Apelin peptides linked to anti-serum albumin domain antibodies retain affinity in vitro and are efficacious receptor agonists in vivo

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Abstract
The apelin receptor is a potential target in the treatment of heart failure and pulmonary arterial hypertension where levels of endogenous apelin peptides are reduced but significant receptor levels remain. Our aim was to characterise the pharmacology of a modified peptide agonist, MM202, designed to have high affinity for the apelin receptor and resistance to peptidase degradation and linked to an anti-serum albumin domain antibody (AlbudAb) to extend half-life in the blood. In competition, binding experiments in human heart MM202-AlbudAb (pKi = 9.39 ± 0.09) bound with similar high affinity as the endogenous peptides [Pyr1]apelin-13 (pKi = 8.83 ± 0.06) and apelin-17 (pKi = 9.57 ± 0.08). [Pyr1]apelin-13 was tenfold more potent in the cAMP (pD2 = 9.52 ± 0.05) compared to the β-arrestin (pD2 = 8.53 ± 0.03) assay, whereas apelin-17 (pD2 = 10.31 ± 0.28; pD2 = 10.15 ± 0.13, respectively) and MM202-AlbudAb (pD2 = 9.15 ± 0.12; pD2 = 9.26 ± 0.03, respectively) were equipotent in both assays, with MM202-AlbudAb tenfold less potent than apelin-17. MM202-AlbudAb bound to immobilised human serum albumin with high affinity (pKD = 9.02). In anaesthetised, male Sprague Dawley rats, MM202-AlbudAb (5 nmol, n = 15) significantly reduced left ventricular systolic pressure by 6.61 ± 1.46 mm Hg and systolic arterial pressure by 14.12 ± 3.35 mm Hg and significantly increased cardiac contractility by 533 ± 170 mm Hg/s, cardiac output by 1277 ± 190 RVU/min, stroke volume by 3.09 ± 0.47 RVU and heart rate by...
INTRODUCTION AND BACKGROUND

The apelin receptor is a class A G protein-coupled receptor first identified in 1993. Its cognate ligand apelin was identified in 1998, and since then, a further ligand, Elabela/Toddler (ELA), has been found. The apelin system has shown potential in the treatment of a number of diseases, particularly cardiovascular diseases, such as heart failure and pulmonary arterial hypertension (PAH) where circulating levels of apelin and ELA are reduced. In the cardiovascular system, the predominant apelin isoform is [Pyr1]apelin-13 and exogenous administration results in vasodilatation and cardiac inotropy without hypertrophy. It has proven beneficial in animal models of both heart failure and PAH. The most abundant form of ELA has not yet been identified; however, exogenous administration of ELA peptides results in similar activity to apelin, with ELA-32, the longest predicted circulating isoform, displaying greater potency and a beneficial role in a rat model of PAH.

Despite the potential benefits of exogenous apelin or ELA administration, they are limited by short half-lives of only a few minutes. To overcome this issue, we have studied cyclisation as well as the addition of unnatural amino acids and polyethylene glycol (PEG) spacers to produce peptide mimetics. A previous study demonstrated that a cyclic peptide based on apelin, MM07, was able to extend plasma half-life in rats to 17 minutes compared to 2 minutes. In this study, we have utilised unnatural amino acid addition and PEG spacers to reduce proteolysis, as has previously been demonstrated. Furthermore, we have conjugated an apelin mimetic peptide to an anti-serum albumin domain antibody (AlbudAb™) using sSMCC (sulphosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) and maleimide chemistry via a (PEG)4 linker at the pyroglutamylate on the N-terminus to retain binding at the apelin receptor as previously outlined in Patent WO 2012022703 (CA2808683A1; Filing data 8/12/2011; Patent pending 8/7/2015). This strategy was selected based on existing extensive structure-activity relationship (SAR) data that indicate that substitution at the N-terminus of the ligand is well tolerated by the apelin receptor (for additional information, see Appendix S1 Methods and Data File, Figures S1 and S2 and Tables S1 and S2). Peptides conjugated to the antibody are referred to as [Pyr1]apelin-13 or MM202 conjugate. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.

