMA400, or vehicle with each dose separated by a 2- to 3-day washout period. Additionally, other groups of CCI rats received single i.p. bolus doses of EMA300 at 1 and 10 mg/kg (N = 5–6 per dose) or vehicle (N = 3).

Pharmacokinetics and Oral Bioavailability in Rats

Blood Sample Collection and Test Item Administration

EMA200 and EMA300. While rats were anesthetized with 3% isoflurane delivered in oxygen, indwelling polyethylene cannulae were implanted into the femoral artery and jugular vein on the day prior to test item administration. Briefly, rats were administered benzylpenicillin (50,000 IU i.p.) to prevent infection. On the right side, a 1-cm incision was made parallel to the trachea, and blunt dissection was used to expose the bifurcation of the internal and external branches of the jugular vein and a small incision was made. The cannula (1.05 mm O.D. × 0.5 mm I.D.) prefilled with heparinized saline (10 IU/mL) was advanced ~1.5–2 cm into the common jugular vein followed by withdrawal of a small amount of blood to verify correct cannula placement. The cannula was flushed with heparinized saline (200 μL), and a loop of tubing was tied into a subcutaneous (s.c.) pocket at the incisional site to allow adequate flexion during movement. The cannula was exteriorized between the scapulae and protected by a stainless steel spring sutured into an s.c. pocket. A 1-cm transverse incision was used to expose the femoral artery,

and it was carefully separated from the femoral vein and sciatic nerve by blunt dissection. A small incision was made in the bifurcation of the internal and external branches of the iliac artery, and the cannula (0.8 mm O.D. × 0.5 mm I.D.) prefilled with heparinized saline was advanced ~1.5–2 cm into the artery. After a small amount of blood was withdrawn to verify correct placement, the cannula was flushed with heparinized saline. A loop of tubing was tied into an s.c. pocket at the incisional site, and the cannula was exteriorized between the scapulae. The incision was closed using sterile silk sutures. Rats were kept warm during surgical recovery, and cannulae patency was maintained by infusion of normal saline (9 mL/24 hours) until commencement of experimentation the following day.

Blood sampling was via the femoral artery cannula. For animals dosed by the i.v. route, the jugular cannula was used for test item administration. Single bolus doses of EMA200 (7 mg/kg) or EMA300 (10 mg/kg; N = 4) were administered by each of the i.v. and oral routes to groups of male SD rats (300–350 g). For blood sample collection, the first 50 μL was discarded to clear the femoral artery cannula of heparinized saline, and the next 200 μL was collected into heparinized 1-mL polypropylene tubes. Blood samples were collected immediately pre-dose and at 0.083, 0.25, 0.5, 1, 2, 5, 8, and 24 hours post-dosing. Blood samples were kept on ice until centrifugation at 4,000 × *g* for 10 minutes at 4°C. The resulting plasma samples were transferred to 0.5-mL polypropylene tubes and stored frozen at approximately –20°C until analysis.

EMA400. Single bolus doses of EMA400 formulated as a solution in DMSO/Solutol HS15/phosphate-buffered saline were administered by the i.v. route at 1 mg/kg (N = 3) to adult male SD rats (180–250 g) via an implanted jugular cannula [14]. After i.v. dosing, blood samples (300–400 μL) were collected into lithium heparin tubes pre-dose and at 0.083, 0.25, 0.5, 1, 2, 4, 6, and 12 hours post-dosing. Following oral dosing at 5 mg/kg (N = 3) by gastric gavage, the blood sampling times were pre-dose and at 0.25, 0.5, 1, 2, 4, 6, 8, and 12 hours post-dosing [14]. Collected blood samples were kept on ice and centrifuged at 2,500 × g for 15 minutes at 4°C within 1 hour of collection. Plasma samples were transferred to clean, labeled tubes and stored frozen at approximately –20°C until analysis [14].

EMA401. Single bolus doses of EMA401 were administered intravenously to adult male SD rats (300–370 g) via the tail vein at 1 mg/kg on day 1 [15]. One week later, on day 8, the same rats received single oral bolus doses of EMA401 at 10 mg/kg by gavage. Blood samples (~500–600 μL) were collected from five cohorts of animals consisting of three animals/sex bled twice to equal 10 time points [15]. After i.v. dosing, blood samples were collected pre-dose and at 0.083, 0.167, 0.5, 1, 2, 4, 8, 12, and 24 hours post-dosing. Following oral dosing, the blood sampling times were collected pre-dose and at 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, and 24 hours post-dosing [15]. Rats were lightly anesthetized by carbon dioxide/oxygen inhalation for brief intermittent periods to facilitate blood sample collection from the orbital sinus [15]. Blood samples were collected into tubes containing tripotassium ethylenediaminetetraacetic acid and placed on ice. The samples were centrifuged at 4–6°C following completion of sample collection at each interval and were stored at approximately –20°C until shipped on dry ice to Micro-Constants, Inc. (San Diego, CA, USA) for analysis [15].

Plasma concentrations of EMA200, EMA300, EMA400, and EMA401 were quantified using the screening LC-MS/MS assay methods outlined in the following section.

LC-MS/MS Bioanalytical Methods

EMA200

Briefly, a screening LC-MS/MS bioanalytical method for EMA200 (PD123,319) using methadone as the internal standard was developed. Rat plasma samples were assayed in batches, and each batch included a standard curve (0.7–353.5 ng/mL) as well as triplicate low (7 ng/mL), medium (35.4 ng/mL), and high (353.5 ng/mL) quality control samples. The internal standard solution concentration was 100 ng/mL.

