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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 ($pK_i = 9.39 \pm 0.09$) bound with similar high affinity as the endogenous peptides [Pyr^1]apelin-13 ($pK_i = 8.83 \pm 0.06$) and apelin-17 ($pK_i = 9.57 \pm 0.08$). [Pyr^1]apelin-13 was tenfold more potent in the cAMP ($pD_2 = 9.52 \pm 0.05$) compared to the β-arrestin ($pD_2 = 8.53 \pm 0.03$) assay, whereas apelin-17 ($pD_2 = 10.31 \pm 0.28$; $pD_2 = 10.15 \pm 0.13$, respectively) and MM202-AlbudAb ($pD_2 = 9.15 \pm 0.12$; $pD_2 = 9.26 \pm 0.03$, 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 ($pK_D = 9.02$). 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

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4.64 ± 2.24 bpm. This study demonstrates that conjugating an apelin mimetic peptide to the AlbudAb structure retains receptor and in vivo activity and may be a new strategy for development of apelin peptides as therapeutic agents.

KEYWORDS
AlbudAb, apelin, cardiovascular, G protein-coupled receptor, in vivo

1 | INTRODUCTION AND BACKGROUND

The apelin receptor is a class A G protein-coupled receptor first identified in 1993.[1] Its cognate ligand apelin was identified in 1998,[2] and since then, a further ligand, Elabela/Toddler (ELA), has been found.[3,4] 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 [Pyr^1]apelin-13[5] and exogenous administration results in vasodilatation[5,6] and cardiac inotropy[8,9] without hypertrophy. It has proven beneficial in animal models of both heart failure[10-12,15] and PAH.[17] The most abundant form of ELA has not yet been identified; however, exogenous administration of ELA peptides results in similar activity to apelin,[18,19] with ELA-32, the longest predicted circulating isoform, displaying greater potency and a beneficial role in a rat model of PAH.[21]

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 minute compared to 2 minute. for [Pyr^1]apelin-13, in addition to introducing a beneficial bias profile to the molecule.[6] In this study, we have utilised unnatural amino acid addition and PEG spacers to reduce proteolysis, as has previously been demonstrated.[22,23] Furthermore, we have conjugated an apelin mimetic peptide to an anti-serum albumin domain antibody (AlbudAb) to extend transit time in the blood. AlbudAbs have previously been conjugated to soluble IL-1Rα,[25] GLP-1[26,27] and TNF-R1[29] and demonstrated half-life extension with retained activity. To our knowledge, this is the first time the AlbudAb moiety has been chemically rather than genetically conjugated to a ligand. Furthermore, it is the first time a non-endogenous peptide or receptor has been used.

Here, we conjugated [Pyr^1]apelin-13 and MM202, an apelin mimetic containing unnatural amino acids, to a PEG linker and an AlbudAb moiety. We pharmacologically characterised the addition of each substituent, illustrating how the binding and signalling profile of the molecules changed. As proof of concept, we carried out additional in vitro and in vivo studies with the MM202-AlbudAb conjugate. This molecule demonstrated the improved half-life desired, while retaining a high binding affinity to the human apelin receptor, as well as functional activity in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Materials

Chemicals were obtained from Sigma-Aldrich Co. Ltd (Poole, UK) unless otherwise stated. [Pyr^1]apelin-13 (Glp-RPRLSHKGPMPF), apelin-17, MM202, [Pyr^1]apelin-13-AlbudAb and MM202-AlbudAb conjugates (Figure 1) were synthesized by Severn Biotech (Kidderminster, UK). [Glp^{65},Nle^{75},Tyr^{77}][^{125}I]apelin-13 was from PerkinElmer (MA).

[Pyr^1]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).[30] 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 [Pyr^1]apelin-13-AlbudAb or MM202-AlbudAb conjugate. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[31] Human tissues were obtained with informed consent from the Papworth Hospital Research Tissue Bank (08/H0304/56) and local ethical approval (05/Q104/142). All experiments conformed to the principles outlined in the Declaration of Helsinki. The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol. All animal studies were ethically reviewed and carried out in accordance with

