Pharmacological Characterization of MK-0974 \([N-(3R,6S)-6-(2,3\text{-difluorophenyl})-2\text{-oxo-1-(2,2,2-trifluoroethyl)}azepan-3-yl]-4-(2\text{-oxo-2,3-dihydro-1H-imidazo}[4,5-b]pyridin-1-yl)piperidine-1-carboxamide]\), a Potent and Orally Active Calcitonin Gene-Related Peptide Receptor Antagonist for the Treatment of Migraine


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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a potent neuropeptide that plays a key role in the pathophysiology of migraine headache. CGRP levels in the cranial circulation are increased during a migraine attack, and CGRP itself has been shown to trigger migraine-like headache. The correlation between CGRP release and migraine headache points to the potential utility of CGRP receptor antagonists as novel therapeutics in the treatment of migraine. Indeed, clinical proof-of-concept in the acute treatment of migraine was demonstrated with an intravenous formulation of the CGRP receptor antagonist BIBN4096BS (olcegepant). Here we report on the pharmacological characterization of the first orally bioavailable CGRP receptor antagonist in clinical development, MK-0974 \([N-(3R,6S)-6-(2,3\text{-difluorophenyl})-2\text{-oxo-1-(2,2,2-trifluoroethyl)}azepan-3-yl]-4-(2\text{-oxo-2,3-dihydro-1H-imidazo}[4,5-b]pyridin-1-yl)piperidine-1-carboxamide]\). In vitro, MK-0974 is a potent antagonist of the human \((K_i = 0.77 \text{nM})\) and rhesus \((K_i = 1.2 \text{nM})\) CGRP receptors but displays \(\leq 1500\)-fold lower affinity for the canine and rat receptors as determined via \(^{125}\text{I}\)-human CGRP competition binding assays. A rhesus pharmacodynamic assay measuring capsaicin-induced changes in forearm dermal blood flow via laser Doppler imaging was utilized to determine the in vivo activity of CGRP receptor antagonism. MK-0974 produced a concentration-dependent inhibition of dermal vasodilation, generated by capsaicin-induced release of endogenous CGRP, with plasma concentrations of 127 and 994 nM required to block 50 and 90% of the blood flow increase, respectively. In conclusion, MK-0974 is a highly potent, selective, and orally bioavailable CGRP receptor antagonist, which may be valuable in the acute treatment of migraine.

CGRP is a 37 amino acid neuropeptide produced by tissue-specific alternative mRNA splicing of the calcitonin gene (Amara et al., 1982) and is a member of the calcitonin family of peptides, which includes calcitonin, amylin, and adrenomedullin. CGRP activity is mediated by the coexpression of a G-protein-coupled receptor, calcitonin receptor-like receptor, a single transmembrane-spanning protein designated receptor activity-modifying protein (RAMP) 1 (McLatchie et al., 1998), and an intracellular protein, receptor component pro-

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; hCGRP, human CGRP; MK-0974, \([N-(3R,6S)-6-(2,3\text{-difluorophenyl})-2\text{-oxo-1-(2,2,2-trifluoroethyl)}azepan-3-yl]-4-(2\text{-oxo-2,3-dihydro-1H-imidazo}[4,5-b]pyridin-1-yl)piperidine-1-carboxamide]; RAMP, receptor activity-modifying protein; BIBN4096BS, olcegepant; HEK, human embryonic kidney; PBS, phosphate-buffered saline; TRPV1, transient receptor potential vanilloid subfamily member 1.
tein, required for G-protein signal transduction (Evans et al., 2000). In addition to forming functional CGRP receptors, the calcitonin receptor-like receptor (CLR) can form a high-affinity adrenomedullin receptor through heterodimerization with RAMP2 or RAMP3 (McLatchie et al., 1998).