Sample Preparation. After thawing, aliquots (50 μL) of rat plasma samples were added to 1.5-mL polypropylene tubes followed by aliquots of the internal standard (50 μL) and deionized water (50 μL), and the tubes were briefly vortex-mixed. After addition of aliquots of acetonitrile (300 μL), the assay tubes were vortex-mixed before being left to stand for 30 minutes at 4°C. Next, the samples were centrifuged, and the supernatants were transferred to fresh 1.5-mL polypropylene tubes before being placed in a Savant evaporative centrifuge until the volume was reduced to ~50 μL. Aliquots (10 μL) of formic acid (1% v/v) were added to each assay tube, and the volume was made up to ~100 μL with deionized water. The samples were transferred to labeled 250 μL polypropylene inserts for analysis. An on-column injection volume of 50 μL was used for the analysis.

Chromatographic Conditions. Chromatographic separation was achieved using an Agilent Zorbax SB-C18 5 μm, 2.1 × 50 mm column, and mobile phase was delivered at 0.20 mL/minute. The mobile phase comprised component A (0.1% v/v formic acid) and component B (0.1% v/v formic acid in 90% v/v acetonitrile : water), and was delivered at 0.20 mL/minute using a stepwise gradient, such that the gradient was initially A : B (100%:0%) for 10 seconds then increased to A : B (60%:40%) for 1.5 minutes, and then to A : B (0%:100%) for 4.4 minutes. Sample analysis was performed by multiple reaction monitoring (MRM) ion monitoring on an Applied Biosystems API3000 triple quadrupole mass spectrometer operating in electrospray mode, using a Turbo Ionspray source, after the LC effluent was split and ~10% introduced into the MS system. The ion transitions used to monitor EMA200 and

the internal standard, methadone, were 509.4/148.1 and 310.2/265.2. Calibration curves constructed using peak area ratios were linear for the concentration range examined. The EMA200 concentrations in the rat plasma samples from the pharmacokinetic study were calculated using inverse prediction from the calibration curve assayed with each batch of samples that were processed.

EMA300

A screening LC-MS/MS bioanalytical method for EMA300 with losartan as the internal standard was developed. Rat plasma samples were assayed in batches, and each batch included a standard curve (1.1–549.5 ng/mL) as well as triplicate quality control samples at 1.1, 11.0, 55.0, and 549.5 ng/mL.

Sample Preparation. Rat plasma samples were thawed, and aliquots (50 μL) were transferred to 1.5-mL polypropylene tubes to which were added aliquots (50 μL) of the internal standard solution (100 ng/mL) followed by 1-methylpiperidine (5 mM). After the tubes were briefly vortex-mixed, aliquots of acetonitrile (300 μL) were added, and the tubes were again vortex-mixed before being left to stand on the bench for ~2–2.5 hours. The tubes were centrifuged at 14,000 rpm for 5 minutes, and the supernatants transferred to clean 1.5-mL polypropylene tubes and evaporated to dryness in a Savant evaporative centrifuge. The residues were reconstituted in aliquots (100 μL) of 10 mM 1-methylpiperidine containing 4.5% (v/v) acetonitrile, briefly vortex-mixed, sonicated for 2 minutes, centrifuged at 3,000 rpm for 2 minutes, and then transferred to labeled 250-μL polypropylene inserts for analysis.

Chromatographic Conditions. Chromatographic separation was performed using an Agilent Zorbax Extend-C18 (2.5 or 5 μm, 2.1 mm ID × 50 mm) column, with Phenomenex Gemini C18 (4.0 × 2.0 mm) SecurityGuard cartridges (Sydney, NSW, Australia) as the pre-column, using a sample injection volume of 50 μL. The mobile phase comprising component A (10 mM 1-methylpiperidine) and component B (10 mM 1-methylpiperidine in 90% [v/v] acetonitrile : water) was pumped at a flow rate of 0.20 mL/minute using gradient conditions such that the mobile phase comprised 95%:5% (A : B) for 2.0 minutes followed by 100% A for 4.1 minutes followed by 95%:5% (A : B) for 4.4 minutes. An Agilent series 1100 binary pump and well plate sampler were used to deliver mobile phase and for sample injection, respectively. The LC effluent was split, and ~10% was introduced into the MS system. Sample analysis was performed by MRM negative ion monitoring using an Applied Biosystems API3000 triple quadrupole mass spectrometer operating in electrospray mode, using a TurboIonSpray source. The ion transitions used to monitor EMA300 and the internal standard were 493.1/299.7 and 420.2/178, respectively. Calibration curves were constructed from the peak area ratios of EMA300 relative to the internal standard. Inverse prediction from the linear standard curve assayed with each batch of rat plasma samples was used to calculate concentrations of EMA300 in rat plasma samples from the pharmacokinetic study.

EMA400

A screening LC-MS/MS bioanalytical method for EMA400 was established at Cerep and used to assay batches of rat plasma samples from the pharmacokinetic study [14]. Each batch of rat samples included a 10-point standard curve (blank and nine concentrations in the range 1.0–5,000 ng/mL) [14].

Sample Preparation. After thawing, aliquots (50 μL) of rat plasma samples containing EMA400 were added to labeled tubes to which were added 200 μL aliquots of acetonitrile [14]. Samples were mixed on a plate shaker for 5 minutes and refrigerated at approximately 4°C for 2 hours to facilitate precipitation of plasma proteins. Samples were centrifuged at 6,000 × *g* for 15 minutes at 4°C, and supernatant aliquots (150 μL) were transferred to clean tubes followed by addition of 100 μL aliquots of de-ionized water and a further period of centrifugation (3,900 × *g* for 15 minutes). Aliquots (40 μL) were injected into the HPLC-MS/MS system [14].

