JPET Fast Forward. Published on January 11, 2010 as DOI: 10.1124/jpet.109.163816
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Title Page

Pharmacological Properties of MK-3207 [2-[(8*R*)-8-(3,5-difluorophenyl)-10-oxo-6,9-diazaspiro[4.5]dec-9-yl]-*N*-[(2*R*)-2'-oxo-1,1',2',3-tetrahydrospiro[indene-2,3'-pyrrolo[2,3-*b*]pyridin]-5-yl]acetamide], a Potent and Orally Active CGRP Receptor Antagonist

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Running Title Page

Running Title: Pharmacological properties of MK-3207

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Number of text pages:

Number of tables: 1

Number of figures: 8

Number of references: 39

Number of words in abstract: 247

Number of words in introduction: 750

Number of words in discussion: 1,490

Abbreviations: AM, adrenomedullin; AMY, amylin; CIDV, capsaicin-induced dermal vasodilation; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CSF, cerebrospinal fluid; CT, calcitonin; CTR, calcitonin receptor; GPCR, G-protein-coupled receptor; hCGRP, human CGRP; hCLR, human CLR; human RAMP1, hRAMP1; MK-3207, [2-[(8*R*)-8-(3,5-difluorophenyl)-10-oxo-6,9-diazaspiro[4.5]dec-9-yl]-*N*-[(2*R*)-2'-oxo-1,1',2',3-tetrahydrospiro[indene-2,3'-pyrrolo[2,3-*b*]pyridin]-5-yl]acetamide]; RAMP, receptor activity-modifying protein

Recommended section: Neuropharmacology

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Abstract

Calcitonin gene-related peptide (CGRP) has long been hypothesized to play a key role in migraine pathophysiology and the advent of small molecule antagonists have clearly demonstrated a clinical link between blocking the CGRP receptor and migraine efficacy. MK-3207 [2-[(8*R*)-8-(3,5-difluorophenyl)-10-oxo-6,9-diazaspiro[4.5]dec-9-yl]-*N*-[(2*R*)-2'-oxo-1,1',2',3-tetrahydrospiro[indene-2,3'-pyrrolo[2,3-*b*]pyridin]-5-yl]acetamide] represents the third CGRP receptor antagonist to display clinical efficacy in migraine trials. Here we report the pharmacological characterization of MK-3207, a potent and orally bioavailable CGRP receptor antagonist. In vitro, MK-3207 is a potent antagonist of the human and rhesus CGRP receptors (K_i = 0.024 nM). In common with other CGRP receptor antagonists MK-3207 displays lower affinity for CGRP receptors from other species including canine and rodent. As a consequence of species selectivity the in vivo potency was assessed in a rhesus pharmacodynamic assay measuring capsaicin-induced changes in forearm dermal blood flow via laser Doppler imaging. MK-3207 produced a concentration-dependent inhibition of dermal vasodilation with plasma concentrations of 0.8 and 7 nM required to block 50 and 90% of the blood flow increase, respectively. A tritiated analog, [^{3}H]MK-3207, was used to study the binding characteristics on the human CGRP receptor. [^{3}H]MK-3207 displayed reversible and saturable binding (K_D = 0.06 nM) and the off-rate was determined to be 0.012 min^{-1} with a $t_{1/2}$ of 59 min. In vitro autoradiography studies on rhesus brain slices identified the highest level of binding in the cerebellum, brainstem, and meninges. Finally, as an index of CNS penetrability, the in vivo CSF/plasma ratio was determined to be 2-3% in cisterna magna-ported rhesus monkeys.

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Introduction

Migraine is one of the most prevalent and disabling neurological disorders with characteristic symptoms that can last for several days. Despite its severity and high prevalence, migraine is not generally recognized as a serious medical condition and the societal burden is not fully appreciated. Migraine often affects people during their most productive years, which in turn burdens families and employers and ultimately impacts the quality of life of the migraine sufferer. Migraine is generally agreed to be under-diagnosed and many migraineurs do not receive appropriate therapy, indicating there is significant room for improvement in the diagnosis and management of migraine.

An overall improvement in migraine treatment occurred with the introduction of the $5HT_{1B/1D}$ receptor agonists, the triptans, which currently represent the antimigraine therapy of choice. However, some patients do not respond optimally to triptans and some only partially respond. Triptans are considered safe when used appropriately but are contraindicated for patients with cardiovascular disease, because they are direct coronary vasoconstrictors. The next generation antimigraine drugs need to improve upon the shortcomings of triptan therapy.

After successful introduction of the triptans evidence began to mount linking CGRP to the pathophysiology of migraine. It was found that plasma levels of CGRP were elevated during the headache phase of migraine (Gallai et al., 1995; Goadsby et al., 1990) and the levels were normalized concomitantly with pain relief (Goadsby and Edvinsson, 1993). Additionally, intravenous administration of CGRP to migraineurs induced a delayed migraine-like headache (Lassen et al., 1998). These observations suggested antagonism of the CGRP receptor might represent a novel approach to migraine treatment.

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More definitive evidence for a role of CGRP in migraine came from the original proof of concept studies with olcegepant (BIBN4096BS; Doods et al., 2000). In this study intravenous administration of olcegepant was shown to be effective in the acute treatment of migraine (Olesen et al., 2004). Subsequently, we described the identification of a novel, orally bioavailable CGRP receptor antagonist, telcagepant (MK-0974; Paone et al., 2007; Salvatore et al., 2008), which was effective as an acute treatment for migraine with efficacy comparable to zolmitriptan (Ho et al., 2008).

CGRP is a 37 amino acid neuropeptide produced by tissue-specific alternative mRNA splicing of the calcitonin gene (Amara et al., 1982) and belongs to the calcitonin family of peptides which includes calcitonin, amylin, and adrenomedullin. The CGRP receptor is heterodimeric and CGRP activity is mediated by the co-expression of a G-protein-coupled receptor, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein designated receptor activity-modifying protein (RAMP) 1 (McLatchie et al., 1998) and an intracellular protein, receptor component protein (RCP) (Evans et al., 2000). The RAMPs comprise a group of three proteins designated RAMP1, RAMP2, and RAMP3. A functional CGRP receptor requires co-expression of CLR and RAMP1, whereas when CLR is co-expressed with RAMP2 or RAMP3 a receptor with high affinity for adrenomedullin (AM) is produced (McLatchie et al., 1998). Additionally, RAMPs can complex with the calcitonin (CT) receptor to form the amylin family of receptors (Christopoulos et al., 1999; Muff et al., 1999).

The distribution of CGRP receptors in the trigeminovascular system is consistent with a role in migraine pathophysiology. CGRP receptor antagonists could act through both peripheral and central sites of action. Blockade of peripheral receptors on blood vessels

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and mast cells could block neurogenic inflammation and normalize dilated blood vessels, whereas blockade of central receptors in the brainstem may inhibit pain transmission. Additionally, CGRP receptors are widely expressed in numerous brain regions including periaqueductal gray, parabrachial nucleus, nucleus solitaris, cerebellum, hippocampus, and amygdala (Sexton et al., 1986; Sexton, 1991; Christopoulos et al., 1995). Finally, CLR and RAMP1 are located in the spinal trigeminal nucleus of rat and are co-localized in the pre-synaptic terminals of the spinal dorsal horn further indicating a potential role in pain transmission (Lennerz et al., 2009; Marvizon et al., 2007).