Although a complete understanding of the pathogenesis of migraine is not clear, several lines of evidence in migraineurs support a role of CGRP as a key mediator in the pathophysiology of migraine. CGRP levels in the cranial circulation are increased during a migraine attack (Goadsby et al., 1990), and intravenous administration of CGRP to migraineurs induced a delayed migraine headache in some patients (Lassen et al., 1998). Nitroglycerine-induced migraine, which is clinically very similar to spontaneous attacks, is also characterized by increased levels of CGRP in plasma (Juhasz et al., 2003). Furthermore, successful treatment of migraine headache pain with the 5-hydroxytryptamine1B/1D agonist sumatriptan resulted in the normalization of CGRP levels (Goadsby and Edvinsson, 1993). Finally, compelling evidence for a role of CGRP in migraine was obtained with the demonstration that intravenous administration of the potent CGRP receptor antagonist olcegepant (BIBN4096BS) was effective in the acute treatment of migraine (Olesen et al., 2000). MK-0974 displayed good oral bioavailability in rats (20%) and dogs (35%). In rats, clearance was low (9.4 ml/min/kg) with a moderate i.v. half-life (1.6 h) and a short oral $T_{\text{max}}$ (0.67 h). In dogs, clearance was moderate at 17 ml/min/kg. Recent clinical results from a Phase 2 study showed that MK-0974 significantly improved migraine pain relief 2 h after dosing compared to placebo, and the relief was sustained throughout 24 h (Ho et al., 2007). The present study examines the in vitro and in vivo pharmacological profile of MK-0974.

Materials and Methods

Expression Vector Constructs. Human cDNA for CLR was provided by Dr. Douglas MacNeil (Merck Research Laboratories, Rahway, NJ) and subcloned as a NheI-Pml fragment into pIREShyg2 (Clontech, Mountain View, CA), which had been digested with Nhel and EcoRV. Human RAMP1, RAMP2, and RAMP3 cDNAs were provided by Dr. Bruce Dougherty (Merck Research Laboratories, Rahway, NJ) and subcloned as Nhel-NotI fragments into pIRE-Spuro2 (Clontech, Mountain View, CA).

Cell Culture and Generation of Recombinant Cell Lines. HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 1 mM sodium pyruvate, and 2 mM glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin and maintained at 37°C, 5% CO2, and 95% humidity. Cells were subcultured with treatment by 0.25% trypsin with 0.1% EDTA in Hanks' balanced salt solution.

For stable transfections 24 h before transfection cells were seeded at 3 x 10^6/T75 flask. Transfections were performed by combining 5 g of human CLR and 5 g of human RAMP with 30 g of Lipo- fectamine 2000 (Invitrogen, Carlsbad, CA). The transfection cocktail was added directly to the medium. Twenty-four hours later, the cells were passaged into fresh medium, and growth medium plus 300 g/ml hygromycin and 1.0 g/ml puromycin was added the following day. Clonal cell populations were generated via single cell sorting and maintained in growth medium containing 150 g/ml hygromycin and 0.5 g/ml puromycin.

Membrane Preparation and Radioligand Binding Studies. Stably transfected HEK293 cells were washed once with PBS and harvested in 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. The pellets were resuspended in harvest buffer plus 250 mM sucrose. Membranes were stored at −70°C as aliquots. Membranes from rat and dog brains were prepared similarly. SK-N-MC membranes were purchased from Receptor Biology, Inc. (Beltsville, MD). Rhesus cerebellum was disrupted using a laboratory homogenizer in 10 mM HEPES and 5 mM MgCl2 and used directly in binding experiments.

Competition binding assays were conducted by combining antagonist and 500 nM Compound 3 (Hershey et al., 2005) or 100 nM unlabeled CGRP for nonspecific binding, 10 pM 125I-hCGRP (GE Healthcare, Piscataway, NJ), followed by 10 g of CLR/RAMP1, 25 g of SK-N-MC, 100 g of brain, or 1 mg of rhesus cerebellum membranes and incubated for 3 h at room temperature in binding buffer (10 mM HEPES, 5 mM MgCl2, and 0.2% bovine serum albumin) in a total volume of 1 ml. Adrenomedullin binding assays were set up as above but with 7.5 g of CLR/RAMP2 or 5 g of CLR/ RAMP3 membranes, 10 pM 125I-human adrenomedullin (GE Healthcare) as the radioligand, and 10 nM unlabeled human adrenomedul- lin for nonspecific binding. Incubations were terminated by filtration through GF/B 96-well filter plates that had been blocked with 0.5% polyethyleneimine. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA), and the $K_i$ was determsted using the equation $K_i = IC_{50}/1 + ([\text{ligand}]/K_d)$. The $K_d$ values...
for the CGRP and adrenomedullin receptors were determined by saturation binding experiments (data not shown).

