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Short communication

Plasma kallikrein cleaves and inactivates apelin-17: Palmitoyl- and PEG-extended apelin-17 analogs as metabolically stable blood pressure-lowering agents

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ABSTRACT

Apelins are human peptide hormones with various physiological activities, including the moderation of cardiovascular, renal, metabolic and neurological function. Their potency is dependent on and limited by proteolytic degradation in the circulatory system. Here we identify human plasma kallikrein (KLKB1) as a protease that cleaves the first three N-terminal amino acids (KFR) of apelin-17. The cleavage kinetics are similar to neprilysin (NEP), which cleaves within the critical 'RPRL'-motif thereby inactivating apelin. The resulting C-terminal 14-mer after KLKB1 cleavage has much lower biological activity, and the presence of its N-terminal basic arginine seems to negate the blood pressure lowering effect. Based on C-terminally engineered apelin analogs (A2), resistant to angiotensin converting enzyme 2 (ACE2), attachment of an N-terminal C16 fatty acid chain (PALMitoylation) or polyethylene glycol chain (PEGylation) minimizes KLKB1 cleavage of the 17-mers, thereby extending plasma half-life while fully retaining biological activity. The N-terminally PEGylated apelin-17(A2) is a highly protease resistant analog, with excellent apelin receptor activation and pronounced blood pressure lowering effect.

1. Introduction

The metabolic syndrome [1] and resulting implications including cardiovascular diseases [2] are significant health concerns and major causes of mortality [3]. In the search for endogenous targets to combat these diseases, the apelinergic system has been identified as key regulating machinery for energy metabolism and cardiovascular health [4,5]. Apelins are endogenous peptide hormones and ligands for APJ, a G-protein-coupled receptor. They are crucial in regulating cardiovascular functions, fluid homeostasis and carbohydrate and fat metabolism [6–8]. Apelin is expressed as a 77-amino acid prepropeptide, that is further processed to bioactive C-terminal fragments including apelin-13 (or the N-terminal pyroglutamate pyr-apelin-13), apelin-17 and apelin-36. Whereas pyr-apelin-13 is the predominant form in human plasma [9], both pyr-apelin-13 and apelin-17 are dominant in rat plasma [10]. Apelin isoforms have an average lifetime in blood plasma of about 5 min [11], rendering their physiological activity profile. Angiotensin converting enzyme 2 (ACE2) [12,13] and the metalloprotease neprilysin (NEP) [14,15] have so far been unveiled as proteases that cleave and partially (ACE2) [16] or fully (NEP) inactivate apelin. Here we describe plasma kallikrein (KLKB1, EC 3.4.21.34) as another major hydrolytic enzyme, that contributes to the rapid destruction of apelin-17. Cleavage by this enzyme produces a C-terminal 14-mer lacking the polar basic KFR head group. *In vivo* tests show, that this apelin-14 peptide lacks significant blood-pressure lowering effects, in contrast to apelin-17. Several strategies, including insertion of non-natural amino acids [17,18], D-amino acid substitution [19], *N*– and α-C methylation [14,20] and internal macrocyclization [21] have been tested and shown beneficial in increasing the metabolic stability of various apelin peptides. The

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recently reported N-terminal PALMytoylation of apelin-13 and PEGylation of apelin-36 increased proteolytic stability and plasma half-life of these analogs [20,22]. However, this strategy has not yet been studied for apelin-17. Here we test N-terminal extension of apelin-17 by a PALM- or PEG-chain in order to increase metabolic stability towards both NEP and KLKB1 and the physiological activity of these novel apelin analogs.

2. Results and discussion

2.1. Elucidation of KLKB1 and kinetics

Our previous studies [14] on apelin-13 and apelin-17 revealed an additional fragment, when apelin-17A2 (compound **2**, Scheme 1) was incubated with human plasma for very short times (10–30 min, Fig. S1). Analysis of major breakdown products of **2** by LC-MS showed a dramatic increase of the 1–3 and 4–17 fragments, disregarding random amino exopeptidase breakdown. MALDI MS analysis confirmed that this C-terminal 14-mer (i.e. compound **4**, cleavage between Arg3-Arg4) was quickly observed even in the presence of the ACE2 inhibitor MLN-4760 (1 mM) [12], the metalloprotease inhibitor EDTA (10 mM) or thiorphan (10 mM, M13 protease family inhibitor), indicating that a different protease than ACE2 or NEP must be responsible for this cleavage.