The work presented here characterizes the preclinical pharmacology of the novel, potent, and orally bioavailable CGRP receptor antagonist MK-3207 (Bell et al., in press). MK-3207 displayed good oral bioavailability in rats (74%), dogs (67%) and rhesus (41%). In rhesus, clearance and i.v. half-life were moderate with values of 15 mL/min/kg and 1.5 h, respectively. Recently, the efficacy of MK-3207 was evaluated in a Phase II adaptive dose ranging trial for the acute treatment of migraine. In this study MK-3207 significantly improved migraine pain relief 2 h after dosing compared to placebo (Hewitt et al., Oral presentation, 14th Congress of the International Headache Society) further pointing to the clinical utility of this mechanism for a future migraine therapy.

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Methods

Expression Vector Constructs and Mutagenesis. Human CLR, RAMP1, RAMP2, and RAMP3 expression vector constructs were previously described by Salvatore et al. (2008). The expression vector construct for the insert-negative human CT receptor (CTR) was described previously by Salvatore et al. (2006). Rat CLR and RAMP1 expression vector constructs were previously described by Mallee et al. (2002). Human RAMP1 site-directed mutagenesis (tryptophan at position 74 was replaced with an alanine) was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

Cell Culture and Generation of Recombinant Cell Lines. HEK293 and COS-7 cells were cultured in DMEM with 4.5 g/L glucose, 1 mM sodium pyruvate and 2 mM glutamine supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/ml streptomycin, and maintained at 37°C, 5% CO_2, and 95% humidity. Cells were subcultured by treatment with 0.25% trypsin with 0.1% EDTA in HBSS.

HEK293 cell lines stably expressing the human CGRP (CLR/RAMP1), AM_1 (CLR/RAMP2), and AM_2 (CLR/RAMP3) receptors were described previously (Salvatore et al., 2008). For transient transfections, 24 h prior to transfection COS-7 or HEK293 cells were seeded in 500 cm^2 dishes. Transfections were performed by combining 60 μg/dish DNA with 180 μg/dish Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Human CTR was transfected singly in HEK293 cells or co-transfected in COS-7 cells with equal amounts of RAMP1 (AMY_1 receptor) or RAMP3 (AMY_3 receptor). Transfection cocktail was added directly to the medium and the cells were harvested for membranes 48 h post-transfection.

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Membrane Preparation and Radioligand Binding Studies. Transiently or stably transfected cells were washed with PBS and harvested in ice-cold harvest buffer containing 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). The cell suspension was disrupted with a laboratory homogenizer and centrifuged at 48,000g to isolate membranes. Membranes from rat and dog brain were prepared similarly. Rhesus cerebellum was disrupted using a laboratory homogenizer in 10 mM HEPES and 5 mM $MgCl_2$ and used directly in binding experiments. SK-N-MC membranes were purchased from Receptor Biology, Inc. (Beltsville, MD).

CGRP and adrenomedullin competition binding assays were conducted as described previously by Salvatore et al. (2008). Amylin binding assays were conducted by combining MK-3207 and 40 pM ^{125}I-rat amylin (PerkinElmer, Waltham, MA), followed by 25 μg of CTR/RAMP1 or 25 μg of CTR/RAMP3 membranes and incubated for 3 h at room temperature in binding buffer (10 mM HEPES, 5 mM $MgCl_2$, 0.2% bovine serum albumin) in a total volume of 1 mL. Calcitonin binding assays were set up as above but with 25 μg CTR membranes and 30 pM ^{125}I-human calcitonin (PerkinElmer) as the radioligand. Incubations were terminated by filtration through GF/B 96-well filter plates that had been blocked with 0.5% polyethylenimine. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) and the K_i was determined using the equation $K_i = IC_{50}/1 + ([\text{ligand}]/K_D)$. The K_D for each receptor was determined by saturation binding experiments (data not shown).

[^{3}H]MK-3207 Saturation Binding and Association and Dissociation Kinetics. Saturation binding studies were performed by combining increasing concentrations of

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[^{3}H]MK-3207 (specific activity, 73.7 Ci/mmol), 1 μM compound 25 (Stump et al., 2009) for non-specific binding, and 50 μg/well SK-N-MC membrane in a total volume of 1 mL binding buffer. Reactions were incubated overnight (18 h) at room temperature. Association kinetic assays were performed by combining 60 pM [^{3}H]MK-3207 with 50 μg/well SK-N-MC membranes in binding buffer and incubating at room temperature for various times from 1 to 300 min. Dissociation kinetic assays were performed by combining 60 pM [^{3}H]MK-3207 with 50 μg/well SK-N-MC membranes in binding buffer and incubating at room temperature for 3 h. At that point, 1 μM compound 25 was added and dissociation was monitored for various intervals from 1 to 390 min. All assays were terminated by filtration through GF/B 96-well filter plates that had been blocked with 0.5% polyethylenimine.

In Vitro Functional Studies. Functional assays were conducted as described previously (Salvatore et al., 2008). Briefly, HEK293 cells stably expressing the human CGRP receptor were plated at a density of 85,000 cells/well in 96-well poly-D-lysine coated plates approximately 19 h before the assay. Cells were washed with PBS and pre-incubated with various concentrations of MK-3207 in the presence or absence of 50% human serum for 30 min at 37°C in a CO_2 incubator. Isobutyl-methylxanthine (300 μM) was added to the cells and incubated for 30 min at 37°C followed by stimulation with 0.3 nM α-CGRP for 5 min at 37°C. Following agonist stimulation cells were washed with PBS and the intracellular cAMP concentration measured using the cAMP SPA Biotrak Direct Screening Assay (GE Healthcare, Piscataway, NJ). Dose response curves were plotted and IC_{50} values determined. Schild analysis was used as a measure of competitive antagonism by plotting log (DR-1) versus log [B] where DR is the ratio of the EC_{50}

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values in the presence and absence antagonist and [B] is the antagonist concentration. The X-intercept is equal to the pA_2 and the K_B calculated using the formula $pA_2 = -\log K_B$.

Functional assays with HEK293 cells transiently expressing the CGRP receptor were conducted using the CisBio HTRF cAMP dynamic assay kit (CisBio, Bedford, MA). Twenty four h prior to transfection, the cells were plated in 10 cm dishes. For the transfection, 4 μg CLR and 4 μg RAMP1 were combined with 23 μg Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 30 min. The transfection mix was added to the cell medium and incubated for 48 h at 37°C in a CO_2 incubator. Cells transiently expressing CLR and RAMP1 were dissociated, centrifuged, and resuspended in assay buffer consisting of Cellgro COMPLETE Media (Mediatech, Manassas, VA) with 300 μM isobutyl-methylxanthine. Cells were added to a 384-well assay plate (Proxiplate plus, PerkinElmer) containing antagonist at a density of 2000 cells/well and incubated at room temperature for 30 min. An EC_{50} concentration of the peptide agonist CGRP was then added and the plate was incubated at room temperature for 20 min. Finally, HTRF reagents were added to the plate, incubated for 1 h at room temperature, and the plate read using an Envision (PerkinElmer) plate reader in the HTRF mode. The raw data were converted to nM cAMP using a standard curve.