MATERIALS AND METHODS

2.1 Materials

Chemicals were obtained from Sigma-Aldrich Co. Ltd (Poole, UK) unless otherwise stated. [Pyr1]apelin-13 (Glp-RPRLSHKGPMPF), apelin-17, MM202, [Pyr1]apelin-13-AlbudAb and MM202-AlbudAb conjugates (Figure 1) were synthesized by Severn Biotech (Kidderminster, UK). [Glp65,Nle75,Tyr77][125I]apelin-13 was from PerkinElmer (MA). [Pyr1]apelin-13 and MM202 were covalently bonded to AlbudAb™ using sSMCC (sulphosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) and maleimide chemistry via a (PEG)4 linker at the pyroglutamate on the N-terminus to retain binding at the apelin receptor as previously outlined in Patent WO 2012022703 (CA2808683A1; Filing data 8/12/2011; Patent pending 8/7/2015). This strategy was selected based on existing extensive structure-activity relationship (SAR) data that indicate that substitution at the N-terminus of the ligand is well tolerated by the apelin receptor (for additional information, see Appendix S1 Methods and Data File, Figures S1 and S2 and Tables S1 and S2). Peptides conjugated to the antibody are referred to as [Pyr1]apelin-13 or MM202 conjugate. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.
guidelines from the local ethics committee (University of Cambridge), the Home Office (UK) under the Animals (Scientific Procedures) Act 1986, the GSK Policy on the Care, Welfare and Treatment of Animals and the ARRIVE guidelines. All animal experiments were performed and analysed by blinded experimenters. Rats were accommodated under standard housing and husbandry conditions under room temperature and normoxia, in individually ventilated cages of four rats each with wood chip bedding, under 12-hour light/12-hour dark cycle with ad libitum access to clean water and normal chow. The rats were monitored daily for signs of sickness and distress. No humane end-point was met during the study.

2.2 | Radioligand competition binding assays

Radioligand competition binding assays were performed in human left ventricular homogenates (1.5 mg/mL) using [Glp⁶⁵,Nle⁷⁵,Tyr⁷⁷][¹²⁵I]apelin-13 (0.1 nmol/L) and increasing concentrations of the unlabelled competitive agonist as previously described. Binding in the presence of 2 µmol/L of [Pyr¹]apelin-13 was considered to be non-specific.

2.3 | Cell-based functional assays

β-Areerstirn recruitment and cAMP inhibition assays in CHO-K1 cells expressing the human apelin receptor (DiscoverX, Fremont, CA) were carried out according to the manufacturer’s instructions and as previously published. Agonist responses were measured as relative light units (RLUs) detected and expressed relative to the maximum response (Eₘₐₓ) of the endogenous agonist, [Pyr¹]apelin-13, in the β-arreerstirn assay or as a percentage relative to the maximal forskolin response in the cAMP inhibition assay. A four-parameter model was used to fit the data and the Eₘₐₓ and pD₂ values (−log₁₀ of the EC₅₀ [concentration producing 50% of the Eₘₐₓ]) calculated and compared.

2.4 | Human serum albumin binding assays

Experiments were performed to determine the binding characteristic of AlbudAb and the MM202-AlbudAb conjugate to human serum albumin (HSA) using the Biacore T200 system (GE Healthcare Life Sciences). Briefly, HSA protein was immobilised via amine coupling onto the Biacore CM5 chip. A multi-cycle kinetic binding study was performed by injecting increasing concentrations of both species. AlbudAb (0.156-80 nmol/L) and MM202-AlbudAb (0.156-160 nmol/L) were tested and data fitted to a 1:1 binding model to calculate binding characteristics including association rates (kₐ), dissociation rates (k₅) and affinity (K_D) within the Biacore evaluation software.

2.5 | In vivo catheterisation for measurement of acute cardiovascular changes upon administration of the MM202-AlbudAb conjugate

