Design, Characterization, and First-In-Human Study of the Vascular Actions of a Novel Biased Apelin Receptor Agonist

Aimee L. Brame,* Janet J. Maguire,* Peiran Yang, Alex Dyson, Rubben Torella, Joseph Cheriyan, Mervyn Singer, Robert C. Glen, Ian B. Wilkinson, Anthony P. Davenport

Abstract—[Pyr1]apelin-13 is an endogenous vasodilator and inotrope but is downregulated in pulmonary hypertension and heart failure, making the apelin receptor an attractive therapeutic target. Agonists acting at the same G-protein–coupled receptor can be engineered to stabilize different conformational states and function as biased ligands, selectively stimulating either G-protein or β-arrestin pathways. We used molecular dynamics simulations of apelin/receptor interactions to design cyclic analogues and identified MM07 as a biased agonist. In β-arrestin and internalization assays (G-protein–independent), MM07 was 2 orders of magnitude less potent than [Pyr1]apelin-13. In a G-protein–dependent saphenous vein contraction assay, both peptides had comparable potency (pD2:[Pyr1]apelin-13 9.93±0.24; MM07 9.54±0.42) and maximum responses with a resulting bias for MM07 of =350- to 1300-fold for the G-protein pathway. In rats, systemic infusions of MM07 (10-100nmol) caused a dose-dependent increase in cardiac output that was significantly greater than the response to [Pyr1]apelin-13. Similarly, in human volunteers, MM07 produced a significant dose-dependent increase in forearm blood flow with a maximum dilatation double that is seen with [Pyr1]apelin-13. Additionally, repeated doses of MM07 produced reproducible increases in forearm blood flow. These responses are consistent with a more efficacious action of the biased agonist. In human hand vein, both peptides reversed an established norepinephrine constrictor response and significantly increased venous flow. Our results suggest that MM07 acting as a biased agonist at the apelin receptor can preferentially stimulate the G-protein pathway, which could translate to improved efficacy in the clinic by selectively stimulating vasodilatation and inotropic actions but avoiding activating detrimental β-arrestin–dependent pathways. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.05099.)

Key Words: β-arrestin G-protein coupled receptors pulmonary arterial hypertension [Pyr1]apelin-13
to design =105 cyclic peptide analogues. These were tested for inhibition of radiolabelled apelin binding followed by a screening cascade comparing G-protein–dependent versus G-protein–independent pathways to identify a biased agonist, MM07. As proof of principle in first-in-human studies, we show MM07, as predicted, is a more effective vasodilator in human vasculature than apelin and increases cardiac output in rodent by echocardiography.

Methods
Additional information is available in the online-only Data Supplement. Animal experiments were performed according to local ethics committee (Universities of Cambridge and London) and Home Office (UK) guidelines under the 1986 Scientific Procedures Act.

Computational Methods
From analysis of molecular dynamics simulations, MM07 (cyclo[1–6][CRPRLCKGPMPF]) was expected to mimic the solution conformation of apelin-13 (NH$_2$-QPRPLSHKGPMPF-COOH) and promote a β-turn conformation at the RPRL motif, suggested to be important for initial recognition and binding at the apelin receptor.22–24

Simulation Protocol
The model of MM07 was constructed using the Biopolymer module of the Sybyl 7.3 program3 and energy minimized in vacuo with the Amber11 FF99SB force field.36 Gromacs version 3.3.127–29 was used for all simulations. Replica exchange molecular dynamics80 was used to explore the conformational space of the peptides.

Homology Modeling of the Apelin Receptor
The modeling template was based on the 2.5 Å resolution crystal structure of the human CXCR4 chemokine receptor. MODELLER9v8 was used to generate homology models of apelin and subsequently refined using molecular dynamics.

Human Tissue Collection
Human tissues were obtained with informed consent (Papworth Hospital Research Tissue Bank REC08/H0304/56) and experiments performed with local ethical approval (REC05/Q0104/142). Saphenous vein was from 19 patients receiving coronary artery bypass grafts. Heart tissues were from 3 patients undergoing cardiac transplantation.

Competition Binding Assays
Initial assays were performed in cells expressing the human apelin receptor (CHO-K1-APJ cells; Cerep, Celle L’Evescault, France). In subsequent experiments, homogenate of human left ventricle was incubated with 0.1 nmol/L [Glp65,Nle75,Tyr77][125I]apelin-13 and with [Pyr1]apelin-13 or MM07 (0.01 nmol/L–100 μmol/L). Nonspecific binding was defined using 1 μmol/L [Pyr1]apelin-13. Data were analyzed (KELL package; Biosoft, UK) to obtain the dissociation constant $K_{d}$ (concentration of ligand occupying 50% of receptors).

Cell-Based Functional Assays
β-Arrestin and receptor internalization assays (AGTR1L; DiscoveRx, Fremont, CA) used cells expressing the human apelin receptor. Agonist concentration-response curves were analyzed to determine values of pD2 ($-\log_{10}$ of the EC$^{50}$ [the concentration of agonist producing 50% of maximum response]) and maximum response ($E_{\text{max}}$) for [Pyr1]apelin-13 and MM07.

Human Saphenous Vein Contraction Study
The predominant effect of apelins in vivo is vasodilatation and apelin peptides are equi-effective dilators of human endothelium-intact arteries in vitro.3 However, removal of the endothelium in saphenous vein unmask apelin contraction,3 which we have exploited as a routine bioassay. Cumulative concentration–response curves to [Pyr1]apelin-13 and MM07 (10$^{-12}$ to 10$^{-7}$mol/L) were expressed as a percent of the maximum response to 100 mmol/L KCl. Data were analyzed as described for the cell based assays.