Rhesus Monkey Pharmacodynamic Assay. All procedures related to the use of animals were approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories, West Point, PA and conform with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, National Research Council, revised 1996). Six adult rhesus monkeys (either sex, range 4.8-12.7 kg) were used for both vehicle and active test agent studies. Anesthesia was induced with ketamine (5-

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30mg/kg, i.m., to effect). Animals were then intubated and anesthesia maintained with isoflurane (1-2% carried in 100% oxygen). Animals were placed on a temperature controlled heating blanket and instrumented with a temporary peripheral catheter for the administration of vehicle or test agent. Four rubber O-rings (8 mm inner diameter) were placed on the ventral side of the forearm and positioned such that they were not directly overlying a visible vessel. Dermal vasodilation was induced within the O-rings in sequential order by topical administration of capsaicin (2 mg in 30% ethanol/30% Tween 20/40% water), and quantitated using a laser Doppler imager (Moor Instruments, Ltd., Millwey, Axminster, Devon, UK), according to the study protocols summarized below. In all vehicle and test agent studies, the order in which capsaicin was administered to the four O-rings was varied from study to study.

To determine the effect of MK-3207 on capsaicin dependent dermal vasodilation, each animal was administered three sequential infusion of vehicle or a MK-3207 treatment regimen following a no treatment capsaicin baseline challenge. Animals were allowed at least a 7 day "washout" between studies. Vehicle administration was as follows: Dose 1, iv bolus 0.5 mL 50% DMSO/50% water followed by 25 min continuous iv infusion of 0.025 mL /min 50% DMSO/50% water (0.625 mL); Dose 2, iv bolus 0.5 mL 50% DMSO/50% water followed by 25 min continuous iv infusion of 0.025 mL /min 50% DMSO/50% water (0.625 mL); Dose 3, iv bolus 0.5 mL 100% DMSO followed by 25 min continuous iv infusion of 0.025 mL /min 100% DMSO (0.625 mL). MK-3207 was administered in three different experiments as three dosing regimes to target a range of plasma exposures from 1 – 300 nM. Each regimen was given once to all rhesus, and was as follows: 1) 0.3 µg/kg iv bolus + 0.008 µg/kg/min, then 0.6 µg/kg iv

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bolus + 0.025 μg/kg/min, then 2.1 μg/kg + 0.084 μg/kg/min; 2) 0.6 μg/kg iv bolus + 0.025 μg/kg/min, then 2.1 μg/kg iv bolus + 0.084 μg/kg/min, then 9.1 μg/kg iv bolus + 0.25 μg/kg/min; 3) 9.1 μg/kg + 0.25 μg/kg/min, then 21.2 μg/kg iv bolus + 0.84 μg/kg/min, then 60.6 μg/kg iv bolus + 2.5 μg/kg/min.

Within each test period the dermal blood flow was measured at 20 min post application of capsaicin (i.e. 25 min after initiation of vehicle or test agent administration) as well as baseline: the basal blood flow value acquired in that ring just prior to the dose of vehicle or test agent. Blood samples for determination of plasma concentration of MK-3207 also were obtained at 20 min post application of capsaicin (i.e. 25 min after initiation of vehicle or test agent administration) during each test agent test period. An empirical E_{max} model was then used to describe the PK vs. efficacy relationship of MK-3207 for inhibition of CIDV in rhesus monkeys. Details of the model structure are provided below. Blood flow is described as a baseline blood flow plus an incremental blood flow as a result of CIDV and blockade of CIDV by MK-3207 through an E_{max} relationship. The model was fit to the rhesus CIDV data pooled across all MK-3207 treatment regimes and model parameters for drug efficacy (E_{max} and EC_{50}) were estimated using least square regression method. Two data points at 0.3 μg/kg dose were excluded from the analysis due to plasma concentrations falling below the limit of quantification (LOQ).

The PK versus efficacy relationship was described using the equation $F = F_0 + (F_{caps} - F_0')*(1-E_{max}*C/(EC_{50}+C))$, where F is the measured laser Doppler flow (LDF) at the end of MK-3207 infusion and 20 min after capsaicin challenge, F_0 is the baseline LDF (no capsaicin or MK-3207), F_{caps} is the LDF at 20 min after capsaicin challenge (no MK-

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3207), F_0' is the baseline LDF measured during the capsaicin response test (where F_{caps} was measured, no capsaicin or MK-3207), E_{max} is the maximal % inhibition by MK-3207, C is the plasma concentration of MK-3207, and EC_{50} is the plasma concentration of MK-3207 corresponding to 50% inhibition of CIDV.

In Vitro Autoradiography Studies in Rhesus Monkey. Brain slices (coronal section; 20 µm thickness) were prepared using a Leica cryostat (CM3050) from a fresh frozen rhesus monkey brain. [^{3}H]MK-3207 (specific activity, 73.7 Ci/mmol) was tested at 0.045 nM. Non-displaceable binding was defined by blocking with 1 µM unlabeled MK-3207 using an adjacent slice. Slices were pre-incubated for 15 min in binding buffer (0.9% NaCl, 50 mM Tris-HCl pH 7.5, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) followed by 90 min incubation with radiotracer at room temperature. Slices were washed 3 times (1 min each wash) in ice-cold buffer (0.9% NaCl, 50 mM Tris-HCl pH 7.5) followed by an ice-cold water rinse for 5 s. Slices were air-dried, then exposed to Fuji Phosphor imaging plates (TR2025) for three weeks and scanned with a Fuji BAS 5000 scanner. Image analysis was carried out with MCID software.

Cisterna Magna-Ported Rhesus Monkey Model for Cerebrospinal Fluid Collection. The chronically implanted cisterna magna catheter and port system (CMP) provides for noninvasive collection of uncontaminated CSF samples and the capability for repeat sampling. A customized flexible silicone catheter (SoloCath™) is freely suspended in the cisterna magna, anchored firmly on both sides of the atlanto-occipital membrane, and tunneled subcutaneously to the midscapular region where it is fed into a surgically implanted port body. CSF was accessed by aseptically inserting a needle through the skin and membrane covering the port into the reservoir of the port body

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(Gilberto et al., 2003); blood samples were collected by peripheral venipuncture. Following oral administration of MK-3207 at 10 mg/kg (0.5% methylcellulose, with an adjusted pH ~ 3) to CMP rhesus monkeys, n = 3, CSF and plasma samples were collected at 0.5, 1, 2, 4, 8, and 24 h and analyzed for compound levels.