Chromatographic Conditions. Processed plasma samples were analyzed by LC-MS/MS using a Phenomenex Synergi-Max RP column (4 μm, 2.0 mm ID × 50 mm) [14]. The HPLC mobile phase comprised component A (13.3 mM ammonium formate/6.7 mM formic acid in water) and component B (6 mM ammonium formate/3 mM formic acid in water/acetonitrile; 1:9, v/v). The mobile phase gradient was 80%:20% (A : B) for 2.5 minutes, 0%:100% (A : B) for 1 minute, and then 80%:20% (A : B) for a further 1 minute. The mobile phase flow rate was 0.5 mL/minute. Sample analysis was performed by selective ion monitoring using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher, Hudson, NH, USA) operating in positive ion mode with capillary temperature at 325°C and capillary voltage at 4.0 kV [14]. The ion transition for EMA400 was 508.3/314.10. The peak areas for EMA400 for the calibration standards in the range 1–1,000 ng/mL were fitted to an appropriate regression equation. Inverse prediction using peak areas of EMA400 from the calibration curve analyzed with each batch of rat plasma samples was used to interpolate the concentration of EMA400 in the samples from the rat pharmacokinetic study [14].

EMA401

An LC-MS/MS bioanalytical method for EMA401 with EMA401-d7 as the internal standard was developed by MicroConstants, Inc. (San Diego, CA, USA) and used to assay batches of rat plasma samples from the pharmacokinetic study [17]. Each batch of rat samples included a standard curve (2.00–10,000.0 ng/mL) as well as triplicate quality control samples at 6.00, 200.0, and 8,000 ng/mL [17].

Sample Preparation. After thawing, rat plasma sample aliquots (25 μL) containing EMA401 were added to labeled 12 × 75-mm glass culture tubes to which were added aliquots (20 μL) of the internal standard solution (EMA401-d7 at 250.0 μg/mL) to give a final concentration of 500 ng/mL [17]. Samples were vortex-mixed for 2 minutes followed by addition of 0.40 mL of phosphate citrate buffer (pH 6) and 2.5-mL aliquots of hexane : 2-butanol (97.5:2.5 v/v) as the extraction solvent. Samples were vortex-mixed for 5 minutes and then centrifuged at 575 × *g* for 5 minutes. Tubes were covered with aluminum foil and placed in a –70°C freezer for at least 20 minutes. The organic layers were carefully transferred to clean labeled glass tubes (16 × 100 mm) and evaporated to dryness under nitrogen (40°C for ~8 minutes) [17]. Dried sample extracts were reconstituted with aliquots (300 μL) of acetonitrile : water (50:50 v/v), vortex-mixed for 3 minutes, and then transferred into labeled polypropylene injection vials for analysis [17].

Chromatographic Conditions. Plasma sample extracts were analyzed by LC-MS/MS using a Phenomenex Max RP column (4 μm, 2.0 mm ID × 150 mm) maintained at 35°C [17]. An Agilent HP1100 series HPLC system was used, and the mobile phase comprised component A (0.05% acetic acid in water; 1,000:0.5 water: glacial acetic acid v/v) and component B (acetonitrile : methanol : glacial acetic acid v/v [700:300:0.5 v/v/v] at a ratio of 17%:83% (A : B) pumped at 0.25 mL/minute. The sample injection volume was 20 μL. Mobile phase was nebulized using heated nitrogen in a Z-spray source/interface, and the ionized compounds were detected using a tandem quadrupole mass spectrometer operating in electrospray mode with negative ion monitoring (Quattro Ultima, Waters, Beverly, MA, USA). The ion transitions for EMA401 and EMA401-d7 were 506.10/312.10 and 513.20/319.20, respectively [17]. The peak height ratios (EMA401/EMA401-d7) of the calibration standards (2.00–10,000.0 ng/mL) were fitted to an appropriate regression equation using MassLynx™ v4.0 (Waters, Milford, MA, USA). Inverse prediction using peak height ratios of EMA401/internal standard from the calibration curve analyzed with each batch of rat plasma samples was used to interpolate the concentration of EMA401 in the samples from the rat pharmacokinetic study [17].

Data Analysis

Radioligand Binding

The IC_{50} values for EMA200, EMA300, EMA400, EMA401, and EMA402 at each of the cloned rat AT_1 and AT_2 receptors were determined using nonlinear regression as implemented in the GraphPad™ Prism software package (v5.03; GraphPad Software, La Jolla, CA, USA). Additionally, the K_D values for angiotensin II at each of the cloned rat AT_1 and AT_2 receptors were determined using GraphPad Prism (v5.03). Similarly, the IC_{50} values for EMA401, EMA402, and saralasin at each of the cloned human AT_1 and AT_2 receptors were determined using nonlinear regression [13].

Efficacy Data in CCI Rats

In vivo efficacy data are presented as mean (±standard error of the mean [SEM]) PWT vs time curves. For CCI rats, the PWT values were normalized by subtracting pre-dosing baseline values for each individual rat to obtain ΔPWT values as follows:

$$\Delta\text{PWT value} = \text{Post-dosing PWT} - \text{pre-dosing baseline PWT}$$

For individual rats, the extent and duration of pain relief (area under the ΔPWT vs time curves; ΔPWT AUC values) produced by each of the test items was calculated using trapezoidal integration, and these values were normalized to percentage of the maximum possible ΔPWT AUC value. Dose-response curves were generated by plotting mean (±SEM) %MAX ΔPWT AUC values vs log dose. ED_{50} values were estimated using nonlinear regression as implemented in the GraphPad Prism software package (v5.03).