**Functional Studies.** HEK293 cells stably transfected with CLR/RAMP1 were plated in complete growth medium at 85,000 cells/well in 96-well poly-d-lysine-coated plates and cultured for ~19 h before assay. Cells were washed with PBS and then incubated with inhibitor in the presence or absence of 50% human serum (SeraCare, Inc., Oceanside, CA) for 30 min at 37°C and 95% humidity in Cellgro Complete Serum-Free/Low-Protein medium (Mediatech, Inc., Herndon, VA) with 1-glutamine and 1 g/l bovine serum albumin. Isobutylmethylxanthine was added to the cells at a concentration of 300 μM and incubated for 30 min at 37°C. Human α-CGRP was added to the cells at a concentration of 0.3 nM and allowed to incubate at 37°C for 5 min. After α-CGRP stimulation, the cells were washed with PBS and processed for cAMP determination using the two-stage assay procedure according to the manufacturer’s recommended protocol (cAMP SPA direct screening assay system; RPA 559; GE Healthcare). Dose-response curves were plotted, and IC50 values were 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 EC50 values in the presence and absence antagonist and [B] is the antagonist concentration. The X-intercept is equal to the pA2, and the Kd was calculated using the formula pA2 = −log Kd.

**Rhesus 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 (Institute of Laboratory Animal Resources, 1996). Rhesus monkeys (male and female) weighing between 4 and 10 kg were anesthetized initially with ketamine (0.1 ml/kg i.m.) and then placed in the supine position on a temperature-controlled water circulating blanket and intubated with a 3-mm tracheal tube connected to 1-liter oxygen/1 to 2% isoflurane gas anesthesia. The right saphenous vein was cannulated for intravenous drug delivery, and blood samples were obtained from the left saphenous artery. Four rubber O-rings (8 mm inner diameter) were placed on the ventral side of the forearm without directly being positioned over a visible vessel. A laser Doppler imager (Moor Instruments, Ltd., Millwey, Axminster, Devon, UK) was used to generate a color-coded image of dermal blood flow and quantitate the vasodilatory response to topically applied capsaicin. Using the manufacturer supplied software, average blood flow, expressed as perfusion units, was determined within the specified region of interest. After a baseline scan, a capsaicin solution (2 mg in 30% ethanol/20% Tween 20) was added inside one of the rings to obtain a control response. The O-rings served both as a well to contain the topically applied solution and also standardized the region of interest during data analysis. The capsaicin-induced changes in dermal blood flow were monitored by obtaining laser Doppler scans at 5, 10, and 20 min post-capsaicin. After obtaining the control capsaicin response, MK-0974 was administered intravenously using a dose-escalating protocol, including a bolus dose delivered in a volume of 500 μl (0.03 to 3.7 mg/kg i.v.) followed by a 25-min continuous infusion (0.13 to 20 μg/kg/min i.v.) at a rate of 0.025 ml/min to target plasma concentrations ranging from 31 nM to 5 μM. Five minutes after the start of each infusion, capsaicin was applied to an unused ring, and the vasodilatory response was measured after 5, 10, and 20 min. The blood flow response at 20 min post-capsaicin was compared to the control response (i.e., before administration of antagonist) to determine the percentage inhibition. Plasma samples were obtained at the 20-min time point to correlate the pharmacodynamic effect to the plasma concentration of MK-0974.

**Results**

**Binding Studies on CGRP Receptors.** Competitive binding experiments were carried out to determine the relative affinity of MK-0974 (Fig. 1) for human, rhesus, rat, and dog CGRP receptors. MK-0974 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 125I-hCGRP binding with Kd values of 0.78 ± 0.05 (n = 10) and 0.77 ± 0.07 nM (n = 13), respectively. MK-0974 displayed a similar affinity (Kd) for the rhesus receptor (1.2 ± 0.08 nM; n = 10) as that for human, but it displayed >1500-fold lower affinity for the canine and rat receptors, with values of 1204 ± 38 (n = 10) and 1192 ± 56 nM (n = 10), respectively (Fig. 2).

**Binding Studies on Human Adrenomedullin Receptors.** Competitive binding experiments were carried out to determine the selectivity of MK-0974 for the human CGRP receptor versus the related human adrenomedullin receptors. MK-0974 displayed little to no affinity for the human adrenomedullin receptors as measured by the ability to compete with 125I-human adrenomedullin, with Kd values of >100 and 29 μM on CLR/RAMP2 and CLR/RAMP3, respectively.