Incubation of **2** with 10 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF) resulted in a disappearance of the 14-mer, suggesting the cleaving enzyme to be a serine protease [23,24]. Upon closer comparison of the cleavage characteristics (Phe in P2, basic Arg in P1 and a polar amino to acid in P1′, compare Figs. S3 and S4) we suspected the unknown protease to be plasma kallikrein (KLKB1) [25]. Indeed, plasma kallikrein has been shown to be involved in the cleavage and activation of various peptide hormones like kininogen [26], factor XII and Neuropeptide Y (NPY) [27], all of which trigger cardiovascular functions. However, no connection to date has been made to its involvement in apelin degradation. In the human body, KLKB1 circulates as zymogen bound to high-molecular-weight kininogen and is activated by coagulation factor XIIa [28]. Once activated, KLKB1 possesses a trypsin like activity, cleaving at a basic residue in P1 position (Arg), whereas the bulky hydrophobic Phe side chain at P2 can be accommodated in the S2 pocket of this enzyme [29]. Monitoring the decrease of parent peptide upon incubation of recombinant human KLKB1 (*rh*KLKB1) with native apelin-17 (compound **1**, Scheme 1) as well as ACE2 and NEP-stabilized apelin analogs **2** and **3** enabled us to extract the kinetic parameters for this cleavage (Table 1, S1). It appears that KLKB1 cleaves as effectively as NEP, and the enzyme processes apelin-17 (**1**) and analogs **2** and **3** with moderate efficiency ($k_{cat}/K_m \sim 10^5$), comparable to other cardiovascular active peptide substrates (Figs. S5–S7, Table S2). As the potent analog **3,** which is resistant to ACE2 and neprilysin, is degraded quickly, this stimulated an endeavor to explore a stabilization strategy closer to the Arg3-Arg4 cleavage site.

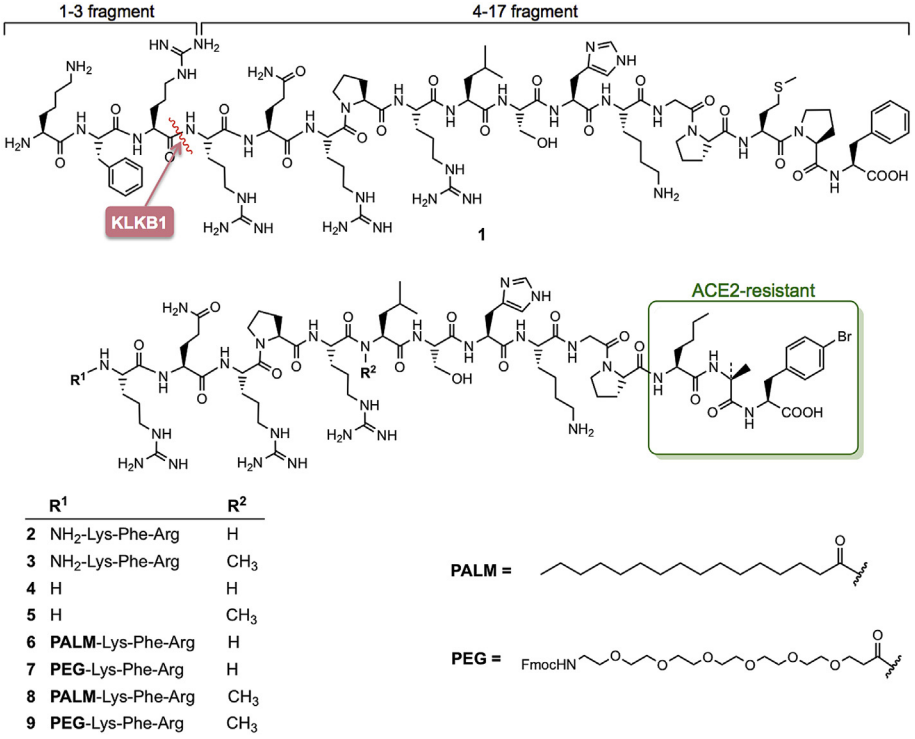
2.2. N-terminally PALMitoylated and PEGylated apelin analogs

We tested if N-terminal extension of apelin-17 by a fatty acid

Table 1
Kinetic parameters for NEP and KLKB1 cleavage of cardiovascular active peptides.