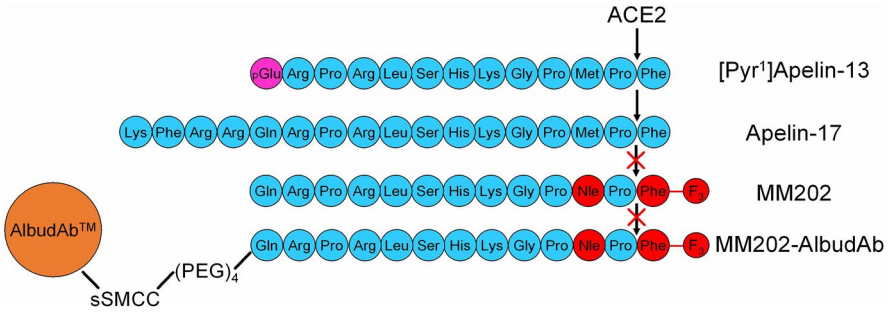


FIGURE 1 The structures of the endogenous peptides, [Pyr^1]apelin-13 and apelin-17, the analogue, MM202, and the MM202-AlbudAb conjugate. Substituted residues in MM202 are shown in red, pyroglutamate in pink and AlbudAb in orange. MM202 features a norleucine substituted for methionine to improve stability by reducing propensity for oxidation during subsequent reaction stages. Meanwhile, the 3,4,5 trifluorophenylalanine improves binding affinity and stability and may reduce C-terminal cleavage by ACE2. In the AlbudAb construct, MM202 has first been linked to a polyethylene glycol (PEG) chain and then via a sulpho-SMCC (sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) linker to the AlbudAb moiety using maleimide chemistry

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 [Glp^{65},Nle^{75},Tyr^{77}][^{125}I]apelin-13 (0.1 nmol/L) and increasing concentrations of the unlabelled competitive agonist as previously described.[6] Binding in the presence of 2 μmol/L of [Pyr^1]apelin-13 was considered to be non-specific.

2.3 | Cell-based functional assays

β-Arrestin 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.[6] Agonist responses were measured as relative light units (RLUs) detected and expressed relative to the maximum response (E_{max}) of the endogenous agonist, [Pyr^1]apelin-13, in the β-arrestin 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 E_{max} and pD_2 values ($-\log_{10}$ of the EC_{50} [concentration producing 50% of the E_{max}]) 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 (K_a), dissociation rates (K_d) 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.[32] 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 [Pyr^1]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 [Pyr^1]apelin-13 from a different batch to which it responded robustly. It was suspected that this first batch of [Pyr^1]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-AbudAb 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 [Pyr^1]apelin-13

Apelin-17 bound to the native human apelin receptor in left ventricular homogenates ($pK_i = 9.57 \pm 0.08$) with approximately sevenfold greater binding affinity than [Pyr^1]apelin-13 ($pK_i = 8.83 \pm 0.06$) (Figure 2 and see also Table S3). In functional assays, whereas [Pyr^1]apelin-13 was approximately tenfold more potent in the cAMP inhibition ($pD_2 = 9.52 \pm 0.05$) than β-arrestin ($pD_2 = 8.53 \pm 0.03$) signalling assay, apelin-17 was equipotent in both ($pD_2 = 10.31 \pm 0.28$ and 10.15 ± 0.13, respectively; Figure 3A,B and see also Table S5).

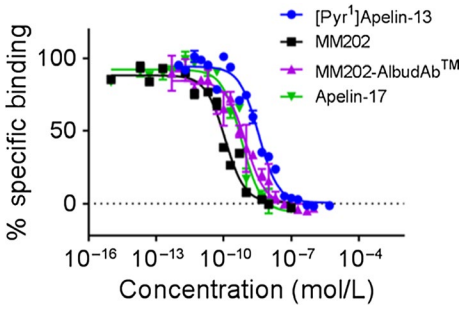


FIGURE 2 Competition radioligand binding of the endogenous peptides [Pyr^1]apelin-13 and apelin-17, as well as the synthetic peptide MM202 and its AlbudAb conjugate against [Glp65,Nle75,Tyr77][^{125}I] apelin-13 in human left ventricular heart homogenates. All peptides competed with similar nanomolar affinities at the human apelin receptor

3.2 | MM202 and the MM202-AlbudAb conjugate bound with high affinity comparable to the endogenous agonist, [Pyr^1]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 ($pK_i = 9.80 \pm 0.06$). This was tenfold more strong than the endogenous peptide, [Pyr^1]apelin-13 ($pK_i = 8.83 \pm 0.06$). Conjugating MM202 to AlbudAb slightly decreased the binding affinity of MM202 ($pK_i = 9.39 \pm 0.09$; Figure 2 and Table S4). Additional data for binding experiments with [Pyr^1]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 [Pyr^1]apelin-13