**Functional Studies on the Human CGRP Receptor.** The effect of MK-0974 on CGRP-induced cAMP production in CLR/RAMP1 cells was investigated. Consistent with the binding data, MK-0974 potently blocked human α-CGRP-stimulated cAMP responses in human CGRP receptor-expressing HEK293 cells with an IC50 of 2.2 ± 0.29 nM (n = 8). The addition of 50% human serum (IC50 = 10.9 ± 2.1 nM; n = 10) reduced the apparent potency of MK-0974 by approximately 5-fold.

Increasing concentrations of MK-0974 caused a dose-dependent rightward shift in the CGRP dose-response curve, with no reduction in the maximal agonist response (Fig. 3A). Schild regression (Fig. 3B) yielded a pA2 value of 8.9 (n = 4; Kd = 1.1 nM).

![Fig. 1. Chemical structure of MK-0974.](image)

![Fig. 2. Concentration-dependent inhibition of 125I-hCGRP binding by MK-0974 from SK-N-MC, CLR/RAMP1, rhesus cerebellum, dog brain, and rat brain membranes. Mean values ± S.E.M.](image)
Effect of MK-0974 on Capsaicin-Induced Vasodilation in Rhesus. The increase in dermal vasodilation in response to capsaicin was found to be concentration- and time-dependent. A dose of 2 mg of capsaicin caused a 30% increase in dermal blood flow 20 min postapplication. Infusion of increasing doses of MK-0974 resulted in the blockade of the capsaicin-induced vasodilation response, illustrated in Fig. 4, affording EC50 and EC90 values of 127 nM or 71.9 ng/ml (95% confidence interval from 56 to 257 nM) and 994 nM or 563 ng/ml (95% confidence interval from 643 to 1600 nM), respectively (Fig. 5A). Further pharmacodynamic studies with MK-0974 (i.v. bolus, 1 mg/kg) demonstrated that the efficacy of this antagonist was time-dependent and correlated with plasma levels (Fig. 5B).

Additionally, MK-0974 was tested in a transient receptor potential vanilloid subfamily, member 1 (TRPV1) electrophysiology assay using a whole-cell patch-clamp technique to ensure that effects on vasodilation were not a result of TRPV1 antagonism. The ability of MK-0974 to inhibit capsaicin-induced (500 nM) TRPV1 currents was evaluated in Chinese hamster ovary-K1 cells stably expressing the human TRPV1 receptor. At a concentration of 1 μM, MK-0974 inhibited only 27.2 ± 6.6% (n = 3 cells) capsaicin-induced current.

Discussion

In this report, we detail the pharmacological profile of MK-0974, a structurally novel CGRP receptor antagonist in clinical development for the acute treatment of migraine. Furthermore, a rhesus dermal vasodilation assay, which relies on the CGRP-mediated response to topically applied capsaicin as a pharmacodynamic measure, was employed to assess the in vivo potency of MK-0974. The affinity of MK-0974 for the human CGRP receptor was established for both the recombinant and native receptors. To confirm that the recombinant CGRP receptor stably expressed in HEK293 cells exhibited physiologically relevant pharmacology, comparisons were made to the native human receptor found in SK-N-MC cells (Semark et al., 1992). MK-0974 displayed equal affinity for the native and cloned receptor as determined by radioligand binding experiments. MK-0974 functioned as a competitive antagonist of CGRP-induced cAMP accumulation in cells expressing the recombinant human CGRP receptor.

MK-0974 was highly selective for the CGRP receptor versus the closely related human adrenomedullin receptors, CLR/RAMP2 and CLR/RAMP3, and displayed no significant activity (IC50 > 10 μM) in a screen of 166 enzyme and binding assays, including the human calcitonin receptor (MDS Pharma Services, Taipei, Taiwan; partial list of assays summarized in supplemental data).