Analysis of MM07 Signaling Pathway Bias
Data from in vitro assays were analyzed as previously described31 to determine the pathway signaling profile for MM07 compared with [Pyr1]apelin-13 to show whether MM07 exhibited additional bias for the individual signaling pathways investigated.

In Vivo Plasma Half-Life of Apelin Peptides
Rats (230–260 g), anaesthetized with 2% isoflurane, were given a single intravenous bolus (600 nmol) of apelin or MM07 (both n=3). Arterial blood samples were taken over the subsequent 30 minutes and plasma concentrations measured using an enzyme immunoassay that detects both peptides, and plasma half-life was determined.

Systemic Infusions in Rat
Surgical preparation and experimental techniques were as described previously.32 After baseline measurements, male Wistar rats (300±25 g body weight) received vehicle (300 μL bolus), then incremental doses of [Pyr1]apelin-13 or MM07 (1–300 nmol/300 μL), followed by S-Nitroso-N-acetyl-D,L-penicillamine (SNAP). Blood pressure (BP) was monitored continuously via an intra-arterial line and echocardiography was used after each bolus to determine changes in heart rateHR, stroke volume, cardiac output (from peak velocity and velocity time interval) and respiratory rate. Rectal temperature was monitored throughout. BP was analyzed using absolute change in mean BP from the baseline taken just before the bolus and area under the curve analysis.

Human Volunteer Studies
All volunteers (n=12 forearm plethysmography, n=10 hand vein studies) gave written informed consent, and studies adhered to the principles of the Declaration of Helsinki and were approved by the National Research Ethics Service Committee East of England–Cambridge Central (REC 11/EE/0305).

Forearm Venous Occlusion Plethysmography
[Pyr1]apelin-13 and MM07 were infused in 3 incremental doses, 6 minutes each, using the previously determined optimal dose range. The order of peptides was randomized, separated by 20 minutes saline infusion, and the study completed by infusion of sodium nitroprusside (3 μg/min for 6 minutes) or saline. Forearm blood flow (FBF) was measured in both arms and expressed as absolute change in FBF in response to agonists. In a second study (n=4), the effect of repeated (3 doses, each for 8 minutes) infusions of MM07 was determined after a 30 minute saline washout.

Aellig Hand Vein Technique Study
After baseline measurements, norepinephrine (1–60 ng/min) was infused in 7 minute increments (7.5 mL/h) to achieve a stable constant of 50% to 80%. [Pyr1]apelin-13 or MM07 (4 incremental doses, each for 8 minutes at 7.5 mL/h) were infused 30 minutes later, with measurements taken in the last 2 minutes of each infusion period. Aellig hand vein measurement was taken by echocardiography.

Statistical Analysis
For in vivo animal and human volunteer studies, variables are reported as mean±standard error of the mean. Data were analyzed using 1- or 2-way ANOVA or 2-way repeated measures ANOVA. Post hoc multiple comparisons were made using Sidak’s or Tukey’s post hoc test as appropriate (Graph-Pad Prism; Graph-Pad Software Inc, San Diego, CA). Student’s 2-tailed t test was also used as appropriate. Statistical significance was taken at the 5% level.
Results

Design and Conformation of MM07
To investigate the receptor bound conformation of MM07, compounds were initially docked into a receptor homology model and the complex refined using molecular dynamics. The apelin and MM07-apelin receptor binding poses are shown in Figure 1 taken from the most representative structure of the principal cluster of the MD simulation trajectory. The results implied sufficient volume was available for the cyclized peptide to occupy a similar binding pose to apelin-13.

MM07 Binds to the Human Apelin Receptor
MM07 competed with nanomolar affinities for binding of [Glp65,Nle75,Tyr77] [125I]apelin-13 to human apelin receptors in CHO-K1 cells (K_D, 300 nmol/L) and human heart (K_D, 172 nmol/L, n=3).

MM07 Is a G-Protein Pathway Biased Agonist In Vitro
In the β-arrestin (Figure 2A) and internalization (Figure 2B) assays, MM07 (β-arrestin pD_2=5.67±0.1, n=6; internalization pD_2=6.16±0.07, n=3) was ≈790- and ≈215-fold less potent than [Pyr1]apelin-13 (β-arrestin pD_2=8.57±0.1, n=6; internalization pD_2=8.49±0.1, n=3), respectively. However, in the saphenous vein contraction assay, the 2 peptides had comparable potency (pD_2 values: [Pyr1]apelin-13, 9.93±0.24, n=11; MM07 9.54±0.42, n=11) and maximum response (E_MAX as %KCl: [Pyr1]apelin-13, 22%±4%; MM07, 17%±3%; Figure 2C). Comparing the relative effectiveness of MM07 to activate the G-protein pathway, saphenous vein contraction compared with the non-G-protein–dependent β-arrestin and internalization pathways yielded bias factors for MM07 compared with [Pyr1]apelin-13 of 1374 and 353, respectively (Table).

Half-Life of [Pyr1]apelin-13 and MM07
Plasma half-life of MM07 was 17.4±0.40 minutes compared with 2.3±0.51 minutes for [Pyr1]apelin-13 (P<0.05, 2-tailed Student’s t test).