Substrate	Enzyme	K_m(μM)[a]	k_{cat} (1/s)[a]	k_{cat}/K_m (1/Ms)[a]
1	NEP	190 ± 40	3.4 ± 0.3	(1.8 ± 0.4) *10^4
1	KLKB1	97 ± 21	13.7 ± 1.3	(1.4 ± 0.3) *10^5
2	KLKB1	30 ± 4	1.4 ± 0.2	(4.7 ± 0.8) *10^4
3	KLKB1	245 ± 75	16.5 ± 2.1	(6.7 ± 1.0) *10^4
Kininogen [25]	KLKB1	0.75	0.031	4.1 *10^4
Factor XII [25]	KLKB1	2.4	0.001	4.2 *10^2

[a] Errors represent standard error of the mean (S.E.M).



Scheme 1. Compounds studied in this paper. KLKB1 cleaves apelin between Arg3-Arg4 (highlighted). Introduction of the ACE2-resistant C-terminus (incorporating L-norleucine, aminoisobutyric acid and 4-bromophenyl alanine) was chosen as basis for the analog design.

chain (PALM, $M_{w\text{-}gain}$ 240 g/mol) or a PEG_6-chain ($M_{w\text{-}gain}$ 558 g/mol) could minimize recognition and proteolytic degradation not just by the newly described KLKB1 but also by NEP. To negate the impact of ACE2 cleavage, the ACE2 resistant "A2" C-terminus, i.e. Nle-Aib-BrF, was incorporated into all modified analogs [12]. Synthetically, Fmoc-*p*-BrPhe-OH was loaded onto 2-chlorotrityl chloride resin and extended by solid phase peptide synthesis to the octapeptide stage. For each peptide, 0.2 mmol of the octapeptide was extended to the full 17-mer or to the 14-mer stage excluding (compounds **2**, **4**, **6**, **7**) or including the NEP-resistant Arg-*N*Me-Leu dipeptide (compounds **3**, **5**, **8**, **9**). The Fmoc-deprotected 17-mers were coupled with palmitic acid (**6**, **8**) or Fmoc-$(PEG)_6$-propanoic acid (**7**, **9**) in order to yield the title N-terminally palmitoylated and pegylated apelin analogs. All peptides were analyzed by MALDI and LC-MS and purified by preparative HPLC.

2.3. In vitro stability

We initially compared the *in vitro* NEP and KLKB1 stability of the PALM/PEG analogs to native apelin-17 (**1**), the A2 substituted analog (**2**) and the NEP-resistant analog **3**. Analogs **6**–**9** were incubated with recombinant human NEP (*rh*NEP, Sino Biological) or KLKB1 (*rh*KLKB1, Novoprotein) at 37 °C for up to 72 h, and the extent of degradation was analyzed via LC–MS because of the overlapping elution patterns of peptide fragments (NEP) (Table 2, Fig. S2). As previously reported, the *N*Me-Leu apelin analog **3** has a pronounced resistance to NEP with a half-life >30 h [15], compared to the native form **1** and the A2-analog **2**. This is also true for the N-terminally PALM/PEG analogs **8** and **9**, that have the *N*Me-Leu substitution. Also the PEGylated analog **7** without the stabilizing *N*Me-Leu has a significantly higher resistance towards NEP cleavage ($t_{1/2}$ >30 h), indicating a size-restriction in the substrate recognition of NEP. It is known, that neprilysin has just a modest active site pocket volume, generally limiting the substrate size to about 2500 g/mol [30]. Therefore it seems, that in addition to previously studied amino acid modification [17,19–21], N-terminal extension of apelins might also benefit NEP resistance. With regard to the cleavage capacity of KLKB1 it becomes obvious from Table 2, that all the N-terminally PALM/PEGylated analogs **6**–**9** have 2–6 fold longer half-lives than the native apelin-17. This effect gets more pronounced in human plasma. Plasma incubation of native apelin **1** and analogs **2**–**9** and subsequent HPLC analysis of the remaining peptide reveals that the half-life of the respective N-terminally extended peptides is 225–1350 times longer than for the native apelin-17 (Table 2). Thus, our findings underline that N-terminal extension is a potent strategy to increase the proteolytic stability of apelin-17, as has been shown in similar fashion for a N-terminally palmitoylated apelin-13 derivative [20]. Variations in the effective KLKB1 concentration and differences in its activation state might explain the discrepancy of apelin stability in plasma vs. isolated enzyme incubation assay.