MM202 was able to recruit β-arrestin to the human apelin receptor in a CHO cell signalling assay ($pD_2 = 10.71 \pm 0.58$) with much greater potency than the endogenous agonist [Pyr^1]apelin-13 ($pD_2 = 8.53 \pm 0.03$) (Figure 3A and Table S5). It was also able to inhibit cAMP stimulated by forskolin ($pD_2 = 9.96 \pm 0.14$) with similar potency to [Pyr^1]apelin-13 ($pD_2 = 9.52 \pm 0.05$; Figure 3B and Table S5). [Pyr^1]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_{max} values for MM202 were similar to [Pyr^1]apelin-13, suggesting it acts as a full agonist through both pathways. Conjugating AlbudAb to MM202 reduced potency in the β-arrestin assay ($pD_2 = 9.26 \pm 0.03$)

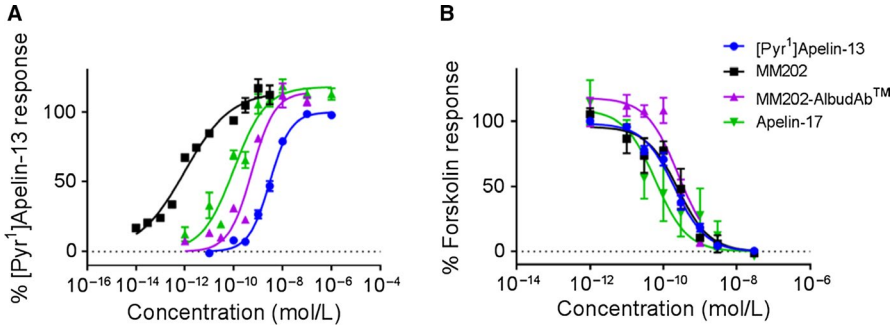


FIGURE 3 The functional activities of the endogenous peptides [Pyr^1]apelin-13 and apelin-17, as well as the synthetic peptide MM202 and its AlbudAb conjugate in human apelin receptor expressing CHO cell. (A) β-arrestin recruitment and (B) cAMP inhibition assays. In the β-arrestin assay, the potencies generally corresponded to the rank order of the binding affinities; however, for cAMP inhibition, they all displayed similar activity

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 sensorgrams 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

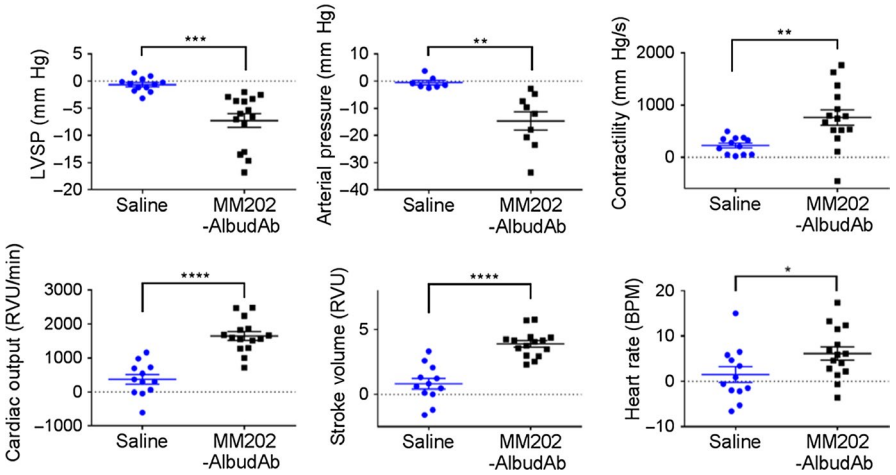


FIGURE 4 In vivo cardiovascular responses in normotensive male Sprague Dawley rats to administration of either saline (n = 12) or MM202-AlbudAb (5 nmol, n = 15). Compared to saline, MM202-AlbudAb reduced the left ventricular systolic pressure (LVSP, 6.61 ± 1.46 mm Hg, $P < 0.001$) and the systolic arterial pressure (14.12 ± 3.35 mm Hg, $P < 0.01$); meanwhile, it increased the dp/dt_{max} (533 ± 170 mm Hg/s, $P < 0.01$), the cardiac output (1277 ± 190 RVU/min, $P < 0.0001$), the stroke volume (3.09 ± 0.47 RVU, $P < 0.0001$) and the heart rate (4.64 ± 2.24 bpm, $P < 0.05$). Data were compared using a two-tailed Student's *t* test, and statistical significance was taken as 5%
* indicates significance of $P<0.05$; **indicates significance of $P<0.01$; ***indicates significance of $P<0.001$; ****indicates significance of $P<0.0001$

(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 but 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, [Pyr^1]apelin-13 (5 0 nmol, 0.5 mL) was administered to all animals. The pattern of responses to [Pyr^1]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.