MM07 Increases Cardiac Output in Rat
There was a trend to increase in cardiac output with the highest dose of [Pyr1]apelin-13, which corresponded to a significant drop in BP (Figure 3A and 3B) and systemic vascular resistance (Figure S3B in the online-only Data Supplement). In contrast, MM07 caused a dose-dependent increase in cardiac output (ANOVA versus baseline, P=0.0074; Figure 3A), and although there was a decrease in vascular resistance (Figure S3B), this was without corresponding effects on BP (Figure 3B). Administration of SNAP produced a profound fall in BP in both [Pyr1]apelin-13 and MM07-treated groups; however, although cardiac output was significantly increased in response to SNAP in the MM07 group, it was significantly reduced in the [Pyr1]apelin-13 group. Neither peptide caused a significant change in heart rate (Figure 3C), respiratory rate (Figure 3D), or temperature (not shown). Both [Pyr1]apelin-13 and MM07 increased peak velocity above basal levels (ANOVA versus baseline P=0.046 and P=0.01, respectively; Figure 3E and 3F).

MM07 Is a Vasodilator in Human Forearm Venous Occlusion Plethysmography Studies
[Pyr1]apelin-13 (Figure 4A) and MM07 (Figure 4B) produced a significant dose-dependent increase in FBF compared with baseline (ANOVA P<0.01). [Pyr1]apelin-13 at 100 nmol/min produced an increase in FBF in the control arm. This was not seen with MM07 (data not shown). Importantly, the maximum dilatation to MM07 was approximately double that seen with

![Figure 1. Representation of the overlap between the calculated poses of apelin-13 (red) and MM07 (blue) binding to the apelin receptor. The disulphide bond of MM07 is indicated in stick, the side chains in lines, and the backbone in cartoon representation. The transmembrane helices of the apelin receptor are indicated by roman numerals.](image1)

![Figure 2. Comparison of in vitro functional activity of [Pyr1]apelin-13 (■) and MM07 (●) in G-protein-independent (A) β-arrestin recruitment and (B) receptor internalization assays and G-protein-dependent (C) saphenous vein contraction assay.](image2)
Contraction vs internalization

**β-Arrestin assay**

- LogR: 8.59±0.11
- ΔLogR: 0.00±0.16
- RE: 1

**Internalization assay**

- LogR: 8.45±0.08
- ΔLogR: 0.00±0.12
- RE: 1

**Contraction vs β-arrestin**

- Bias factor: 1
- LogR: 0.00±0.36
- ΔLogR: 3.14±0.35

**Contraction vs internalization**

- Bias factor: 1
- ΔLogR: 0.00±0.35
- 2.55±0.33

**β-Arrestin vs internalization**

- Bias factor: 1
- ΔLogR: 0.00±0.19
- -0.59±0.19

**MM07 and Venous Tone**

Responses in resistance and capacitance vessels do not always correlate; we therefore conducted further studies in peripheral veins. After the arterial studies, we repeated the dose-ranging for both peptides and performed our definitive studies at a higher dose range than previously reported.2 In the human hand vein, MM07 and [Pyr1]apelin-13 reversed an established norepinephrine constriction and significantly increased flow in the venous bed with no adverse events observed. In contrast to arterial studies, both peptides were equally effective in the vein comparable to our in vitro contraction assay. The magnitudes of the responses were comparable with acetylcholine and sodium nitroprusside, producing significant reversal of preconstriction.35,36 MM07 is therefore an effective peripheral venodilator. The effects of apelin peptides on the central capacitance vessels have not been tested, and further studies are required to determine the in vivo effects of endogenous apelin and related peptides on the systemic vasculature.

**Systemic Effects of MM07 in Rat**

Apelin is reported to be the most potent inotropic agent measured in human isolated heart in vitro3 with significant effects on cardiac contractility in vivo animal models,36,37 including ischemic cardiomyopathy38 and myocardial injury.39 The hemodynamic goals in patients with ventricular failure secondary to pulmonary hypertension are decreased pulmonary vascular resistance and augmented cardiac output, which may potentially be achieved with an apelin agonist. Importantly, in heart failure patients, systemic infusion of [Pyr1]apelin-13 caused a beneficial peripheral and coronary vasodilation with increased cardiac output.40 These actions were sustained in the presence of angiotensin II and sodium depletion.40 To explore the systemic effects of MM07 safely, we used hemodynamic measurements and echocardiography in rats. Echocardiography in...
is the mainstay of the noninvasive assessment of cardiac function in rodent models and has the advantage of clear translation to humans in whom the same parameters are used. In our study, [Pyr1]apelin-13 augmented cardiac output that was accompanied by significant hypotension, whereas MM07 caused a significant increase in cardiac output without any evidence

![Figure 3](image1.png)

**Figure 3.** Response to [Pyr1]apelin-13 (●, n=6) and MM07 ( ■, n=5) in anaesthetized rats showing changes in cardiac output (CO; A), change in blood pressure (BP) from baseline (B), heart rate (HR; C), and respiratory rate (RR; D). Echocardiograms showing an increase in peak velocity across the left ventricular outflow tract after bolus administration of [Pyr1]apelin-13 (E) and MM07 (F). Horizontal line indicates baseline (BL) peak velocity. Significantly different from baseline *P<0.05, **P<0.01 or [Pyr1]apelin-13 #P<0.05 (ANOVA).