Pharmacokinetic Data in Rats

Following i.v. administration of each of EMA200, EMA300, and EMA400 in rats, the concentrations at time zero (C_0) were determined for individual data sets by extrapolating the natural log linear transformed values for the first two plasma concentrations at 0.083 and 0.25 hours back to 0 hour. For EMA401, C_0 was estimated from the mean values of the first two plasma concentrations at 0.083 and 0.167 hours back to 0 hours. For the time points sampled following oral administration, peak plasma concentrations (C_{max}) and the corresponding times to achieve peak plasma concentrations (T_{max}) were determined by visual inspection of the plasma concentration vs time plots. The value of the last quantifiable plasma concentration was referred to as C_{last}; the time of C_{last} was T_{last}. The area under the plasma drug concentration vs time curve from time zero to the time of C_{last} ($AUC_{0\text{-}t}$) was determined by linear trapezoidal integration.

The elimination rate constant (β) was estimated from three or four points in the terminal linear portion of the natural log-transformed plasma drug concentration vs time curve for each individual data set for EMA200, EMA300, and EMA400, and from the mean data set for EMA401. Estimates of β were not performed on data sets where there were insufficient points (fewer than 3) in the terminal linear portion of the natural log-transformed concentration vs time curve.

The terminal elimination half-life ($t_{1/2\ elim}$) was subsequently calculated from β using the following equation [18]:

$$t_{1/2\ elim} = \ln 2/\beta \quad (1)$$

The area in the tail of the plasma drug concentration vs time curve ($AUC_{t\text{-}\infty}$) was estimated from C_{last} by the following equation [18]:

$$AUC_{t-\infty} = C_{last}/\beta \quad (2)$$

The total area under the plasma drug concentration vs time curve ($AUC_{0-\infty}$) was determined by the addition of AUC_{0-t} and $AUC_{t-\infty}$. The clearance (Cl) was calculated as follows [18]:

$$Cl = dose/AUC_{0-\infty} \quad (3)$$

The apparent volume of distribution (Vd) was determined using the following equation [18]:

$$Vd = Cl/\beta \quad (4)$$

The oral bioavailability (F) of each test item was calculated from the following equation [18]:

$$F = (AUC_{0-\infty}\ \text{oral}/AUC_{0-\infty}\ \text{i.v.}) \times (\text{i.v. dose}/\text{oral dose}) \quad (5)$$

Dose-normalized values of C_0, C_{max}, and $AUC_{0-\infty}$ were calculated by dividing the non-normalized values by the administered dose in mg/kg.

Results

Radioligand Binding

The relative binding affinities of EMA200, EMA300, EMA400, EMA401 (*S*-enantiomer of EMA400), EMA402 (*R*-enantiomer of EMA400), and angiotensin II for cloned rat AT_1 and AT_2 receptors are shown in Table 1. The binding affinity of angiotensin II showed ~10-fold greater selectivity for the AT_2R (K_D = 1.6 nM) *c.f.* the AT_1R (K_D = 14.9 nM) (Table 1) in a manner similar to previous reports [19–21]. Consistent with earlier work by others [19–21], the binding affinity (IC_{50} values) for each of EMA200, EMA300, and EMA400 at the AT_2R was <100 nM (Table 1) showing that their binding was three to four orders of magnitude higher at the AT_2R than at the AT_1R (Table 1). Importantly, the IC_{50} values for EMA401 at the rat and human AT_2 receptors were similar at 39 nM (Table 1). The binding affinities of EMA402 at the rat and human AT_2R were approximately 20- to 30-fold lower (IC_{50} values >800 nM) than the respective values for EMA401 (Table 1). The binding affinities of EMA401 and EMA402 at both the rat and human AT_1R were very low (IC_{50} values >50 μM) (Table 1), reinforcing the AT_2R specificity of these compounds.

Selective AT_2R Antagonists Produce Pain Relief in CCI Rats

Mechanical allodynia was fully developed in the ipsilateral hindpaws of all CCI rats herein by 14 days after CCI surgery in agreement with others [16].

EMA200

In CCI rats with fully developed mechanical allodynia in the ipsilateral hindpaws, single i.p. bolus doses of EMA200 at

Table 1 Radioligand binding affinities and relative selectivities at cloned rat and human AT_2 and AT_1 receptors

	AT_2R		AT_1R		
	Mean	SEM	Mean	SEM	Binding Affinity Selectivity AT_2R/AT_1R
Cloned rat receptors*					
Ligand	K_D (nM)		K_D (nM)		
Angiotensin II	1.6	1.0	14.9	1.1	9.3
	IC_{50} (nM)		IC_{50} (μM)		
EMA200	71.7	14.5	210.5	121.5	~3,000
EMA300	46.5	3.3	49.9	10.2	>1,000
EMA400	75.2	10.0	2,918	1,270	>30,000
EMA401	39.5	5.2	408	335	>10,000
EMA402	804		85.4		106
Cloned human receptors					
Ligand	IC_{50} (nM)		IC_{50} (nM)		
Saralasin‡	0.19		0.48		0.40
	Mean IC_{50} (nM)		Mean IC_{50} (μM)		
EMA401†	39		n.c.		>10,000 but n.c.
EMA402†	1,100		59		53.6

* N = 3–4 separate experiments performed in duplicate except for EMA402 (*R*-enantiomer of EMA400) where N = 2 separate experiments each performed in duplicate.
For the human receptors, N = 1† or 2‡ performed in duplicate.
AT_2R = angiotensin II type 2 receptor; AT_1R = angiotensin II type 1 receptor; n.c. = not calculable; SEM = standard error of the mean.