4 | DISCUSSION

4.1 | 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 [Pyr^1]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 [Pyr^1]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.

4.2 | 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]

4.3 | 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.

4.4 | 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 conjugate is available for binding 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 molecule, 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 in vitro cell screening assays. Meanwhile, the AlbudAb moiety 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 up 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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DISCLOSURES

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REFERENCES

1. O'Dowd BF, Heiber M, Chan A, et al. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene*. 1993;136:355-360.
2. Tatemoto K, Hosoya M, Habata Y, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun*. 1998;251:471-476.
3. Chng SC, Ho L, Tian J, Reversade B. ELABELA: a hormone essential for heart development signals via the apelin receptor. *Dev Cell*. 2013;27:672-680.
4. Pauli A, Norris Ml, Valen E, et al. Toddler: an embryonic signal that promotes cell movement via Apelin receptors. *Science*. 2014;343:1248636.
5. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension*. 2009;54:598-604.
6. Brame AL, Maguire JJ, Yang P, et al. Design, characterization, and first-in-human study of the vascular actions of a novel biased Apelin receptor agonist. *Hypertension*. 2015;65:834-840.
7. Tatemoto K, Takayama K, Zou MX, et al. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept*. 2001;99:87-92.
8. Szokodi I, Tavi P, Földes G, et al. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res*. 2002;91:434-440.
9. Perjés A, Skoumal R, Tenhunen O, et al. Apelin increases cardiac contractility via protein kinase Cepsilon- and extracellular signal-regulated kinase-dependent mechanisms. *PLoS ONE*. 2014;9:e93473.
10. Berry MF, Pirolli TJ, Jayasankar V, et al. Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation*. 2004;110:187-193.
11. Atluri P, Morine K, Liao G, et al. Ischemic heart failure enhances endogenous myocardial apelin and APJ receptor expression. *Cell Mol Biol Lett*. 2007;12:127-138.