![Figure 4](image2.png)

**Figure 4.** Absolute change in forearm blood flow (FBF) during intrabrachial infusion of [Pyr1]apelin-13 (A) and MM07 (B). C, Repeated doses of MM07 produce a significant and reproducible response to 100 nmol/min (P<0.05). D, Concentration-dependent dilatation in dorsal hand veins after infusions of [Pyr1]apelin-13, MM07, and saline control. Significantly different from baseline *P<0.05, **P<0.01 (ANOVA).
of positive chronotropy or hemodynamic instability, and the inotropic effects were comparable with commonly used inotropes, such as dobutamine, mithramycin, and isoprenaline. Some of the apparent cardiac effect of [Pyr1]apelin-13 may be attributable to a reduction in afterload secondary to peripheral vasodilatation, shown by the drop in BP and systemic vascular resistance (Figure S3B). In contrast, MM007 did reduce vascular resistance (though to a lesser extent than [Pyr1]apelin-13), but had no effect on BP or heart rate; we hypothesize that this may be a result of an additional load-independent inotropic effect. In support of our hypothesis, in isolated perfused rat heart and paced human atrial tissue, apelin caused a sustained increase in contractility, independent of preload and autonomic reflexes. This is predicted to occur by increasing the calcium sensitivity of the contractile machinery via activation of myosin light chain kinase.

Apelin Agonists in Cardiovascular Disease?

Data suggest that activating the apelin pathway may be valuable in conditions, such as heart failure and pulmonary arterial hypertension. After an initial rise in the early stages of heart failure, plasma levels of peptide tend to decrease in later stages. Although there is downregulation of the apelin receptor pathway in the human heart, the favorable systemic actions of apelin on peripheral vasodilatation and cardiac output remain. In patients with pulmonary arterial hypertension, >70% have reduced bone morphogenetic protein receptor-2. Disruption of a bone morphogenetic protein receptor–mediated transcriptional complex between PPARγ and β-catenin reduced apelin levels in pulmonary arterial endothelial cells from pulmonary arterial hypertension patients, and apelin deficiency also causes proliferation in smooth muscle cells. Apelin-null mice developed more severe pulmonary hypertension compared with wild-type when exposed to chronic hypoxia, and crucially injections of apelin attenuated myocardial hypertrophy in the monocrotaline-treated rat. We therefore speculate that apelin agonists, such as MM07, that may have a sustained effect at the apelin receptor would be beneficial in these conditions.

Perspectives

The results suggest that agonists acting at the apelin receptor can preferentially stimulate the G-protein pathway over β-arrestin, which can translate to improved efficacy in the clinic. The use of biased agonists to selectively stimulate vasodilatation and inotropic action but avoid activating the β-arrestin–dependent stretch response within the heart represents a new therapeutic strategy in the treatment of cardiovascular disease.

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Disclosure

None.

References

Novelty and Significance

What Is New?

- We report the discovery and first in human in vivo characterization of MM07, a biased agonist for the apelin receptor.

What Is Relevant?

- The apelin system is downregulated in pulmonary arterial hypertension and heart failure; therefore, targeting the apelin receptor using novel agonists is an attractive therapeutic strategy.

Summary

Our results suggest that apelin agonists that preferentially stimulate G-protein over β-arrestin pathways could translate into improved clinical efficacy by selectively stimulating beneficial vasodilata-

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Supplementary Information File

Design, characterization and first-in-human study of the vascular actions of a novel ‘biased’ apelin receptor agonist

Running title: Cardiovascular action of biased apelin agonist

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Methods

**MM07 peptide synthesis**
MM07 was custom synthesized at Cambridge Peptides (Cambridge, UK) and was purified through HPLC with the sequence confirmed by electrospray ionization mass spectrometry (Figure S1A-C).

**Computational methods**
Reported binding affinities of modified apelin-13 sequences, using alanine replacement, implied that introduction of cysteines at positions e.g. one, six and seven of apelin-13 could be well tolerated and have a minimal impact on receptor binding affinity. Interestingly, alanine substitution at position six showed good affinity while having lower levels of receptor internalization, compared to e.g. seven substitution, which could be a possible pointer towards signaling bias, which made one and six substitution particularly interesting to explore. However, the introduction of cyclisation at positions 1 and 6 of apelin-13 between the cysteine thiol side chains (linked with a disulfide bridge) required analysis of the dynamics of the system to ascertain the effects of cyclisation on the geometry of the compounds, and how this compared to apelin-13 itself.

**Simulation protocol**
Replica exchange molecular dynamics was used to explore the conformational space of the peptides. Sixteen replicas were employed for each simulation with a temperature distribution spanning a physiologically relevant range (298.0–338.3K). Trajectories were clustered using the method of Daura et al and a backbone RMSD cutoff of 0.2 nm. Representative conformations were taken from the middle structure of the most populated cluster. Secondary structure analysis was performed on each trajectory using the DSSP program. β-Turns were defined as having a Cα distance between the first (i) and last (i+3) residues in the turn of less than 0.7 nm. The same distance was used to identify α-turns, where measurements are taken between the first (i) and last (i+4) Cα atoms of the five-residue turn. The dihedral angles were also used to calculate γ-turns.