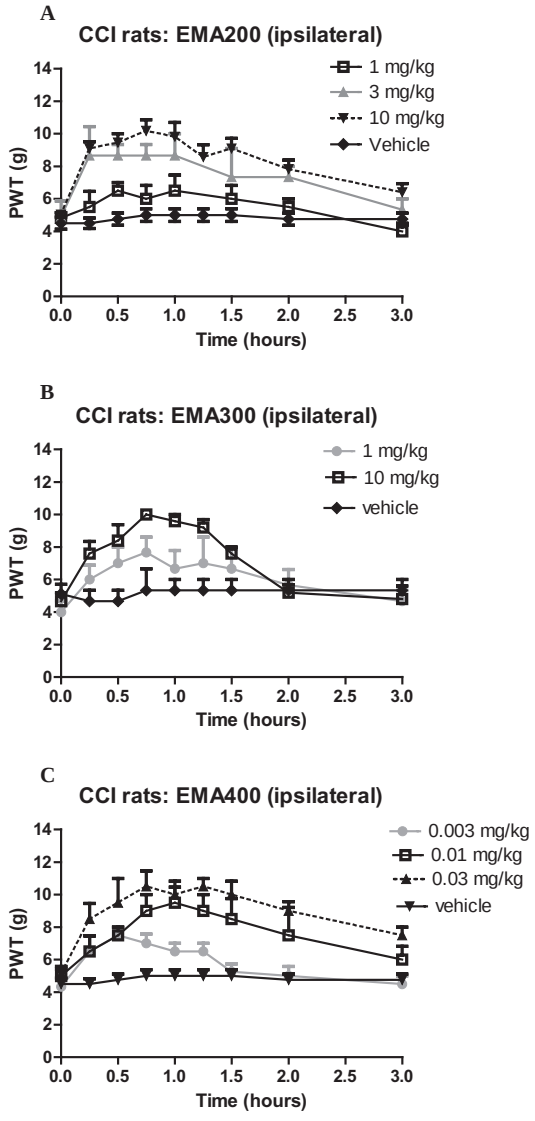

Figure 2 Small molecule angiotensin II type 2 receptor (AT_2R) antagonists produced analgesia in the chronic constriction injury (CCI) rat model of neuropathic pain. Single intraperitoneal (i.p.) bolus doses of the AT_2R antagonists, EMA200 (A), EMA300 (B), and EMA400 (C) produced dose-dependent pain relief in the ipsilateral (injured) hindpaws of CCI rats.

1–10 mg/kg evoked dose-dependent pain relief, whereas vehicle was inactive (Figure 2A). EMA200 pain relief was characterized by a rapid onset of action with mean peak analgesia observed at 30–45 minutes post-dosing, and at the highest dose tested (10 mg/kg), the mean duration of action was >3 hours. The mean ED_{50} was 3.2 (95% confidence interval [CI] 2.0–5.1) mg/kg (Table 2). At the doses tested in CCI rats herein, there were no discernible behavioral side effects.

EMA300

Single i.p. bolus doses of EMA300 at 1 and 10 mg/kg produced dose-dependent relief of mechanical allodynia in the ipsilateral hindpaws of drug-naïve CCI rats, whereas single i.p. bolus doses of vehicle were inactive (Figure 2B). EMA300-mediated anti-allodynia was characterized by a rapid onset of action with mean peak effects observed at ~45 minutes post-dosing (Figure 2B). The mean duration of action was ~2 hours, and there were no discernible behavioral side effects produced. The mean ED_{50} was 0.78 (95% CI 0.08–7.7) mg/kg showing that EMA300 is ~fourfold more potent than EMA200 for producing anti-allodynia in CCI rats (Table 2).

EMA400

Similar to each of EMA200 and EMA300, single i.p. bolus doses of EMA400 at 0.003–0.03 mg/kg produced dose-dependent relief of mechanical hypersensitivity in the ipsilateral hindpaws of CCI rats, whereas vehicle was inactive (Figure 2C). Following administration of bolus doses of EMA400, there was a rapid onset of anti-allodynia in the ipsilateral hindpaws with mean peak analgesia observed at 30–75 minutes post-dosing. At the highest dose tested (0.03 mg/kg), the mean duration of action was ~3 hours, and EMA400 did not evoke discernible behavioral side effects at the doses tested in these animals. The mean ED_{50} was 0.01 (95% CI 0.01–0.02) mg/kg showing that it is ~250-fold more potent than EMA200 and ~60-fold more potent than EMA300 for the relief of mechanical allodynia in the ipsilateral hindpaws CCI rats (Table 2).

Pharmacokinetics and Oral Bioavailability in Rats

The mean (±SEM) plasma concentration vs time curves for each of EMA200, EMA300, EMA400, and EMA401 after i.v. administration to male SD rats are shown in Figure 3A–D. The corresponding derived pharmacokinetic parameters are shown in Table 3.

Table 2 Mean ED_{50} values and 95% confidence intervals for each of EMA200, EMA300, and EMA400 for producing pain relief in the chronic constriction injury rat model of neuropathic pain

	ED_{50} (mg/kg)	
AT_2R Antagonist	Mean	95% Confidence Interval
EMA200	3.22	2.02 to 5.14
EMA300	0.78	0.08 to 7.68
EMA400	0.013	0.008 to 0.021