It is well documented that small-molecule antagonists of the CGRP receptor exhibit species-selective pharmacology (Doods et al., 2000; Edvinsson et al., 2001; Hasbak et al., 2001; Mallee et al., 2002), and MK-0974 is no exception. MK-0974 displayed marked species-selectivity, exhibiting approximately 1500-fold higher affinity for the human and rhesus CGRP receptors compared to the rat and dog CGRP receptors. We have previously demonstrated that RAMP1 is responsible for the high-affinity binding of the BIBN4096BS class of antagonist (Mallee et al., 2002) via in vitro transient mixed species cotransfections of CLR and RAMP1. MK-0974 was evaluated in a similar manner, and it was determined that RAMP1 governs species selectivity of MK-0974 (data not shown).

![Fig. 3. Concentration-response curve and Schild plot analysis of MK-0974.](image-url)

**Fig. 3.** Concentration-response curve and Schild plot analysis of MK-0974. 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-0974 (n = 4). B, Schild plot showing the effect of MK-0974 on cAMP production in HEK293 cells stably expressing human CLR/RAMP1.

![Fig. 4. Laser Doppler images of dermal blood flow in the rhesus forearm in response to topical application of capsaicin before and after administration of MK-0974.](image-url)

**Fig. 4.** Laser Doppler images of dermal blood flow in the rhesus forearm in response to topical application of capsaicin before and after administration of MK-0974. Dermal blood flow during baseline (A) and 20 min following topical application of capsaicin (B), which produced a 47% increase in blood flow within the highlighted O-ring. After obtaining the control response to capsaicin, MK-0974 was administered intravenously (0.91 mg/kg i.v. bolus, followed by 4.9 μg/kg/min i.v. infusion) and dermal blood flow measured in the highlighted O-ring before (C) and 20 min (D) after capsaicin application. There was only a 3% increase in capsaicin-induced dermal blood flow (D) in the presence of MK-0974 (973 nM plasma).
The pronounced species-selectivity exhibited by MK-0974 required the utilization of nonhuman primate to assess in vivo pharmacological activity. Thus, pharmacological studies were conducted in rhesus monkey utilizing a capsaicin-induced dermal vasodilation assay (Hershey et al., 2005). Topical application of capsaicin to the rhesus forearm resulted in an increase in dermal blood flow directly measurable via laser Doppler imaging. The increased blood flow in response to the topical application of capsaicin is a direct result of endogenous CGRP release via activation of TRPV1 receptors (Akerman et al., 2003), and the ability of a CGRP receptor antagonist to block capsaicin-induced vasodilation provides pharmacodynamic evidence of in vivo receptor blockade. MK-0974 produced a concentration-dependent inhibition of capsaicin-induced dermal vasodilation in the rhesus forearm. Furthermore, a pharmacokinetic-pharmacodynamic relationship was observed between plasma concentrations of MK-0974 and inhibition of dermal vasodilation, suggesting a predictable pharmacokinetic/pharmacodynamic relationship. The plasma concentration of MK-0974 required to block rhesus dermal vasodilation is higher than anticipated when based solely upon the intrinsic affinity (rhesus $K_i = 1.2 \text{nM}$) of the molecule. When evaluated in the human in vitro functional assay, MK-0974 exhibited a 5-fold reduction in potency in the presence of serum ($IC_{50} = 2.2$ and $10.9 \text{nM}$ in the absence and presence of serum, respectively), suggesting a significant degree of associative plasma protein binding. Taking into account the loss in potency in the presence of serum, there is only a 10-fold difference between the human serum-shifted in vitro $IC_{50}$ and the rhesus in vivo $EC_{50}$. MK-0974 displayed approximately the same affinity for the rhesus and human receptors; therefore, comparing the human $IC_{50}$ to the rhesus in vivo $EC_{50}$ is probably a valid comparison. Additionally, one cannot rule out the potential of a central nervous system mechanism playing a role in the capsaicin response via dorsal root reflexes (Lin et al., 1999). In summary, the rhesus model is nonterminal, noninvasive, rapid, and quantitative, making it an ideal pharmacodynamic model. It is noteworthy that this model was translated to the clinical setting and served as a valuable tool for dose selection for the clinical development of MK-0974 (Sinclair et al., 2007).

In conclusion, we have identified a potent, highly selective, and orally bioavailable small molecule CGRP receptor antagonist. CGRP receptor antagonists offer a novel mechanism of action for the management of migraine. Phase 3 clinical studies are ongoing with MK-0974 to better understand the potential promise of this class of molecules.

References


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