Male Sprague Dawley rats (266 ± 5g, n = 27; Charles River Laboratories, Margate, UK) were induced (5%) and maintained (1.5%‐2%) under anaesthesia using isoflurane carried by oxygen (1.5 L/min). Animals were kept on a heat mat, and their temperature was monitored throughout the study by use of a rectal probe. Cessation of pain reflexes was tested by use of a hind-paw pinch test. Depth of anaesthesia was monitored throughout by breathing rate and, once catheterised, heart rate. Left ventricular catheterisation was performed as previously described. In brief, the jugular vein was isolated, flushed with heparin solution (2%, 0.9% saline, Macopharma) and cannulated for bolus drug delivery, the right common carotid artery was then isolated, and a pressure-volume catheter
(Millar Inc, SPR-869) was inserted and advanced into the left ventricle to record cardiac parameters. In later experiments, after heart catheterisation, the femoral artery on the left leg was then exposed and a catheter inserted to measure arterial pressure. A dose of either saline (0.9%, 0.5 mL, Macopharma, n = 12) or the MM202-AlbudAb conjugate (5 nmol, 0.5 mL, n = 15) was first administered to randomly chosen animals. After recovery of baseline measurements, some animals then received a second dose consisting of [Pyr1]apelin-13 (50 nmol, 0.5 mL, n = 12). All responded except the first two animals, one of which was then given an additional dose of [Pyr1]apelin-13 from a different batch to which it responded robustly. It was suspected that this first batch of [Pyr1]apelin-13 had expired and these two animals were excluded from the second dose analysis. All doses were followed by a saline flush (0.9%, 0.1 mL; Macopharma) to clear the cannula. Data were acquired using the MPVS Ultra system (ADInstruments), and analysis was performed with LabChart 8 (ADInstruments). The maximal change in left ventricular systolic pressure (LVSP), systolic arterial pressure, stroke volume, cardiac output, heart rate, contractility (dp/dt_max) and lusitropy (dp/dt_min) from baseline was calculated from the traces. Euthanasia was by exsanguination under high-flow isoflurane (5%).

2.6 | Statistical analysis

All data are presented as mean ± SEM, and statistical analysis was performed with GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA) unless otherwise stated. Binding and cell-based functional assays were performed in triplicate where possible. To test binding to HSA, two Biacore chips were used per compound. For in vivo analysis, cardiovascular parameters obtained in response to MM202-AlbudAb administration were compared to saline controls using a two-tailed Student's t test. Statistical significance was taken as 5%.

3 | RESULTS

3.1 | Apelin-17 displayed similar binding and potency in functional assays to [Pyr1]apelin-13

Apelin-17 bound to the native human apelin receptor in left ventricular homogenates (pKᵦ = 9.57 ± 0.08) with approximately sevenfold greater binding affinity than [Pyr1]apelin-13 (pKᵦ = 8.83 ± 0.06) (Figure 2 and see also Table S3). In functional assays, whereas [Pyr1]apelin-13 was approximately tenfold more potent in the cAMP inhibition (pD₂ = 9.52 ± 0.05) than β-arrestin (pD₂ = 8.53 ± 0.03) signalling assay, apelin-17 was equipotent in both (pD₂ = 10.31 ± 0.28 and 10.15 ± 0.13, respectively; Figure 3A,B and see also Table S5).

3.2 | MM202 and the MM202-AlbudAb conjugate bound with high affinity comparable to the endogenous agonist, [Pyr1]apelin-13, at the native human apelin receptor

In radioligand competition assays, MM202 bound to the native human apelin receptor in left ventricular homogenates with high nanomolar affinity (pKᵦ = 9.80 ± 0.06). This was tenfold more strong than the endogenous peptide, [Pyr1]apelin-13 (pKᵦ = 8.83 ± 0.06). Conjugating MM202 to AlbudAb slightly decreased the binding affinity of MM202 (pKᵦ = 9.39 ± 0.09; Figure 2 and Table S4). Additional data for binding experiments with [Pyr1]apelin-13 and MM202 conjugated to spacers/linkers are given in Figure S3, Tables S3 and S4.

3.3 | MM202 and the MM202-AlbudAb conjugate displayed functional activity in cell-based signalling assays with similar or greater potency than the endogenous agonist [Pyr1]apelin-13

MM202 was able to recruit β-arrestin to the human apelin receptor in a CHO cell signalling assay (pD₂ = 10.71 ± 0.58) with much greater potency than the endogenous agonist [Pyr1]apelin-13 (pD₂ = 8.53 ± 0.03) (Figure 3A and Table S5). It was also able to inhibit cAMP stimulated by forskolin (pD₂ = 9.96 ± 0.14) with similar potency to [Pyr1]apelin-13 (pD₂ = 9.52 ± 0.05; Figure 3B and Table S5). [Pyr1]apelin-13 was therefore tenfold more potent in the G protein-coupled assay compared to the β-arrestin recruitment assay, whereas MM202 displayed a degree of β-arrestin signalling bias compared to the endogenous peptide. In both assays, the $E_{\text{max}}$ values for MM202 were similar to [Pyr1]apelin-13, suggesting it acts as a full agonist through both pathways. Conjugating AlbudAb to MM202 reduced potency in the β-arrestin assay (pD₂ = 9.26 ± 0.03).
but to a lesser extent in the cAMP inhibition assay ($pD_2 = 9.15 \pm 0.12$), leading to a reduction in the degree of biased signalling at the receptor. The conjugate also acted as a full agonist at the receptor. Data for [Pyr$^1$]apelin-13 and MM202 conjugated to spacers/linkers are also given in Table S5.