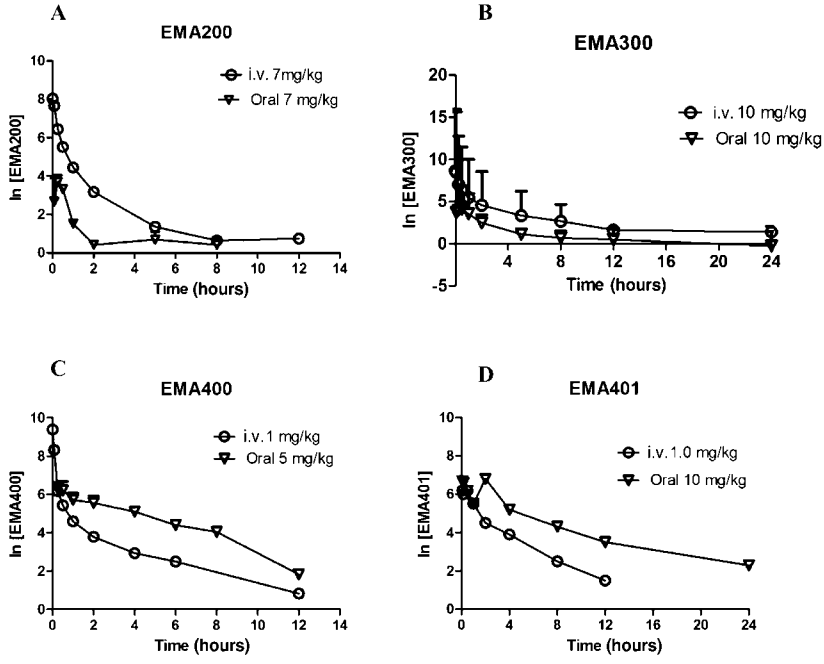

Figure 3 Mean (±standard error of the mean) ln plasma concentration vs time curves for single intravenous (i.v.) and oral bolus doses of EMA200 (A), EMA300 (B), and EMA400 (C) in adult male Sprague-Dawley (SD) rats. Mean ln plasma concentration time vs curves for single i.v. and oral bolus doses of EMA401 (D) in adult male SD rats.

Table 3 Mean (±SEM) pharmacokinetics and oral bioavailability of EMA200, EMA300, EMA400, and EMA401 (*S*-enantiomer of EMA400) in adult male SD rats

Parameter i.v.	EMA200 N = 4	EMA300 ss N = 3	EMA400 N = 3	EMA401 ss N = 3
Dose (mg/kg)	7	10	1.0	1.0
C_0 (ng/mL)	2,837.4 ± 255.7	5,736 ± 1,468	12,216 ± 1,351	590.5
C_0/dose (kg/mL)	405.3	573.6	2,443.2	590.5
K_{elim} (hour^{-1})	0.22 ± 0.04	0.09 ± 0.01	0.12 ± 0.004	0.30
$t_{1/2}$ (hour)	3.6 ± 0.8	8.2 ± 1.0	5.9 ± 0.2	2.3
$AUC_{0\text{-}t}$ (ng.hour/mL)	745.2 ± 31.2	1,889.7 ± 655.1	1,404.9 ± 94.1	868.2
$AUC_{0\text{-}\infty}$ (ng.hour/mL)	758.3 ± 31.2	1,942 ± 671	1,424.9 ± 91.5	907
$AUC_{0\text{-}\infty}$/dose (hour.kg/mL)	108.3	194.2	1,424.9	907
V_d L/kg	47.3 ± 8.0	76.8 ± 30.3	6.0 ± 0.51	3.67
Cl L/hour/kg	9.3 ± 0.4	6.1 ± 1.8	0.71 ± 0.05	1.10
p.o.	N = 4	N = 3	N = 3	N = 3
Dose (mg/kg)	7	10	5	10
C_{max} (ng/mL)	51.4 ± 1.2	67.7 ± 19.1	652 ± 182	885
T_{max} (hour)	0.23 ± 0.1	0.36 ± 0.13	0.33 ± 0.083	2.0
C_{max}/dose (kg/mL)	7.3	6.8	130.4	88.5
K_{elim} (hour^{-1})	n.c.	0.067 ± 0.017	0.153 ± 0.006	0.122
$t_{1/2}$ (hour)	n.c.	11.9 ± 3.3	4.6	5.7
$AUC_{0\text{-}t}$ (ng.hour/mL)	45.2 ± 6.5	123.9 ± 22.6	1,951 ± 275.8	
$AUC_{0\text{-}\infty}$ (ng.hour/mL)	n.c.	138.6 ± 25.3	1,993 ± 269.1	3,010
$AUC_{0\text{-}\infty}$/dose (hour.kg/mL)	6.5*	13.9	398.6	301
F (%)	5.9	7.1	28 ± 3.6	33.2

* Value based on $AUC_{0\text{-}t}$ for EMA200.

AUC = area under the curve; i.v. = intravenous; n.c. = not calculable; p.o. = oral; SD = Sprague-Dawley; SEM = standard error of the mean; ss = sodium salt.

EMA200

After i.v. administration of EMA200 at 7 mg/kg in adult male SD rats, the mean (±) terminal elimination half-life was 3.6 (±0.8) hours, and the mean (±SEM) systemic exposure ($AUC_{0-\infty}$) was 758.3 (±31.2) ng.hour/mL. The mean (±SEM) Vd of EMA200 in rats was high at 47.3 (±8.0) L/kg. The corresponding mean (±SEM) plasma Cl was 9.3 (±0.4) L/hour/kg (Table 3).

Following oral administration of bolus doses of EMA200 in adult male SD rats, the mean (±SEM) C_{max} was 51.4 (±1.2) ng/mL, and the corresponding mean (±SEM) T_{max} was 0.23 (±0.1) hours. The mean (±SEM) systemic exposure was 45.2 (±6.5) ng.hour/mL, and the corresponding mean oral bioavailability was low at 5.9% (Table 3).

EMA300

Following i.v. administration of single bolus doses of EMA300 sodium salt at 10 mg/kg in adult male SD rats, the mean (±SEM) elimination half-life ($t_{1/2}$) was 8.2 (±1.0) hours, and the mean (±SEM) systemic exposure ($AUC_{0-\infty}$) was 1,942 (±671) ng.hour/mL (Table 3). The mean (±SEM) plasma Cl was 6.1 (±1.8) L/hour/kg. The mean (±SEM) Vd was high at 76.8 (±30.3) L/hour/kg (Table 3) in a manner similar to its structural analog, EMA200.