In Vitro P-glycoprotein Transport Studies. Cells were plated on 96-well filters at 85,000 cells/ 0.17 mL/ well (multiscreen Caco-2 96-well device, porous size 0.40 µm, polycarbonate membrane, Millipore Corporation, Bedford, MA) five days prior to the transport studies. Prior to initiating the transport studies, the culture media was replaced with 10 mM HEPES buffered Hank's balanced salt solution pH 7.4 (HBSS) and then aspirated after 30 min equilibrating incubation. Transport studies were initiated by adding 0.15 mL of HBSS to the receiver side and 0.15 mL HBSS containing 1 µM or 5 µM MK-3207 to the donor side (in which the donor side is the apical compartment for A to B transport and the basolateral side for B to A transport). Directional transport of 1 µM verapamil, a known substrate of P-gp, was evaluated in parallel as a positive control. The plates and filters were placed in a 37 °C incubator for 3 h, after which the filter units were separated from the bottom plates and the samples were collected for analysis. Compound was detected and quantitated via LC/MS/MS using a triple quadripole mass spectrometer. The appearance of compound in the opposite compartment represents the functional activity of P-gp.

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Results

Binding Studies on CGRP Receptors. Competitive binding experiments were carried out to determine the relative affinity of MK-3207 for human, rhesus, rat, and canine CGRP receptors. MK-3207 (Fig. 1) displayed high affinity for the native human CGRP receptor in SK-N-MC cells and for the recombinant human receptor as measured by the ability to compete with ^{125}I-hCGRP binding with K_i values of 0.024 ± 0.001 nM (n = 3) and 0.022 ± 0.002 nM (n = 14), respectively. MK-3207 displayed a similar affinity (K_i) for the rhesus receptor (0.024 ± 0.001 nM; n = 14) as for human, but displayed > 400-fold lower affinity for the canine and rat receptors with values of 10 nM (n = 2) and 10 ± 1.2 nM (n = 4), respectively (Fig. 2).

Binding Studies on Human Adrenomedullin, Calcitonin and Amylin Receptors. Competitive binding experiments were carried out to determine the selectivity of MK-3207 for the human CGRP receptor versus the related human AM, CT, and AMY receptors (Fig. 3). MK-3207 was highly selective versus the human AM_1 (CLR/RAMP2) and AM_2 (CLR/RAMP3) receptors with K_i values of 16,500 nM (n = 2) and 156 ± 17 nM (n = 7), respectively. MK-3207 maintained a high degree of selectivity versus human CTR with a K_i value of 1.9 ± 0.58 μM (n = 5). MK-3207 also displayed good selectivity versus the AMY_3 (CTR/RAMP3) receptor with a K_i value of 128 ± 25 nM (n = 3), but was less selective versus the AMY_1 (CTR/RAMP1) receptor with a K_i value of 0.75 ± 0.13 nM (n = 3).

Saturation and Kinetic Binding Studies with [^{3}H]MK-3207. Saturation binding experiments using [^{3}H]MK-3207 were conducted on SK-N-MC membranes to determine the K_D and B_{max}. [^{3}H]MK-3207 displayed saturable binding with a K_D of 60 pM and B_{max}

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of 350 fmol/mg protein (Fig. 4A). Kinetic binding experiments were performed to determine the on- and off-rates for [^{3}H]MK-3207 on SK-N-MC membranes. In the association assay, [^{3}H]MK-3207 reached saturation very quickly with an apparent k_{on} of 1.5 x 10^9 M^{-1} min^{-1} (Fig. 4B). For the dissociation of [^{3}H]MK-3207, the k_{off} was calculated as 0.012 min^{-1} with a $t_{1/2}$ of 59 min (Fig. 4C).

Functional Studies on the human CGRP Receptor. The effect of MK-3207 on CGRP-induced cAMP production in CLR/RAMP1 cells was investigated. Consistent with the binding data, MK-3207 potently blocked human α-CGRP stimulated cAMP responses in human CGRP receptor expressing HEK293 cells with an IC_{50} of 0.12 ± 0.02 nM (n = 6). Addition of 50% human serum (IC_{50} = 0.17 ± 0.02 nM; n = 6) had little effect on the apparent potency of MK-3207.

Increasing concentrations of MK-3207 caused a dose-dependent rightward shift in the CGRP dose response-curve with no reduction in the maximal agonist response (Fig. 5A). Schild regression (Fig. 5B) yielded a pA_2 value of 10.3 (n = 2; K_B = 0.05 nM).

Functional Studies on Mixed-Species CGRP Receptors. To determine whether the species selectivity exhibited by MK-3207 is derived from RAMP1, hybrid human/rat CGRP receptors were generated by transiently transfecting human CLR with rat RAMP1. Human CLR co-expressed with rat RAMP1, or the human RAMP1 mutant which replaces tryptophan-74 with an alanine, resulted in a similar decrease in potency (pIC_{50} = 8.12 ± 0.1 and 8.66 ± 0.18, respectively (n = 3) versus the wild type human receptor (pIC_{50} = 9.75 ± 0.03; n = 3). MK-3207 displayed significantly lower potency for the rat CGRP receptor with a pIC_{50} = 7.31 ± 0.09 (n = 3; Fig. 6).

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Effect of MK-3207 on Capsaicin-Induced Vasodilation in Rhesus. Administration of vehicle alone resulted in somewhat variable and non-time-dependent changes in CIDV responses that were not considered significant from pre-vehicle infusion CIDV responses. Administration of MK-3207 resulted in an exposure dependent decrease in CIDV. Plasma exposure obtained in this study ranged from less than 1 nM (obtained in the lowest dosing regimen of 0.3 µg/kg iv bolus + 0.008 µg/kg/min for 25 min) to approximately 260 nM (obtained in the highest dosing regimen of 60.6 µg/kg iv bolus + 2.5 µg/kg/min for 25 min). An empirical E_{max} model was used to describe the PK vs. efficacy relationship of MK-3207 for inhibition of CIDV in rhesus monkeys. The results suggest that MK-3207 has EC_{50} and E_{max} values of approximately 0.8 nM (± SE: 0.3 nM) and 81% (± SE: 5%), respectively, for inhibition of CIDV in rhesus monkeys. The expected EC_{90} value in rhesus is therefore about 7 nM (9-fold higher than the estimated EC_{50} value). The model fit is presented in Fig. 7.

Autoradiographic Studies in Rhesus Monkey Brain. MK-3207 was labeled with tritium to high specific activity, and used for in vitro autoradiographic studies for binding site localization in rhesus monkey brain slices. The [^{3}H]MK-3207 concentration selected for these studies was 0.045 nM, about 2-fold greater than its affinity value (K_i = 0.024 nM). As shown in Fig. 8B, [^{3}H]MK-3207 displayed minimal non-displaceable binding, defined on an adjacent brain slice by 1 µM self block. In the absence of competing unlabeled ligand, high binding densities were observed in several brain regions examined, including the cerebellum, brainstem, and meninges (Fig. 8A). In the cerebellum, high binding density is mainly located in the gray matter such as cerebellar cortex, with minimal binding in the white matter, consistent with receptor localization in the

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cerebellum. In the brainstem, high density binding sites are non-uniformly distributed to regions essential for processing nociceptive stimuli. Interestingly, [^{3}H]MK-3207 also showed high binding density in the meninges, but minimal binding to the cerebral cortex, providing evidence that a high density of CGRP receptors are localized in the meninges. [^{3}H]MK-3207 binding sites were also observed in the hypothalamus and hippocampus with minimal binding in the caudate putamen (data not shown; Sur et al., 2009).