**Homology modeling of the apelin receptor**
To gain more information about the interaction between MM07 and the apelin receptor, computational approaches have been applied to this system. Owing to the absence of available X-ray structures of the apelin receptor, a homology modeling approach has been used, using MODELLER 9v8. As a starting point, the placement of the apelin-13 ligand in the homology model of the apelin structure utilized the hypothesis that Phe-13 at the C-terminal of apelin-13 may embed in a hydrophobic pocket formed by F257 and W261. GOLD v5.1 has been used to dock MM07 in the apelin receptor cavity, using as a restraint the interaction between F13 on apelin with F257 and W261 on the apelin receptor. The modeling template was based on a modified 2.5 Å resolution crystal structure of the human CXCR4 chemokine receptor (PDB code 3ODU). MODELLER 9v8 was used for generating homology models of apelin. The refinement was guided by the conserved motifs in every TM helix for class A GPCRs which are Asn46 (N1.50) in TM-I, Asp74 (D2.50) in TM-II, Arg126 (R3.50) in TM-III, Trp153 (W4.50) in TM-IV, Pro207 (P5.50) in TM-V, Pro255 (P6.50) in TM-VI and Pro299 (P7.50) in TM-VII. Several functional micro-domains, which were considered to be conserved across the members of the class A GPCR superfamily, were used to improve the sequence alignment, such as the LAxxD motif in TMII, the D/ERY motif in TMIII, and the NPxxY motif in TMVII (Figure S1D). During the construction of models, each model was first optimized with the variable target function
method (VTFM) with conjugate gradients (CG), and was then refined using molecular dynamics (MD) with simulated annealing (SA).

Docking: The apelin receptor and MM07 were used a starting point for further docking analysis, using GOLD v5.1. Particularly, an in-house script was created to make a constraint between the side-chains of F257 and W261 (apelin receptor) and F13 on MM07. No other constraints were applied to the complex.

MD simulations protocol: An equilibrated POPC membrane system using the CHARMM-36 all-atom force field was used for all simulations. To efficiently insert a protein into an equilibrated and fully hydrated membrane with minimal membrane perturbation a computational tool was employed, g_membed, which is part of the GROMACS suite of programs. After energy minimization, 10 ns equilibration was performed to obtain a stable system. The MD simulation system was configured using the new CHARMM-36 parameter set and prepared using the CHARMM package. Simulations were performed using the AMBER11 package, for a total length of 300 ns (3 replica of 100 ns each). Analyses of the simulations have been performed using the GROMACS and AMBER software suites, as described in reference 15.

Competition binding assays
Preliminary assays were performed in CHO-K1 cells exogenously expressing the human apelin receptor (CHO-K1-APJ cells) by Cerep (Celle L’Evescault, France). An initial assay was carried out to confirm that MM07 (10 μmol/L) competed for [Glp^{65},Nle^{75},Tyr^{77}]^{[125]I}apelin-13 binding. Subsequently, in a competition assay CHO-K1-APJ cells were incubated for 120 min in assay buffer (mmol/L: HEPES 50, EDTA 1, NaCl 100, MgCl₂ 5, KCl 10, pH 7.4, 22°C) with 0.03 nmol/L [Glp^{65},Nle^{75},Tyr^{77}]^{[125]I}apelin-13 and increasing concentrations of [Pyr^1]apelin-13 or MM07 (0.3 nmol/L-10 μmol/L). Non-specific binding was defined using 1 μmol/L [Pyr^1]apelin-13. IC₅₀ values were obtained from which the peptide affinities were derived using the Cheng and Prusoff equation. To determine the affinity of MM07 for native human apelin receptors, homogenate of human left ventricle was prepared and stored at -70 °C until use. Homogenate was incubated for 90 min with 0.1 nmol/L [Glp^{65},Nle^{75},Tyr^{77}]^{[125]I}apelin-13 in assay buffer (mmol/L: Tris 50, MgCl₂ 5, pH 7.4, 22°C), in the presence of increasing concentrations of [Pyr^1]apelin-13 or MM07 (0.01 nmol/L-100 μmol/L). Non-specific binding was defined using 2 μmol/L [Pyr^1]apelin-13. Equilibrium was broken by centrifugation (20,000g for 10 min, 4°C). Pellets were washed with Tris-HCl buffer (50 mmol/L, pH 7.4, 4 °C), re-centrifuged and bound radioactivity in final pellets counted. Data were analyzed using the iterative non-linear curve fitting programs EBDA and LIGAND (KELL package, Biosoft, UK) to obtain values for the dissociation constant (K_D, the concentration occupying 50% of available receptors) of MM07 and [Pyr^1]apelin-13.

β-Arrestin assay
Experiments were carried out using the DiscoveRx PathHunter® eXpress β-Arrestin GPCR Assay (AGTRL1). CHO-K1 cells expressing human apelin receptor were thawed, re-suspended in Cell Plating media, seeded into 96 well plates and incubated for 48 hours, 37°C, in 5% CO₂. Serial dilutions of [Pyr^1]apelin-13 and MM07 stock solutions (10⁻³ mol/L) were made in Cell Plating media and 10μl added to wells to give final concentrations of 10⁻¹¹–3x10⁻⁸ mol/L [Pyr^1]apelin-13 or 10⁻⁸–3x10⁻⁴ mol/L MM07. Concentration-response curves to agonists were typically performed in triplicate. Cells were incubated for 90 min at 37°C before Detection Medium was added and cells incubated for a further 2 hours at room temperature. The resulting chemiluminescent signal was measured (LumiLITE™ Microplate
Reader, DiscoveRx, Fremont, CA) and agonist concentration response curves measured as relative light units, were normalized to the mean maximum response obtained to in the same experiment. Resulting agonist data were fitted to a 4 parameter logistic equation using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). Values of pD2 (−log10 of the EC50 (the concentration of agonist producing 50% of maximum response)) and maximum response (EMAX) were determined for MM07 and compared to control responses to [Pyr1]apelin-13.