After oral administration of single bolus doses of EMA300 sodium salt at 10 mg/kg in adult male SD rats, the mean (±SEM) T_{max} was 0.36 (±0.14) hours, and the corresponding mean (±SEM) C_{max} was 67.7 (±19.1) ng/mL (Table 3). The mean (±SEM) $t_{1/2}$ was 11.9 (±3.3) hours, and the mean systemic exposure was 123.9 (±22.6) ng.hour/mL. The mean oral bioavailability of EMA300 was low at 7% (Table 3).

EMA400

For male SD rats administered single i.v. bolus doses of EMA400 at 1.0 mg/kg, the mean (±SEM) $t_{1/2}$ was 5.9 (±0.2) hours, and the mean (±SEM) systemic exposure ($AUC_{0-\infty}$) was 1,424.9 (±91.5) ng.hour/mL (Table 3). The mean (±SEM) plasma Cl was 0.71 (±0.05) L/hour/kg, and the corresponding mean (±SEM) Vd was 6.0 (±0.51) L/hour/kg (Table 3). The mean Vd is approximately 8- and 13-fold lower than that for EMA300 and EMA-200, respectively.

After oral administration of bolus doses of EMA400 at 5 mg/kg in male SD rats, the mean (±SEM) C_{max} and T_{max} were 652 (±182) ng/mL and 0.33 (±0.08) hours, respectively, and the mean (±SEM) $t_{1/2}$ was 0.15 (±0.01) hours. The mean (±SEM) systemic exposure was 1,993 (±269.1) ng.hour/mL, and the mean oral bioavailability of EMA400 was 28% (Table 3).

EMA401

In male SD rats administered single i.v. bolus doses of EMA401 sodium salt at 1.0 mg/kg, the mean $t_{1/2}$ was 2.3 hours, and the mean systemic exposure ($AUC_{0-\infty}$) was 907 ng.hour/mL (Table 3). The mean plasma Cl was 1.10 L/hour/kg, and the corresponding mean Vd was 3.67 L/hour/kg (Table 3), which is approximately 10- and 20-fold lower than that for EMA300 and EMA200, respectively.

Following oral administration of EMA401 at 10 mg/kg in male SD rats, the mean C_{max} and T_{max} were 885 ng/mL and 2.0 hours, respectively, and the mean $t_{1/2}$ was 5.7 hours. The mean systemic exposure was 3,010 ng.hour/mL, and the mean oral bioavailability of EMA400 was 33.2% (Table 3).

Comparative Pharmacokinetics of EMA200, EMA300, EMA400, and EMA401 Following i.v. Administration

Although the mean dose-normalized values of C_0 for each of EMA200, EMA300, and EMA401 administered by i.v. bolus were similar, the corresponding mean dose-normalized systemic exposure for each of i.v. EMA400 and EMA401 were 7.3- and 4.7-fold higher than that for EMA300, respectively, and 13.2- and 8.4-fold higher than that for EMA200, respectively (Table 3). The larger systemic exposure of each of i.v. EMA400 and EMA401 relative to EMA300 and EMA200 is underpinned by their order of magnitude lower plasma Cl relative to EMA300 and EMA200, respectively, as well as the smaller mean apparent Vd of EMA400 and EMA401 at 6.0 and 3.67 L/hour/kg, respectively, which are an order of magnitude lower than the corresponding values for EMA200 and EMA300 (Table 3). The mean elimination $t_{1/2}$ for i.v. bolus doses of EMA300 at 8.2 hours was approximately twice that of EMA200 at 3.6 hours, 1.4-fold longer than that of EMA400, and three times longer than that of EMA401 at 2.3 hours (Table 3).

Comparative Pharmacokinetics of EMA200, EMA300, EMA400, and EMA401 Following Oral Administration

After oral administration, the mean dose-normalized values of C_{max} for EMA400 and EMA401 were 10- to 20-fold higher ($P < 0.05$) than the respective values for each of EMA300 and EMA200 ($P < 0.05$) in adult male SD rats. There was a rapid attainment of mean peak plasma concentrations of EMA200, EMA300, and EMA400 after oral dosing, with peak concentrations observed at 15–20 minutes post-dosing. By contrast, mean peak plasma concentrations of EMA401 were not achieved until 2.0 hours after oral dosing in male rats. Additionally, the mean dose-normalized systemic exposure ($AUC_{0-\infty}$) for each of oral EMA400 and EMA401 in male rats was approximately 20- to 30-fold higher than that for EMA300 ($P < 0.05$) and 50- to 60-fold higher than that for EMA200 ($P < 0.05$), consistent with the lower Cl and smaller Vd of each of EMA400 and EMA401 *c.f.* EMA300 and EMA200 in male rats.

Discussion

Here, we show for the first time that selective small molecule antagonists of the AT_2R, viz. EMA200, EAM300 and

EMA400, evoked dose-dependent pain relief in CCI rats, a widely utilized rat model of neuropathic pain. Importantly, our radioligand binding data confirm that these AT_2R antagonists have >1,000-fold higher binding specificity over the AT_1R (Table 1) in agreement with others [19–21]. Comparison of the binding affinities of the *S*- and *R*-enantiomers of EMA400 (EMA401 and EMA402, respectively) shows that the binding affinity of EMA401 is 20- to 30-fold higher than that for EMA402 at the AT_2R with no differences between the rat and human AT_2R (Table 1). The binding affinities of EMA401 and EMA402 at both the rat and human AT_1R were very low (IC_{50} values >50 μM) (Table 1), further emphasizing the AT_2R specificity of these compounds.