2.4. Binding affinity of apelin analogs

We evaluated the ability of apelin-17 (**1**) or the different apelin analogs (**2** to **8**) to displace [^{125}I]-(Glp [1], Nle [11], Tyr [13]) apelin-13 (0.1 nM) binding on membrane preparations from CHO cells stably expressing the rat ApelinR-EGFP. As shown in Fig. 1, the apelin analogs corresponding to the compounds **2**, **3**, **4**, **5**, **7** and **9** were able to displace the radioligand from the rat apelin receptor-EGFP with IC_{50} values in the subnanomolar range similar to that of compound **1** ($IC_{50} = 0.025 \pm 0.013$ nM). The compounds **6** and **8** displayed IC_{50} values around the nanomolar range. Altogether, these data showed that all the apelin-17 analogs were able to interact with the rat apelin receptor at the same binding site than the natural ligand and are thus orthosteric ligands. In addition, N-terminal extension of apelin-17 by a palmitoyl or PEG chain does not impair receptor binding as previously shown with the addition of a fluorocarbon chain at the N-terminal part of apelin-17 [31].

2.5. Ca^{2+} mobilization assay

To study the extend to which the newly derived analogs trigger APJ-receptor activation, we used a fluorescence coupled calcium release assay including a recombinant human APJ-Ga16 receptor cell line. Reference apelin-17 (**1**) has the lowest EC_{50} value, comparable to its shorter isoform Apelin-13 [20]. Compounds **6**–**9** feature EC_{50} values in the low nanomolar range comparable to the N-terminally free apelin analogs **1**–**3** (Table 2, Fig. S8). This is in accordance with the results obtained for a N-terminally extended apelin-13 and apelin-36 analog by different groups [20,22]. Unlike palmitoylation within the apelin peptide sequence, which can be detrimental for receptor binding [20], N-terminal extension by a

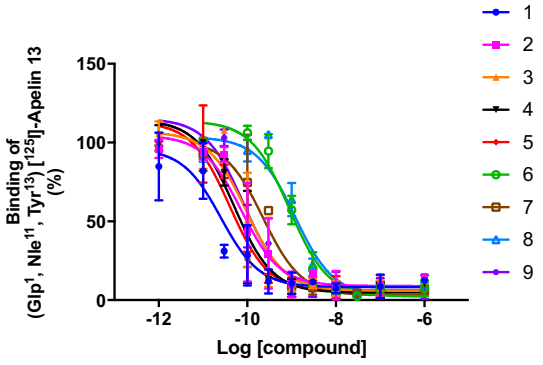


Fig. 1. Binding inhibition curves with apelin-17 analogs. Membranes of CHO cells stably expressing the rat ApelinR were incubated with 0.1 nM (Glp [1], Nle [11], Tyr [13])[^{125}I]-Apelin-13 in the presence of increasing concentrations of each peptide (from 1 pM to 1 μM). Data are expressed as percentage of maximal binding of (Glp [1], Nle [11], Tyr [13])[^{125}I]-Apelin-13 in the absence of any nonradiolabeled ligand ($n = 3$).

Table 2
Peptide half-life and receptor activation of studied compounds.