MK-3207 Cerebrospinal Fluid Levels in Rhesus Monkey. Phamacokinetic parameters were determined in CSF and plasma following oral dosing in cisterna magna-ported rhesus monkey. Following an oral dose of 10 mg/kg MK-3207 the CSF/plasma ratio is 2-3% (Table 1). However, the CSF/plasma ratio is approximately 30% of the unbound fraction in plasma indicating that the central and peripheral compartments are not freely equilibrating.

In Vitro P-glycoprotein Transport Studies. MK-3207 is a substrate for human and mouse P-gp at 1 and 5 µM, as indicated by B-to-A/A-to-B transport ratios of 33 and 50 in L-MDR1 and L-mdr1a cell lines, respectively. MK-3207 has high passive permeability of 24 x 10^{-6} cm/s (Table 1).

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Discussion

Approximately 20 years ago CGRP was first postulated to play a role in the pathophysiology of migraine. It is now evident that CGRP is not simply a migraine biomarker but is an important player in migraine pathogenesis. Three CGRP receptor antagonists (olcegepant, telcagepant, and MK-3207) have displayed efficacy in the treatment of migraine and in this report we detail the pharmacological characterization of MK-3207, the second orally bioavailable CGRP receptor antagonist to be evaluated in the clinic for the acute treatment of migraine. One potential benefit of the new CGRP receptor antagonist class of antimigraine treatments is the absence of vasoconstriction (Petersen et al, 2003; Lynch et al., submitted), a current liability of the triptans. Telcagepant, the first orally bioavailable CGRP receptor antagonist tested in clinical trials, has been shown to be effective as an acute treatment of migraine with efficacy comparable to that of zolmitriptan (Ho et al., 2008). MK-3207, our second orally bioavailable antagonist, is structurally distinct from telcagepant (Fig. 1) and is 50- to 100-fold more potent both in vitro and in vivo. In an adaptive dose ranging trial MK-3207 demonstrated a positive response on the primary 2-h pain freedom endpoint and the secondary endpoint of 2-h pain relief.

Numerous antagonists of the CGRP receptor exhibit species-selective pharmacology (Doods et al., 2000; Edvinsson et al., 2001; Hasbak et al., 2001) and MK-3207 is no exception. MK-3207 displays approximately 400-fold higher affinity for the human and rhesus CGRP receptors compared to the rat and canine receptors. We previously identified RAMP1 as the primary driver of the species selectivity of olcegepant and telcagepant (Mallee et al., 2002; Salvatore et al., 2008). Additionally, we identified W74 of hRAMP1 as a key residue for olcegepant binding (Mallee et al., 2002) and W74 was shown to have a similar effect on telcagepant (Miller et al., 2010). Co-expression of the W74A hRAMP1 mutant or rRAMP1 with hCLR resulted in a 10- to 40-fold reduction in potency implicating RAMP1, specifically residue 74, as playing a key role in MK-3207 binding. The potency of MK-3207 is further reduced on the rat CGRP receptor, suggesting that both RAMP1 and CLR are involved in binding.

CLR can heterodimerize with RAMP2 and RAMP3 to produce high affinity adrenomedullin receptors. Adrenomedullin is a widely expressed, potent vasodilator which, when knocked-out in mice, results in death at mid-gestation due to extreme hydrops fetalis and cardiovascular abnormalities (Caron and Smithies, 2001). Studies to determine which RAMP is responsible for survival identified RAMP2 as being important for survival (Dackor et al., 2007; Ichikawa-Shindo et al., 2008); whereas the absence of RAMP3 had little or no effect until old age (Dackor et al., 2007). Based upon these observations it was important to maintain selectivity for the CGRP receptor versus the related adrenomedulin receptors, most notably AM_1. Since adrenomedullin receptors contain CLR we initially thought that developing highly selective antagonists would be challenging. However, the RAMP1-dependence displayed by MK-3207 provided selectivity for the CGRP receptor versus both AM_1 (> 600,000-fold selective) and AM_2 (6,500-fold selective) receptors.

RAMPs can also complex with the related calcitonin receptor (CTR) to form the amylin (AMY) family of receptors (Christopoulos et al., 1999; Muff et al., 1999). The most well characterized AMY receptors are heterodimers of CTR and RAMP1 (AMY_1) and RAMP3 (AMY_3). MK-3207 displayed excellent selectivity versus the AMY_3

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receptor (> 5,000-fold selective), but that selectivity was greatly reduced against the AMY_1 receptor. Perhaps this observation should not be surprising since the AMY_1 receptor requires co-expression of RAMP1. The RAMP1-dependence which may have helped confer selectivity versus the AM receptors, could also be responsible for the reduced selectivity seen against the AMY_1 receptor.

MK-3207 was evaluated in a screen of 169 enzyme and binding assays (MDS Pharma Services,Taipei, Taiwan; partial list of assays is summarized in the supplemental data). MK-3207 was highly selective for the CGRP receptor with no activity (IC_{50} < 5 μM) in any assay except for the human calcitonin receptor (IC_{50} = 1.75 μM), which is consistent with our in-house determination.

To better understand the binding characteristics of MK-3207 a tritiated analog, [^{3}H]MK-3207, was used to define MK-3207 affinity and binding kinetics. Binding was assessed on membranes from the SK-N-MC cell line which constitutively expresses the human CGRP receptor (Semark et al., 1992). [^{3}H]MK-3207 binding was found to be saturable with an apparent K_D of 60 pM which is in agreement with the K_i values for unlabeled MK-3207 in competition studies using [^{125}I]CGRP as the radioligand. The association of [^{3}H]MK-3207 to SK-N-MC membranes was fast with equilibrium reached quickly, but the dissociation was significantly slower ($t_{1/2}$ = 59 min) compared to telcagepant ($t_{1/2}$= 1.3 min; Moore et al., 2009), a likely consequence of the greatly enhanced affinity for the CGRP receptor. The high affinity (K_D) and slower dissociation kinetics of MK-3207 could result in a low dose being required for migraine efficacy, but this can only be answered through large clinical efficacy trials.

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The CGRP receptor is a Class B GPCR which are characterized by having peptide agonists which occupy extensive binding sites. Due to the putative large agonist binding site there is some discussion centered on whether an antagonist of Class B receptors can be strictly competitive. We have previously shown that telcagepant displays behaviors consistent with competitive antagonism (Salvatore et al., 2008; Moore et al. 2009) and therefore interrogated MK-3207 in a similar fashion. Increasing concentrations of MK-3207 caused parallel rightward shifts in the CGRP dose-response curves in a cAMP functional assay and the dose-ratio plot displays a straight line with a slope of 1.1 and a pA2 of 10.3. These behaviors are consistent with competitive antagonism; however it is difficult to determine conclusively whether MK-3207 is strictly a competitive antagonist.