Our *in vivo* efficacy data show that single i.p. bolus doses of each of EMA200, EMA300, and EMA400 produced dose-dependent relief of hypersensitivity in the ipsilateral (injured side) hindpaw of CCI rats. Additionally, the analgesic potency of EMA300 was approximately fourfold higher than that of EMA200 and ~60-fold lower than that of EMA400 (Table 2). Importantly, single bolus doses of EMA200, EMA300, or EMA400 did not produce discernible side effects at the doses tested in CCI rats.

Investigation of the pharmacokinetics and oral bioavailability of the aforementioned small molecule AT_2R antagonists in adult male rats showed significant between-analog differences. Specifically, the mean oral bioavailability of EMA200 and its structural analog, EMA300 (sodium salt), are low at 5.9% and 7.1%, respectively. The low oral bioavailability of EMA200 is likely due to incomplete absorption across the gastrointestinal mucosa due to the fully ionized carboxylic acid group at the pH of the upper small intestine. In addition, first-pass N-demethylation of the dimethylamino group in its chemical structure may be a significant contributing factor.

Replacement of the dimethylamino substituent in EMA200 by a methoxy group in EMA300 gave only a marginal improvement in oral bioavailability in male rats. This finding suggests that the low oral bioavailability of EMA300 in rats is likely due to incomplete absorption across the gastrointestinal mucosa as the carboxylic acid group in its structure will be fully ionized at the pH of the upper small intestine in a manner similar to EMA200. Interestingly, the terminal elimination half-life of EMA300 was approximately twofold longer at 8.2 hours *c.f.* 3.6 hours for EMA200. The net result was an ~twofold increase in the dose-normalized systemic exposure of EMA300 *c.f.* EMA200 for both the i.v. and oral dosing routes in male rats. One possible explanation for the higher systemic exposure of EMA300 *c.f.* EMA200 in adult male rats is that O-demethylation of the methoxy group in EMA300 may have occurred at a significantly lower rate than N-demethylation of the corresponding dimethylamino group in the structure of EMA200. The larger systemic exposure of EMA300 *c.f.* EMA200 in adult male rats herein is underpinned by an ~60% increase in the apparent Vd and an ~35% decrease in the plasma Cl (Table 3).

Encouragingly, the oral bioavailabilities of EMA400 and its *S*-enantiomer, EMA401 (sodium salt) in adult male SD rats at 28% and 33%, respectively, were approximately fourfold to fivefold and fivefold to sixfold higher than the respective values for EMA300 and EMA200, which may reflect superior absorption of EMA400/EMA401 across the gastrointestinal mucosa in the rat and/or enhanced metabolic stability *c.f.* EMA200 and EMA300. Importantly, the dose-normalized systemic exposure of EMA400 and EMA401 after oral dosing in male rats was 50- to 60-fold and 20- to 30-fold higher than that of orally administered EMA200 and EMA300, respectively (Table 3). This is underpinned by an order of magnitude lower plasma Cl of EMA400/EMA401 *c.f.* EMA300 and EMA200 in adult male rats together with an order of magnitude smaller Vd of EMA400/EMA401 *c.f.* EMA300 and EMA200.

Together, our data on the analgesic efficacy of single i.p. bolus doses of EMA400 relative to EMA200 and EMA300 in the CCI rat model of neuropathic pain herein show that the analgesic potency of EMA400 is approximately 60-fold higher than that of EMA300 and 250-fold higher than that of EMA200. This analgesic potency rank order of EMA400 > EMA300 > EMA200 in adult male CCI rats is aligned with the markedly higher dose-normalized systemic exposure of single i.v. and oral bolus doses of EMA400 and its *S*-enantiomer EMA401 relative to each of EMA300 and EMA200 in adult male rats.

Of interest, the oral bioavailabilities of the small molecule AT_2R antagonist, EMA400, and its *S*-enantiomer, EMA401, are similar to that of the AT_1R antagonist, losartan, in adult male rats [22], with losartan being used in the clinical setting for the treatment of hypertension in humans [23]. Hence, it is plausible that the oral bioavailability of EMA401 at 33% in the male rat will translate to acceptable oral bioavailability of this compound in humans.

Although angiotensin II signaling via the AT_1R is well known for its role in the regulation of blood pressure [24], angiotensin II signaling via the AT_2R does not modulate blood pressure *in vivo*, and its physiological role remains enigmatic [24]. Here, we show for the first time that several small molecule AT_2R antagonists produced pain relief in a widely utilized rat model of neuropathic pain. Our present findings together with work by others showing that angiotensin II-induced excitability of cultured adult rat DRG neurons is blocked by AT_2R antagonism suggest that small molecule AT_2R antagonists may alleviate neuropathic pain in the clinical setting.

In conclusion, our findings show that potent, small molecule AT_2R antagonists with >1,000-fold selectivity over the AT_1R produced dose-dependent analgesia in the CCI rat model of neuropathic pain. The analgesic potency rank order of EMA400 > EMA300 > EMA200 in nerve-injured rats was aligned with the dose-normalized systemic exposure rank order of these compounds in adult male SD rats.

The high potency and superior pharmacokinetics of EMA400 and its *S*-enantiomer, EMA401, relative to each of EMA200 and EMA300 in rats together with EMA401's high binding selectivity for the AT_2R *c.f.* the AT_1R, underpin the choice of EMA401 as a lead compound for further investigation. EMA401 is currently in clinical development as a novel analgesic for the relief of neuropathic pain in humans.

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Note

1. TIU = tripsin inhibitor unit.

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