### 3.4 Both AlbudAb and the MM202-AlbudAb conjugate bound to immobilised human serum albumin

Both AlbudAb and MM202-AlbudAb bound to the directly immobilised HSA on the chip surface and were not limited by mass transport as demonstrated by the sensograms obtained (Figure S4A,B). AlbudAb bound with high nanomolar affinity ($pK_D = 9.27, n = 2$), and conjugating MM202 to the AlbudAb molecule only very slightly reduced the binding affinity to HSA ($pK_D = 9.02, n = 2$; Table S6).

### 3.5 Acute cardiovascular responses to the MM202-AlbudAb conjugate in normotensive male Sprague Dawley rats

Bolus injections of the MM202-AlbudAb conjugate (5 nmol, 0.5 mL) through the jugular vein resulted in robust cardiovascular responses compared to saline in normotensive male Sprague Dawley rats (Figure 4). There was a small but significant difference in weight between the two groups with the treatment group 24.7 ± 8.60g ($P < 0.01$) heavier than the saline group. The left ventricular systolic pressure...
(LVSP) was reduced by 6.61 ± 1.46 mm Hg (P < 0.001), and the systolic arterial pressure underwent a greater drop of 14.12 ± 3.35 mm Hg (P < 0.01). The dp/dt max, a measure of cardiac contractility, increased by 533 ± 170 mm Hg/s (P < 0.01); meanwhile, the dp/dt min, a measure of cardiac relaxation (where a negative value indicates greater relaxation), increased by a smaller margin of 381 ± 178 mm Hg (P < 0.05, data not shown). Additionally, the cardiac output increased by 1277 ± 190 RVU/min (P < 0.0001) and this was mirrored by an increase in stroke volume of 3.09 ± 0.47 RVU (P < 0.0001), as well as a small but significant increase in heart rate of 4.64 ± 2.24 bpm (P < 0.05). These effects were very similar to those obtained with MM202 alone at the same concentration (Methods and Data File, Table S8).

Following either the saline or MM202-AlbudAb conjugate, [Pyr1]apelin-13 (5.0 nmol, 0.5 mL) was administered to all animals. The pattern of responses to [Pyr1]apelin-13 was similar to that of the MM202-AlbudAb conjugate, and there were no significant differences between those that had received saline first or MM202-AlbudAb (Figure S7). The LVSP was reduced by 12.89 ± 2.21 mm Hg and 11.64 ± 2.58 mm Hg and the systolic arterial pressure by 24.75 ± 1.47 mm Hg and 31.18 ± 5.73 mm Hg in the saline and MM202-AlbudAb groups, respectively. Similarly, the dp/dt max was increased by 1660 ± 210 mm Hg/s and 890 ± 434 mm Hg/s and the dp/dt min by 658 ± 499 mm Hg/s and 1553 ± 494 mm Hg/s, respectively. Once again, the cardiac output increased, 3143 ± 707 RVU/min and 2492 ± 620 RVU/min, with a concurrent stroke volume increase, 7.93 ± 1.96 RVU and 6.38 ± 1.32 RVU, and a heart rate increase, 3.43 ± 2.91 bpm and 2.98 ± 7.29 bpm, respectively.

## DISCUSSION

### Spacer and apelin-17

The 17 amino acid peptide corresponding to the sequence 61-77 of pre-proapelin (apelin-17) bound to the native human apelin receptor with slightly higher affinity than [Pyr1]apelin-13 (corresponding to the N-terminally pyroglutamated form of 65-77 of pre-proapelin). In functional assays, apelin-17 was similarly slightly more potent than [Pyr1]apelin-13 but displayed a different signalling profile as has previously been suggested.33 The fact that additional amino acids did not reduce binding affinity or functional activity in cell-based signalling assays confirms tolerance to the addition of a spacer to apelin-13 at the N-terminus, if required. Additionally, extensive homology modelling implies that the amino acids at the N-terminus added to the RPRL recognition sequence project beyond the receptor binding region into solvent and associate with the extracellular loop of the apelin receptor. This suggests that an appropriate linker at the N-terminus should allow apelin binding while not causing interference by the conjugated antibody.