12. Jia Y-X, Pan C-S, Zhang J, et al. Apelin protects myocardial injury induced by isoproterenol in rats. *Regul Pept*. 2006;133:147-154.
13. Ashley E, Powers J, Chen M, et al. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. *Cardiovasc Res*. 2005;65:73-82.
14. Japp AG, Cruden NL, Amer D, et al. Vascular effects of apelin in vivo in man. *J Am Coll Cardiol*. 2008;52:908-913.
15. Koguchi W, Kobayashi N, Takeshima H, Ishikawa M, Sugiyama F, Ishimitsu T. Cardioprotective effect of apelin-13 on cardiac performance and remodeling in end-stage heart failure. *Circ J*. 2012;76:137-144.
16. Pang H, Han B, Yu T, Zong Z. Effect of apelin on the cardiac hemodynamics in hypertensive rats with heart failure. *Int J Mol Med*. 2014;34:756-764.
17. Falcão-Pires I, Gonçalves N, Henriques-Coelho T, Moreira-Gonçalves D, Roncon-Albuquerque R Jr, Leite-Moreira AF. Apelin decreases myocardial injury and improves right ventricular function in monocrotaline-induced pulmonary hypertension. *Am J Physiol Heart Circ Physiol*. 2009;296:H2007-H2014.
18. Yang P, Maguire JJ, Davenport AP. Apelin, Elabela/Toddler, and biased agonists as novel therapeutic agents in the cardiovascular system. *Trends Pharmacol Sci*. 2015;36:560-567.
19. Perjés Á, Kilpiö T, Ulvila J, et al. Characterization of apela, a novel endogenous ligand of apelin receptor, in the adult heart. *Basic Res Cardiol*. 2016;111:2.
20. Murza A, Sainsily X, Coquerel D, et al. Discovery and structure-activity relationship of a bioactive fragment of ELABELA that modulates vascular and cardiac functions. *J Med Chem*. 2016;59:2962-2972.
21. Yang P, Read C, Kuc RE, et al. Elabela/Toddler is an endogenous agonist of the Apelin APJ receptor in the adult cardiovascular system, and exogenous administration of the peptide compensates for the downregulation of its expression in pulmonary arterial hypertension. *Circulation*. 2017;135:1160-1173.
22. Murza A, Parent A, Besserer-Offroy E, et al. Elucidation of the structure-activity relationships of apelin: influence of unnatural amino acids on binding, signaling, and plasma stability. *Chem Med Chem*. 2012;7:318-325.
23. Murza A, Belleville K, Longpre JM, Sarret P, Marsault E. Stability and degradation patterns of chemically modified analogs of apelin-13 in plasma and cerebrospinal fluid. *Biopolymers*. 2014;102:297-303.
24. Jia ZQ, Hou L, Leger A, et al. Cardiovascular effects of a PEGylated apelin. *Peptides*. 2012;38:181-188.
25. Holt LJ, Basran A, Jones K, et al. Anti-serum albumin domain antibodies for extending the half-lives of short lived drugs. *Protein Eng Des Sel*. 2008;21:283-288.
26. O'Connor-Semmes RL, Lin J, Hodge RJ, et al. GSK2374697, a novel albumin-binding domain antibody (AlbudAb), extends systemic exposure of exendin-4: first study in humans–PK/PD and safety. *Clin Pharmacol Ther*. 2014;96:704-712.
27. Bao W, Holt LJ, Prince RD, et al. Novel fusion of GLP-1 with a domain antibody to serum albumin prolongs protection against myocardial ischemia/reperfusion injury in the rat. *Cardiovasc Diabetol*. 2013;12:148.
28. Lin J, Hodge RJ, O'Connor-Semmes RL, Nunez DJ. GSK2374697, a long duration glucagon-like peptide-1 (GLP-1) receptor agonist, reduces postprandial circulating endogenous total GLP-1 and peptide YY in healthy subjects. *Diab Obes Metab*. 2015;17:1007-1010.
29. Goodall LJ, Ovecka M, Rycroft D, et al. Pharmacokinetic and pharmacodynamic characterisation of an anti-mouse TNF receptor 1 domain antibody formatted for in vivo half-life extension. *PLoS ONE*. 2015;10:e0137065.
30. De Angelis E, Enever C, Liu H, Pupecka-Swider M, Schon O. Improved anti-serum albumin binding variants. Google Patents. 2012.
31. Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J. Basic & clinical pharmacology & toxicology policy for experimental and clinical studies. *Basic Clin Pharmacol Toxicol*. 2018;123:233-235.
32. Pacher P, Nagayama T, Mukhopadhyay P, Batkai S, Kass DA. Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc*. 2008;3:1422-1434.
33. El Messari S, Iturrioz X, Fassot C, De Mota N, Roesch D, Llorens-Cortes C. Functional dissociation of apelin receptor signaling and endocytosis: implications for the effects of apelin on arterial blood pressure. *J Neurochem*. 2004;90:1290-1301.
34. Read C, Fitzpatrick CM, Yang P, et al. Cardiac action of the first G protein biased small molecule apelin agonist. *Biochem Pharmacol*. 2016;116:63-72.
35. Murza A, Besserer-Offroy É, Côté J, et al. C-Terminal modifications of apelin-13 significantly change ligand binding, receptor signaling, and hypotensive action. *J Med Chem*. 2015;58:2431-2440.
36. Iturrioz X, Gerbier R, Leroux V, Alvear-Perez R, Maigret B, Llorens-Cortes C. By interacting with the C-terminal Phe of apelin, Phe255 and Trp259 in helix VI of the apelin receptor are critical for internalization. *J Biol Chem*. 2010;285:32627-32637.
37. Ceraudo E, Galanth C, Carpentier E, et al. Biased signaling favoring gi over beta-arrestin promoted by an apelin fragment lacking the C-terminal phenylalanine. *J Biol Chem*. 2014;289:24599-24610.
38. Yang P, Read C, Kuc RE, et al. Cyclic biased agonist of the Apelin receptor, MM07, is disease modifying in the rat monocrotaline model of pulmonary arterial hypertension. *Br J Pharmacol*. 2019. https//:doi.org/10.1111/bph.14603. [Epub ahead of print].

SUPPORTING INFORMATION

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

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