Apelin	Modification	$t_{1/2}$ (*rh*NEP, h)	$t_{1/2}$ (*rh*KLKB1, h)	$t_{1/2}$ (*h*plasma, h)	EC_{50}(nM)[a]	$\Delta MABP_{max}$ (mmHg)
1	Apelin-17	0.4	0.4	0.02	1.9 ± 1.0	−20
2	Apelin-17A2	1.0	0.7	0.3	4.1 ± 1.0	−32
3	NMeLeu-17A2	>30	0.9	1.2	4.9 ± 1.0	−36
4	Apelin-14A2	–	–	–	568 ± 34	+3
5	NMeLeu-14A2	–	–	–	176 ± 21	−15
6	PALM-17A2	1.5	1.0	4.5	2.6 ± 0.8	−41
7	PEG-17A2	>30	1.4	18	6.3 ± 1.0	−48
8	NMeLeu-PALM17A2	>30	2.1	20.5	11.3 ± 2.1	−12
9	NMeLeu-PEG17A2	>30	2.9	27	2.5 ± 1.5	−53

[a] Errors represent standard error of the mean (S.E.M).

palmitoyl or PEG chain seems not to affect receptor binding and internalization. To our surprise, the 14-mer left after KLKB1 cleavage, seems to be rather inactive featuring a 200-times higher EC_{50} than the 17-mers.

2.6. Physiological test—Blood pressure assay

Synthetic analogs **6**–**9** as well as the KLKB1-cleavage fragments **4** and **5** were tested for blood pressure lowering abilities in anesthetized mice. Apelin peptide analogs were delivered systemically via the right internal jugular vein while blood pressure (BP) and heart rate (HR) were continuously monitored in the aorta cannulated via the right carotid artery. All N-terminally extended apelin analogs, except **8**, show pronounced and time-stable blood pressure lowering effects (Fig. 2). Among the newly synthesized N-terminally extended apelin-17 analogs, **8** triggers also the lowest Ca^{2+} mobilization and features the weakest receptor binding. Interestingly, the heart rate increasing effect is restored in this compound, perhaps suggesting alternate activation pathways. In this regard, compounds **6, 7** and **9** are more potent than native apelin-17 (**1**) and the metabolically stabilized analogs **2** and **3**. Importantly, both PEGylated analogs **7** and **9** show the most stable and potent effect. In contrast, both the fragments left after KLKB1 cleavage (compounds **4** and **5**) are inactive or nearly so in their cardio-physiological effects. This inactivity correlates with the results obtained from the Ca^{2+} mobilization assay but contrasts with the receptor binding assay, where both C-terminal cleavage fragments show strong binding capability. This discrepancy between receptor binding and downstream effects may be due to different abilities of analogs to induce conformational changes in the receptor and/or induce alternative downstream activation pathways.

3. Conclusions

Human plasma kallikrein (KLKB1) has been identified as human protease, that cleaves apelin-17 relatively quickly between Arg3-Arg4. The resulting C-terminal 14-mer appears to be inactive with regard to Ca^{2+} mobilization (APJ receptor binding unaffected) and is lacking cardiophysiological effects. Hence KLKB1 degradation inactivates apelin 17. N-terminal extension by a palmitoyl or PEG_6 chain yields apelin analogs that are more cleavage resistant towards both KLKB1 and NEP proteases and have prolonged plasma half-life. Combined with their potent and time-stable blood pressure lowering effect, these new apelin analogs present a promising new target for the development of cardiovascular active peptide drugs. Further physiological studies and other synthetic stabilization approaches are currently underway to obtain a better understanding of the impact of this new cleavage site for the design of novel drug targets.

4. Experimental

4.1. General chemistry and peptide synthesis

General chemistry and full details of the synthesis and purification methods of analogs **4**–**9** can be found in the supplementary material. The attachment of the palmitoyl and PEG chain followed a literature procedure [20].

4.1.1. Apelin-14A2 (*4*)

Resin bound BrF was subjected to manual SPPS, introducing amino acids in the following order: Fmoc-Aib-OH, Fmoc-Nle-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)—OH, Fmoc-His(Trt)—OH, Fmoc-Ser(t-Bu)—OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)—OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)—OH, Fmoc-Glu(Trt)—OH, Fmoc-Arg(Pbf)—OH). A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and purified using a C18 RP-HPLC analytical column (method A), eluting at 10.0 min. The desired peptide was isolated as a white solid after lyophilization (9.0 mg, 11%). Monoisotopic MW calculated for $C_{75}H_{124}BrN_{27}O_{17}$1754.8925, found (FTICR-ESI-MS) 1754.8943 $(M+H)^+$.