**Internalization assay**

Experiments were carried out using the DiscoveRx PathHunter® eXpress Activated GPCR Internalization Assay (AGTRL1, DiscoveRx, Fremont, CA) according to the manufacturer’s instructions. Briefly, U2OS cells expressing human apelin receptor were thawed, resuspended in Cell Plating media, seeded into 96 well plates and incubated for 48 hours at 37°C, in 5% CO2. [Pyr1]apelin-13 and MM07 stock solutions (10⁻³ mol/L) were made in Cell Plating media and 10μl added to wells to give a final concentrations of 10⁻¹¹–3x10⁻⁷ mol/L [Pyr1]apelin-13 or 10⁻⁸–3x10⁻⁴ mol/L MM07. Concentration-response curves to [Pyr1]apelin-13 and MM07 were carried out as for the β-arrestin assay but with a 3 hour incubation at 37°C before Detection Medium was added. Cells incubated for a further 2 hours at room temperature. The resulting chemiluminescent signal was measured and data analyzed as for the β-arrestin assay.

**Human saphenous vein contraction study**

Experiments were carried out as previously described. Briefly, 4mm rings of endothelium-denuded human saphenous vein were set up for isometric force recordings (F30 force transducers; Hugo Sachs, March-Hugstetten, Germany) in 5 mL organ baths containing oxygenated (95 % O2/5 % CO2) modified Krebs solution (mmol/L: NaCl, 90; NaHCO3, 45; KCl, 5; MgSO4.7H2O, 0.5; Na2HPO4.2H2O, 1; CaCl2, 2.25; fumaric acid, 5; glutamic acid, 5; glucose, 10; sodium pyruvate, 5; pH 7.4) at 37 ºC. Data output was to a chart recorder and MP100 data acquisition system (BIOpac Systems Inc., CA, USA). Optimum basal tension was determined by repeated administration of KCl (100 mmol/L) at increasing levels of basal tension until no further increase in developed isometric force was obtained. The absence of functional endothelium was determined by constricting with 10 μmol/L phenylephrine and testing for lack of endothelium dependent relaxation (<15 %) to acetylcholine (1 μmol/L). Concentration-response curves were then constructed to [Pyr1]apelin-13 and MM07 (10⁻¹²-10⁻⁷ mol/L), once the maximum response to each agonist had been achieved the curve was terminated by the addition of 100mmol/L KCl to determine the maximal possible contractile response for each preparation. Agonist data were expressed as a % of the terminal KCl response and analyzed using the non-linear iterative curve-fitting program GraphPad Prism to give values of pD2 and EMAX (maximum response to an agonist expressed as a %KCl).

**Peptide in vivo half life determination**

Male Sprague Dawley rats (n=6, 240±4g) were anaesthetised with 2% isoflurane and the left femoral vein and artery were cannulated for compound administration and blood collection, respectively. A 500μL bolus (600 nmoles) of [Pyr1]apelin-13 (n=3) or MM07 (n=3) were given intravenously. Over the following 35 minutes seven blood samples (~200ul each) were collected at approximately 0.5, 1.5, 2.5, 4.5, 8.5, 15.5 and 30.5 min into heparin-coated tubes. Samples were centrifuged at 2000g for 5 min and plasma concentrations of apelin peptides were determined using an enzyme immunoassay (Phoenix Pharmaceuticals, EK057-23), carried out according to the manufacturer’s instructions. Standards curves were obtained using serial dilutions of [Pyr1]apelin-13 and MM07. Each plasma sample was assayed at three dilutions (1:10-1:10,000 as appropriate). Sample peptide concentrations were
interpolated from the corresponding standards curve and corrected for dilution if appropriate. Half-life values were calculated from two-phase decay curves using GraphPad Prism.

**Systemic infusions in rat**

All experiments were performed according to local ethics committee (University College London) and Home Office (UK) guidelines under the 1986 Scientific Procedures Act. Spontaneously breathing male Wistar rats (300±25g body weight) were anaesthetized with 5% isoflurane and maintained on 2% isoflurane during instrumentation with 0.96 mm external diameter polyvinyl chloride tubing (Biocorp, Huntingdale, Australia) inserted into the left common carotid artery and right internal jugular vein. The arterial line was connected to a pressure transducer (Powerlab, AD Instruments, Chalgrove, UK) for continuous measurement of mean arterial pressure (MAP). A tracheostomy was sited using 2.08 mm external diameter polythene tubing (Portex Ltd, Hythe, UK) to secure and suction the airway; this was connected to a T-piece to maintain anesthesia. A rectal probe was inserted to measure core body temperature. Isoflurane was maintained at 2% for the remainder of the experiment. After a 30 min stabilization period, baseline hemodynamic measurements were recorded. Data was recorded using a 16-channel Powerlab system and Chart 7.0 acquisition software (AD Instruments, Chalgrove, UK).

**Rat echocardiography**

Thoracic echocardiography was performed using a 14 MHz probe scanning at 0–2 cm depth (Vivid 7 Dimension, GE Healthcare, Bedford, UK). Aortic blood flow velocities were determined in the aortic arch using pulsed-wave Doppler. Stroke volume (SV) was determined as the product of the velocity–time integral (VTI) and vessel cross-sectional area. Rats of this age have a reported aortic diameter of 0.26 cm, thus a cross-sectional area of \((0.13)^2 \times \pi\) was assumed for all animals studied. Heart rate (HR) and peak velocity (PV) (the latter a marker of left ventricular contractility) were determined by measuring the time between and maximum blood flow velocity, respectively, of six consecutive cardiac cycles. Cardiac output was calculated as the product of SV and HR. Respiration rate was determined from diaphragmatic movement, captured using time-motion (M)-model. Baseline measurements and dosing protocol are shown in Figure S2A.