The pronounced species-selectivity exhibited by MK-3207 required the utilization of nonhuman primate to assess in vivo pharmacological activity. Therefore, pharmacological studies were conducted in rhesus monkey based upon capsaicin-induced dermal vasodilation (Hershey et al., 2005). Topical application of capsaicin to the rhesus forearm resulted in an increase in dermal blood flow, a direct result of endogenous CGRP release, which is directly measurable via laser Doppler imaging. MK-3207 produced a concentration-dependent inhibition of capsaicin-induced dermal blood flow in the rhesus forearm, affording EC_{50} and EC_{90} values of 0.8 and 7 nM, respectively. MK-3207 (IC_{50} = 0.17 nM) is approximately 65-fold more potent than telcagepant (IC_{50} = 10.9 nM) in the human serum-shifted in vitro functional assay. This in vitro gain in functional potency is maintained upon translation to in vivo activity where MK-3207 is approximately 100-fold more potent in the rhesus CIDV assay versus telcagepant (EC_{90} = 994 nM).

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The prevailing view is that migraine is a neurological disorder, where the primary site of dysfunction resides in the brain. Numerous lines of preclinical and clinical evidence support this hypothesis including: (1) brainstem activation during a migrainous attack (Weiller et al., 1995; Afridi et al., 2005); (2) peripheral application of CGRP to the meningeal dural mater caused an increase in blood flow in rats, but did not sensitize meningeal nociceptors (Levy et al., 2005); and (3) intravenous infusion of vasoactive intestinal peptide (VIP) to migraineurs caused a marked dilation in cranial arteries, but did not induce migraine (Rahmann et al., 2008). Although these observations are intriguing, the interpretation of the clinical migraine efficacy results with CGRP receptor antagonists is not clear cut. To better define potential central nervous system (CNS) sites of action for MK-3207, in vitro autoradiography mapping studies were conducted in rhesus brain slices. Highest expression of [^{3}H]MK-3207 binding sites were found in the cerebellum, brainstem, and meninges. In the cerebellum, high binding density is mainly located in the gray matter with minimal binding in the white matter. In the brainstem, high density binding sites are non-uniformly distributed to regions essential for processing nociceptive stimuli. The next question is does MK-3207 get into the brain at levels which can block the central CGRP receptor binding sites at clinically relevant plasma concentrations? The in vivo CSF level of MK-3207 was evaluated in cisterna magna-ported rhesus monkeys as a surrogate to the clinical experience. A CSF/plasma ratio following oral dosing was computed as an index of CNS penetrability. The CSF/plasma ratio was approximately 2% based upon C_{max} values (20 nM CSF; 979 nM plasma), suggesting MK-3207 has brain penetration potential. At face value one could conclude MK-3207 is CNS-penetrant and therefore significantly engaging central

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receptors. This data must be interpreted carefully since MK-3207 is a Pgp-substrate, and therefore CSF levels cannot be equated to receptor occupancy.

In conclusion, we have identified a structurally novel, potent, selective, and orally bioavailable CGRP receptor antagonist. Although the exact mechanism linking CGRP and migraine is yet to be identified, it is quite clear that CGRP is not simply a migraine biomarker, but is a key player in migraine pathogenesis. Three CGRP receptor antagonists to date have demonstrated clinical efficacy for the treatment of migraine and considered together offers hope for a highly effective new therapy for migraine sufferers.

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Legends for Figures

Fig. 1. Chemical structure of MK-3207 and telcagepant.

Fig. 2. Concentration-dependent inhibition of ^{125}I-hCGRP binding by MK-3207 from SK-N-MC, human (cloned), rhesus cerebellum, dog brain, and rat brain membranes. Mean values ± S.E.M.

Fig. 3. Concentration-dependent inhibition of ^{125}I-rat amylin (AMY_1 and AMY_3), ^{125}I-human calcitonin (CT), or ^{125}I-human adrenomedullin (AM_1 and AM_2) binding by MK-3207 from stably or transiently expressing cell membranes. Mean values ± S.E.M.

Fig. 4. Saturation binding and association / dissociation kinetics of binding of [^{3}H]MK-3207. A, Saturation binding curve for [^{3}H]MK-3207 to 50 µg/well SK-N-MC membranes. Symbols (■ total binding; ▲ non-specific binding; ● specific binding) and error bars represent mean and standard deviation from 5 separate experiments. B, Association kinetics of [^{3}H]MK-3207 (60 pM) binding to 50 µg/well SK-N-MC membranes at room temperature for 300 min. Symbols and error bars represent the mean and standard deviation from 5 replicates. C, Dissociation kinetics of [^{3}H]MK-3207 (60 pM) binding to 50 µg/well SK-N-MC membranes. Incubations proceeded for 3 h at room temperature and dissociation monitored for 390 min after the addition of compound 25. Symbols and error bars represent the mean and standard deviation from 8 replicates.

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Fig. 5. Concentration-response curve and Schild plot analysis of MK-3207. A, Concentration response curves of CGRP-induced cAMP production in HEK293 cells stably expressing human CLR/RAMP1 in the absence or presence of increasing concentrations of MK-3207 (n = 2). B, Schild plot showing the effect of MK-3207 on cAMP production in HEK293 cells stably expressing human CLR/RAMP1.

Fig. 6. Antagonism of CGRP-induced cAMP production in HEK293 cells transiently expressing CLR and RAMP1 by various concentrations of MK-3207. Experiments were conducted 48 h after transfection of HEK293 cells with (●) human CLR + human RAMP1 (hCLR/hRAMP1); (□) human CLR + rat RAMP1 (hCLR/rRAMP1); (▲) human CLR + human W74A RAMP1 (hCLR/hW74A); and (▼) rat CLR + rat RAMP1 (rCLR/rRAMP1). Mean values ± S.E.M. of combined data from at least three separate experiments.

Fig. 7. Effects of MK-3207 on capsaicin-induced dermal vasodilation in rhesus monkey. Administration of MK-3207 resulted in an exposure dependent decrease in capsaicin-induced dermal vasodilation in the rhesus forearm. An empirical E_{max} model was used to describe the PK vs. efficacy relationship of MK-3207 for inhibition of CIDV in rhesus monkeys. The results suggest that MK-3207 has EC_{50} and E_{max} values of approximately 0.8 nM (± SE: 0.3 nM) and 81% (± SE: 5%), respectively, for inhibition of CIDV in rhesus monkeys.

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Fig. 8. Distribution of [^{3}H]MK-3207 binding to rhesus coronal brain slices. A, [^{3}H]MK-3207 (0.045 nM) displayed high binding densities in the cerebellum, brainstem, and meninges. B, [^{3}H]MK-3207 displayed minimal non-displaceable binding in the presence of 1 μM unlabeled MK-3207 on an adjacent brain slice.

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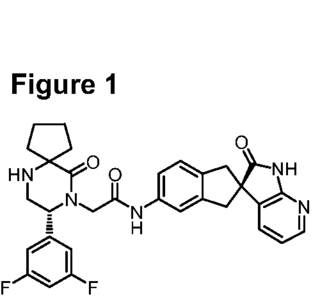
Tables

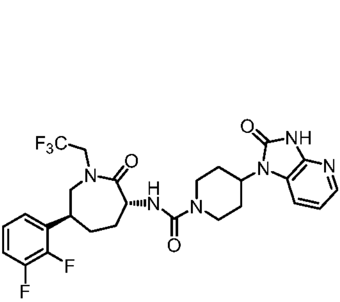
Table 1. Comparison of in vitro and in vivo CNS penetration properties of MK-3207. MK-3207 was dosed orally to cisterna magna-ported rhesus monkeys at 10 mg/kg in 0.5% methylcellulose. Plasma and CSF samples were collected at 0.5, 1, 2, 4, 8, and 24 h post dose. Data are represented as mean ± S.E.M. of three separate experiments.