### MM202 and MM202-AlbudAb bound to the human apelin receptor and show functional activity

MM202 bound to the apelin receptor with high affinity and showed activity in cell-based functional assays. The high activity in the β-arrestin assay likely correlated with the higher binding affinity and is something we have observed before, for instance, with apelin-17. It is possible that the longer peptides, which have increased binding affinity, stabilise regions of the apelin-13 sequence within the binding pocket (the message in the “message-address hypothesis”34) which increases β-arrestin signalling.33,35,36 In the apelin-13 sequence, we hypothesise that the address is the RPRL region and the message is the HKGPMPF region. Similarly, this may explain why smaller molecules, such as the small molecule CMF-019, display such remarkably high G protein bias as they are unable to stabilise the required conformation of the apelin GPCR for β-arrestin signalling.34

The addition of various linker motifs to MM202 did not result in a large loss in binding affinity at the apelin receptor, nor did combining these with AlbudAb. This suggests the linkers did not directly affect binding to the receptor but were able to provide sufficient mobility with reduced steric hindrance around the apelin binding pocket. In fact, although conjugating AlbudAb to MM202 reduced the binding affinity to the receptor slightly, it led to a much greater decrease in β-arrestin signalling compared to cAMP signalling. In this case, it is probable that the steric bulk of the AlbudAb molecule would partially restrict access of MM202 to the receptor, though this would largely be negated by the PEG spacer, resulting in reduced signalling of the message sequence and a consequent small loss in affinity but a proportionally larger decrease in β-arrestin signalling. Fortuitously, reduced signalling through the β-arrestin pathway is something that has been considered beneficial for apelin mimetics.6,34,38

### Conjugating AlbudAb to apelin peptides retained AlbudAb activity at HSA

The ability of the AlbudAb moiety to bind to HSA was retained as shown by its binding to immobilised HSA on the Biacore T200 chip. These results are consistent with previous AlbudAb conjugates which also showed retained activity.

### MM202-AlbudAb was active acutely in vivo

Injecting MM202-AlbudAb intravenously through the jugular vein resulted in robust cardiovascular responses as expected for an apelin agonist. The LVSP was reduced, and...
this was likely due to a decrease in arterial pressure, leading to a reduced afterload on the heart. At the same time, the cardiac output increased in concert with the stroke volume and there was a small increase in heart rate, though this was probably not the driving factor in increasing cardiac output. Finally, the contractility of the heart (measured as change in maximal pressure over time, $dP/dt_{max}$) was also increased, as predicted for a positive inotrope. These cardiovascular changes (apart from the small increase in heart rate) were also seen when [Pyr$^1$]apelin-13 was given following the initial dose of either saline or MM202-AlbudAb. The fact that there was little difference in the [Pyr$^1$]apelin-13 responses following either treatment suggests that there was little desensitisation of the receptor by MM202-AlbudAb. The responses to 5 nmol MM202-AlbudAb were smaller than those to 50 nmol [Pyr$^1$]apelin-13; this is not surprising as MM202-AlbudAb displayed similar activity in binding to the receptor and in functional assays. Doses higher than 5 nmol could not be delivered as the AlbudAb conjugate was limited by its solubility in saline. Moreover, one would expect that the high HSA binding of MM202-AlbudAb could limit the amount able to reach the cardiovascular tissues acutely, though this could have been balanced by protection from degradation. Importantly, the magnitude of the effects of 5 nmol MM202-AlbudAb was comparable to those obtained with 5 nmol MM202, suggesting that the plasma protein–bound AlbudAb molecule also retained the ability to bind to the receptor.

The aim of the study was to pharmacologically characterise a chemically conjugated apelin mimetic peptide containing unnatural amino acids linked to an AlbudAb moiety, in order to demonstrate retained activity of both constituent parts. Such a construct would represent a significant advance in use of the AlbudAb platform which has previously only used genetic conjugation of endogenous molecules, for example, the GLP-1 AlbudAb construct GSK2374697. In this study, it has been demonstrated that MM202-AlbudAb retained a high nanomolar binding affinity to the human apelin receptor, as well as activity in vitro cell screening assays. Meanwhile, the AlbudAb conjugate also retained the ability to bind to human serum albumin in vitro and displayed an improved plasma half-life over both its parent compound and the endogenous agonist. Finally, MM202-AlbudAb was active in vivo and demonstrated similar activity to both the endogenous molecule, [Pyr$^1$]apelin-13, and MM202. Crucially, such a study opens the possibility that additional modified peptides could be used in concert with the AlbudAb platform by chemical rather than genetic conjugation, therefore allowing for the use of proteolytically protected molecules. Hopefully, with such advances, other short peptide agonists can be adapted into more suitable therapeutics by avoiding both peptidases and renal filtration.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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