**Forearm blood flow**

All human studies were performed in the University of Cambridge Vascular Research Unit, Addenbrooke’s Hospital, Cambridge, UK. Volunteer characteristics are given in Table S1. Vasoactive medications were avoided within 48 hours of study. \([\text{Pyr}^1]\)apelin-13 and MM07 were custom synthesized to Good Manufacturing Practice (Severn Biotech, Kidderminster, UK). Exclusion criteria were: hypertension (sustained BP >160/100mmHg); ischaemic heart disease; renal, respiratory or neurological disease; diabetes mellitus; BMI >30, BMI <18; pregnant; smoker; use of vasoactive medication or NSAIDS/ aspirin within 48 hours of study; current involvement in other research studies. Blood pressure and heart rate were recorded at baseline and every 6 min using an Omron HEM705CP oscillimetric sphygmomanometer in the contralateral arm using a cuff size appropriate for the arm circumference. This method has been validated and approved by the British Hypertension Society. Venous return was interrupted by intermittently inflating a cuff to 40 mmHg around the upper arm with a typical inflation period of 8 seconds and deflation period of 4 seconds. The elbows were placed on foam blocks and the hands on pillows to ensure forearms were above the level of the heart. During a 3 min measurement period the wrist cuffs were inflated to 200 mmHg in order to exclude hand circulation. Changes in forearm volume were measured by mercury-in-silastic strain gauges (sized by measuring forearm circumference at the widest part) and connected as
one arm of a Wheatstone bridge. FBF was expressed as ml/100 ml forearm volume per min. The brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper’s Needle Works, Birmingham, UK) under local anesthesia (lignocaine 1%, Hameln Pharmaceuticals Ltd., Gloucester, UK) to allow infusion of the peptides via a 16-gauge catheter (Portex, Kent, UK). A 20g venous cannula was placed under local anesthesia into a dorsal hand vein of the infused arm to allow for collection of effluent blood. FBF was measured in both arms and the data presented as absolute change in forearm blood flood in response to agonists, and percentage change from baseline in the ratio of FBF in infused to non-infused arms.16

The study peptides were supplied in sealed glass vials and stored at -40°C. Before each study peptides were allowed to warm to room temperature and diluted as per protocol with physiological saline to produce stock solutions. These were then filtered using a 0.2 micron flat filter (Portex, Hythe, UK) before undergoing serial dilutions with saline. Samples were tested post-filtration to ensure activity was unchanged.

On each visit [Pyr¹]apelin-13 and MM07 were infused in three incremental doses, each using the optimal dose range determined in pilot studies. The order in which the peptides were infused was randomized. Incremental doses of peptides were infused for 6 min each. After the first agonist, saline was infused for a period of 20 min before the next agonist dose was infused (Figure S2B). Venous blood was drawn after each peptide for estimation of plasma apelin levels. At the end of the peptide infusion, sodium nitroprusside was infused at 3µg/min for 6 min as a positive control, and saline infusion as a negative control.

In a second study repeated infusions of MM07 were given to seek evidence of receptor desensitization. This study used 4 healthy subjects with MM07 infused in 3 incremental doses, each for 8 min. After a 30 min saline washout the MM07 infusions were repeated (Figure S2C).

Forearm plethysmographic data was extracted from Chart data files and forearm blood flow was calculated for individual cuff inflations. Usually the last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. The ratio of flows in the two arms was calculated for each time point; in effect using the non-infused arm as a contemporaneous control.22

Aellig hand vein technique study
Healthy volunteers were seated in an upright position in a quiet, temperature controlled (23-25°C) room for the duration of the study. The left forearm was placed on a cushioned, upward sloping (45°) wooden block with the hand positioned on a horizontal support above the level of the heart to allow for complete emptying of superficial hand veins. The vein selected for study was non-branching for at least 2cm from the site of cannulation. A 23g butterfly cannula (Terumo, Leuven, Belgium) connected to a 10cm epidural catheter (Portex, Hythe, UK) to minimize dead space, was inserted into the vein and saline infused via a syringe driver at a rate of 15 ml/hr to ensure cannula patency. A tripod containing the linear variable differential transformer (LVDT) was mounted with the steel solenoid core on the summit of the vein, 1-2 cm distal to the point of the butterfly needle. Continuous recordings of the position of the core were made before and after inflation of the sphygmomanometer cuff to 50mmHg.23, 24 The difference between the peak and trough measurements was the diameter of the studied vein under a congestion pressure of 50 mmHg. Twenty min after saline infusions were commenced and baseline measurements recorded, norepinephrine (1-60 ng/min) was infused in seven-min increments at a rate of 7.5 ml/hr to achieve a stable constriction of 50-80%. Thirty min after the start of the norepinephrine infusion, four incremental doses (0.1,1,10,100 nmol/min) of either MM07 or [Pyr¹]apelin-13 were infused at 7.5 ml/hr, each dose for eight min, and measurements taken in the last two min of each
infusion period. Agonists were followed by a final 30 min saline infusion (Figure S2D). It was not possible to achieve the concentration of peptide required for a 300nmol/min dose in hand veins.

Materials
Chemicals were obtained from Sigma Aldrich Co. Ltd. (Poole, UK). Unless otherwise stated, MM07 was custom synthesized at Cambridge Peptides (Cambridge, UK), [Pyr1] apelin-13 was from Peptide Institute (Osaka, Japan), [Glp65,Nle75,Tyr77]125Iapelin-13, was from Perkin Elmer (MA, USA).