	C_{max} (nM)	$AUC_{0\text{-}24\ h}$ (nM·h)
CSF	20.0 ± 13.5	96.4 ± 41.8
Plasma	979 ± 570	3285 ± 1205
CSF/Plasma Ratio	2.0%	2.9%
Rhesus Plasma Unbound Fraction	9.4%	
P-gp (BA/AB)		
Human	33	
Mouse	50	
P_{app} (10^{-6} cm/s)	24	

Figure 1

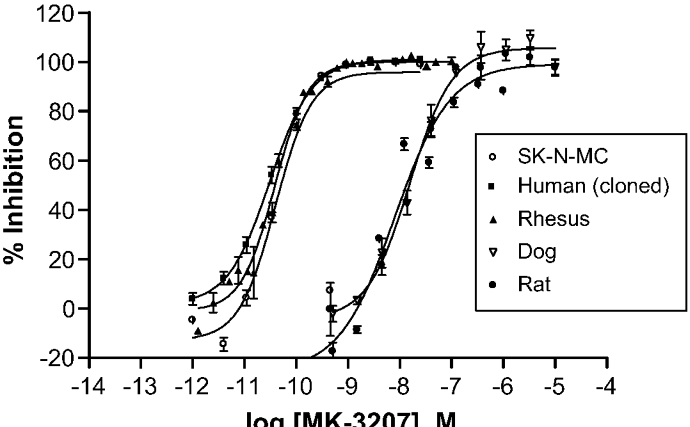


MK-3207



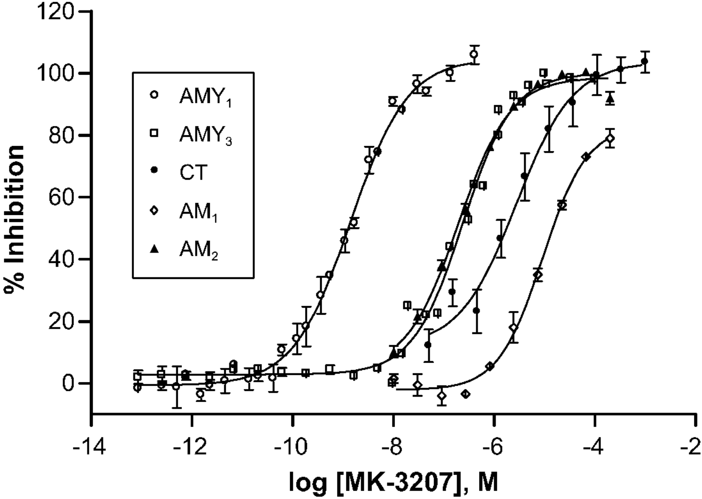
telcagepant

Figure 2



% Inhibition
120
100
80
60
40
20
0
-20
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [MK-3207], M
SK-N-MC
Human (cloned)
Rhesus
Dog
Rat

Figure 3



% Inhibition
120
100
80
60
40
20
0
-14
-12
-10
-8
-6
-4
-2
log [MK-3207], M
AMY1
AMY3
CT
AM1
AM2

Figure 4

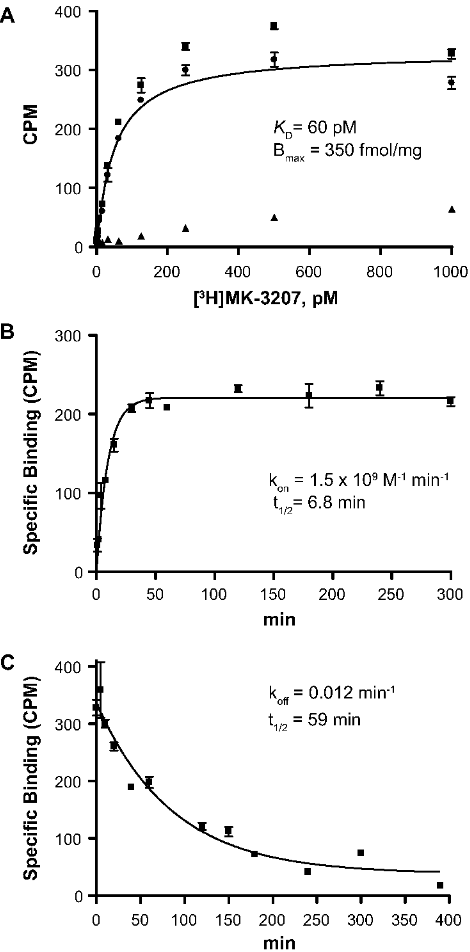

A
400
300
200
100
0
CPM
KD= 60 pM
Bmax = 350 fmol/mg
0
200
400
600
800
1000
[3H]MK-3207, pM
B
300
200
100
0
Specific Binding (CPM)
kon = 1.5 x 109 M-1 min-1
t1/2= 6.8 min
0
50
100
150
200
250
300
min
C
400
300
200
100
0
Specific Binding (CPM)
koff = 0.012 min-1
t1/2 = 59 min
0
50
100
150
200
250
300
350
400
min

Figure 5

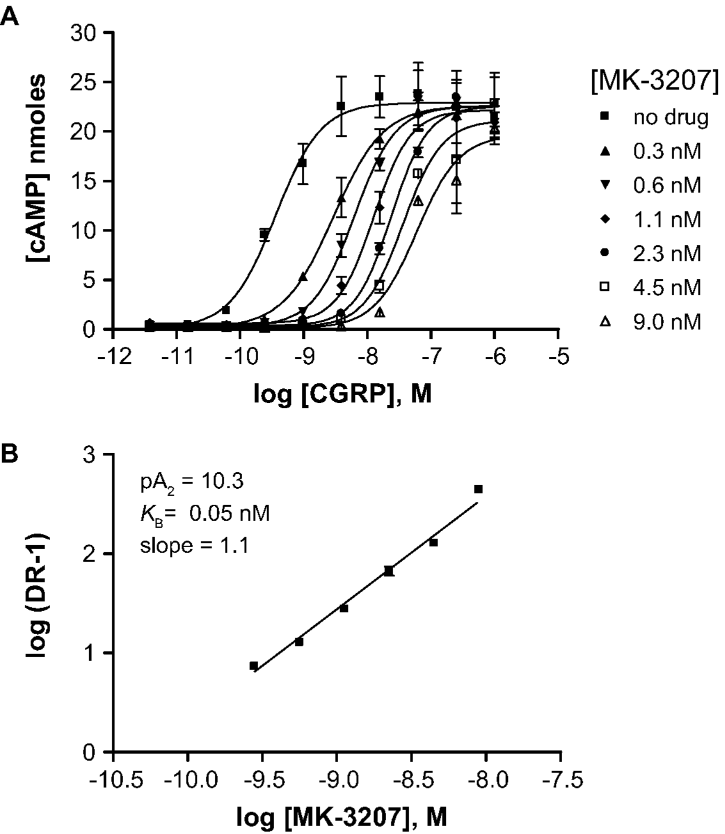
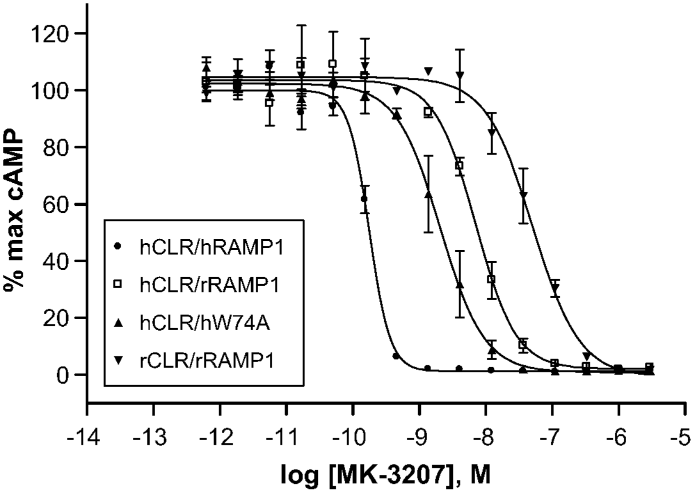
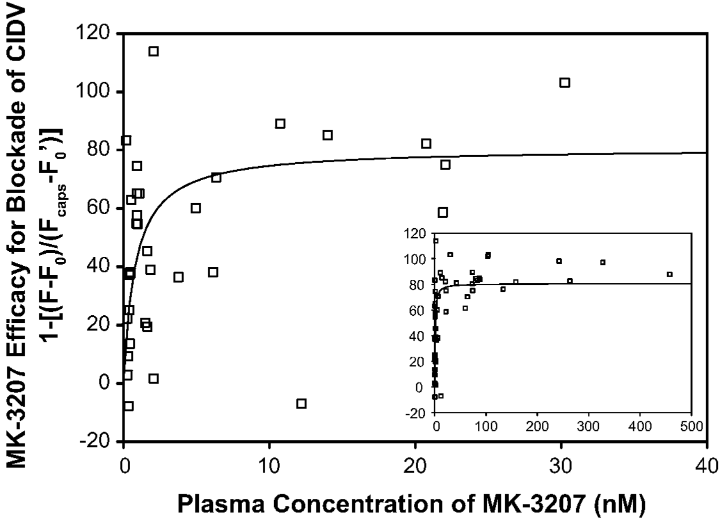

A
[cAMP] nmoles
log [CGRP], M
[MK-3207]
no drug
0.3 nM
0.6 nM
1.1 nM
2.3 nM
4.5 nM
9.0 nM
B
log (DR-1)
log [MK-3207], M
pA2 = 10.3
KB= 0.05 nM
slope = 1.1

Figure 6



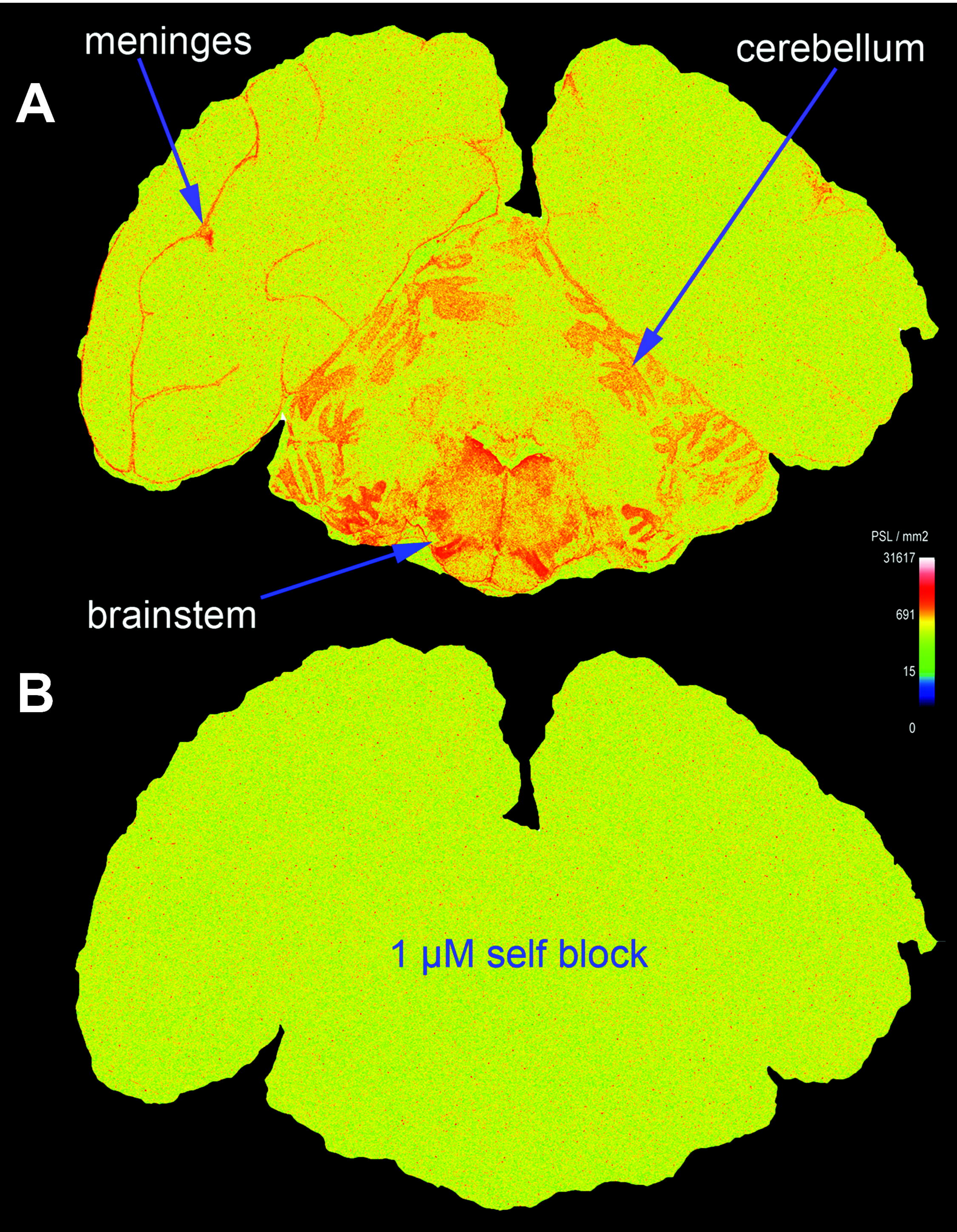
% max cAMP
120
100
80
60
40
20
0
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
log [MK-3207], M
hCLR/hRAMP1
hCLR/rRAMP1
hCLR/hW74A
rCLR/rRAMP1

Figure 7



MK-3207 Efficacy for Blockade of CIDV
1-[(F-F0)/(Fcaps-F0'))]
120
100
80
60
40
20
0
-20
0
10
20
30
40
Plasma Concentration of MK-3207 (nM)
120
100
80
60
40
20
0
-20
0
100
200
300
400
500

Figure 8


A
meninges
cerebellum
brainstem
B
1 μM self block
PSL / mm2
31617
691
15
0