Results
Design and conformation of MM07
Our hypothesis was that the apelin-13 peptide binds to the apelin receptor in a two-stage process involving initial recognition of the RPRL β-turn motif25-27 followed by insertion of the remainder of the peptide into the receptor transmembrane pore. We investigated both the solution and the receptor bound conformations, primarily to determine the propensity to form an RPRL β-turn and if sufficient receptor volume was available for the one-six cyclicised peptide.

Molecular simulation in aqueous solution showed that hydrogen-bonded turns, bends, and disordered loops dominated the conformations of MM07 and C-terminal residues displayed highly disordered conformations (Figure S1E). To gain a deeper insight into specific types of turns at these sequences, the β-turn content was calculated for these motifs. Throughout the simulation of MM07, two β-turns were identified. The Cα distance between R2-L5 and C6-G9 exceeded 0.7 nm in only 3% of conformations (Figure S1F and S1G), indicating that RPRL and CHKG (Figure S1H and S1I) β-turns formed in practically the entire structural ensemble of MM07. This confirmed that the RPRL β-turn conformation in solution was conformationally accessible for the recognition step.

Effect of [Pyr1]apelin-13 and MM07 on hemodynamic in anaesthetized rat
[Pyr1]apelin-13 produced a decrease in BP whereas MM07 had no effect (Figure S3A). Whilst a central venous pressure was not measured, it was possible to calculate an index of systemic vascular resistance using mean arterial blood pressure and calculated cardiac output (Index of SVR=MAP/CO). [Pyr1]apelin-13 produced a significant drop in both blood pressure and systemic vascular resistance (Figure S3B). In contrast, MM07 caused a gradual decrease in SVR (Figure S3B) but BP was maintained (Figure 3B) as a result of the direct increase in cardiac contractility and output.

Effect of [Pyr1]apelin-13 and MM07 on blood pressure and heart rate in human volunteers
There was no significant effect of MM07 on blood pressure (Figure S3C) or heart rate (Figure S3D) during infusions into the forearm at any dose.


Table S1. Human Volunteer Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Forearm</th>
<th>Hand vein</th>
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<tbody>
<tr>
<td>Number of volunteers</td>
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<td>10</td>
</tr>
<tr>
<td>Age (y)</td>
<td>27 ± 2</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/6</td>
<td>6/3</td>
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<tr>
<td>Height (cm)</td>
<td>173 ± 3</td>
<td>176 ± 3</td>
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<tr>
<td>Weight (kg)</td>
<td>70.4 ± 2.8</td>
<td>74.7 ± 4.4</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>24.2 ± 1.1</td>
</tr>
<tr>
<td>HR (bpm)</td>
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</tr>
<tr>
<td>SBP (mmHg)</td>
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<tr>
<td>DBP (mmHg)</td>
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</tr>
<tr>
<td>MAP (mmHg)</td>
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</table>

Values are mean ± SEM
Figure S1. (A) HPLC analysis and (B) MS data for MM07. Images provided by Cambridge Peptides (Cambridge, UK). (C) Table shows a summary of electrospray ionization mass spectrometry data for MM07. (D) Sequence alignment of the apelin and CXCR4 receptor. Identical residues are colored in red, conservative mutations are colored in yellow and semi-conservative mutations are colored in blue. The sequence identity between apelin and CXCR4 is 28.3% and the similarity is 53.8%. (E) Representative conformation of MM07 showing the salt bridge between K8 and the C-terminus. The distance as time evolved between (F) the Cα of R2-L5 and (G) C6-G9, respectively. Representative conformations of MM07 displaying the β-turn at (H) the RPRL motif and at (I) the CHKG motif.
Figure S2. (A) Rat Study Protocol: Baseline measurements were obtained followed by a 300μl bolus of vehicle followed by incremental doses of [Pyr\(^1\)]apelin-13 or MM07 (1, 3, 10, 30, 100, 300 nmol), in volumes of 300μl each followed by a bolus dose of 300μl S-Nitroso-N-acetyl-D,L-penicillamine (SNAP). (B) In human forearm [Pyr\(^1\)]apelin-13 and MM07 and were infused for 6 min for each of three incremental doses with the order of peptide infusion randomized. After the first agonist, saline was infused (20 min) prior to the second peptide. Finally sodium nitroprusside was infused at 3µg/min for 6 min as a positive control and saline infusion as a negative control. (C) Repeated doses of MM07 were infused into the human forearm MM07 was infused in 3 incremental doses, each for 8 min. After a 30 min saline washout the MM07 infusions were repeated to determine whether desensitization occurs. (D) Baseline measurements were recorded; noradrenaline (1-60ng/min) was infused in 7 min increments (7.5ml/hr) to achieve a stable constriction of 50-80%. [Pyr\(^1\)]apelin-13 or MM07 (4 incremental doses, 7.5ml/hr ,each dose for 8 min) were infused 30 min later, with measurements taken in the last 2 min of each infusion period followed by a final 30 min saline infusion.
Figure S3. In anaesthetized rat, (A) the effect of [Pyr1]apelin-13 and MM07 on BP was significantly different 300nmol (p=0.005, ANOVA). (B) Index of systemic vascular resistance (BP/CO) following systemic administration of [Pyr1]apelin-13 (■, n=6) and MM07 (○, n=5). Significantly different from baseline *p<0.05, **p<0.01 (ANOVA). Lack of effect of MM07 on (C) blood pressure and (D) heart rate during infusions of 1, 10 and 100 nmol/min in human forearm.