4.1.2. Apelin-NMe14A2 (*5*)

Resin bound BrF was subjected to manual SPPS, introducing amino acids in the following order: Fmoc-Aib-OH, Fmoc-Nle-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)—OH, Fmoc-His(Trt)—OH, Fmoc-Ser(t-Bu)—OH, Fmoc-Arg(Boc_2)-NMeLeu-OH [15], Fmoc-Pro-OH, Fmoc-Arg(Pbf)—OH, Fmoc-Glu(Trt)—OH, Fmoc-Arg(Pbf)—OH). A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and purified using a C18 RP-HPLC analytical column (method A), eluting at 10.1 min. The desired peptide was isolated as a white solid after lyophilization (7.5 mg, 9%). Monoisotopic MW calculated for $C_{76}H_{127}BrN_{27}O_{17}$ 1768.9091, found (FTICR-ESI-MS) 1768.9119 $(M+H)^+$.

4.1.3. PALM-17A2 (*6*)

Compound **4** was extended by SPPS, introducing amino acids in the following order: Fmoc-Arg(Pbf)—OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)—OH, palmitic acid. A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and purified using a BiPhe RP-HPLC analytical column (method C), eluting at 12.0 min. The desired peptide was isolated as a white solid after

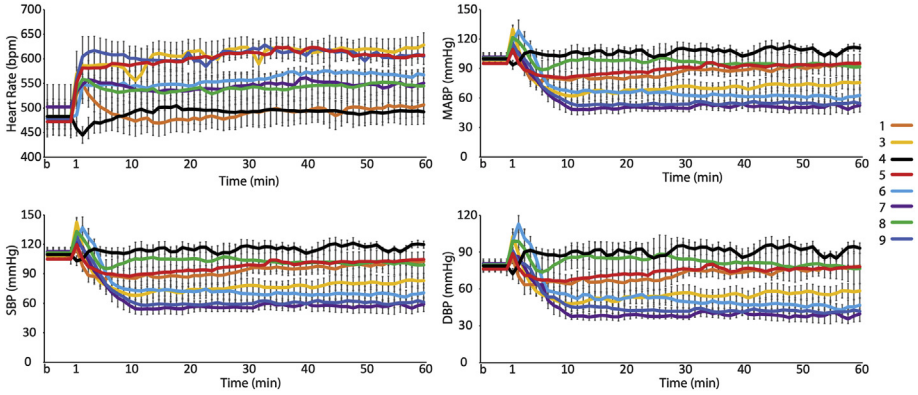


Fig. 2. *In vivo* mean arterial blood pressure (MABP), systolic (SBP), diastolic (DBP), and heart rate (HR) analyses following injection of native apelin-17 (**1**) or analogs **3**–**9** in anesthetized mice ($n = 5$).

lyophilization (5.0 mg, 8%). Monoisotopic MW calculated for $C_{112}H_{188}BrN_{34}O_{21}$ 606.8521, found (FTICR-ESI-MS) 606.8524 $(M + 4H)^{4+}$.

4.1.4. PEG-17A2 (**7**)

Compound **4** was extended by SPPS, introducing amino acids in the following order: Fmoc-Arg(Pbf)–OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)–OH, Fmoc-$(PEG)_6$ propionic acid. A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and purified using a BiPhe RP-HPLC analytical column (method C), eluting at 11.1 min. The desired peptide was isolated as a white solid after lyophilization (6.5 mg, 9%). Monoisotopic MW calculated for $C_{126}H_{197}BrN_{35}O_{29}$ 686.6103, found (FTICR-ESI-MS) 686.6093 $(M + 4H)^{4+}$.

4.1.5. PALM-NMe17A2 (**8**)

Compound **5** was extended by SPPS, introducing amino acids in the following order: Fmoc-Arg(Pbf)–OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)–OH, palmitic acid. A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and purified using a BiPhe RP-HPLC analytical column (method B), eluting at 11.9 min. The desired peptide was isolated as a white solid after lyophilization (5.5 mg, 7%). Monoisotopic MW calculated for $C_{113}H_{190}BrN_{34}O_{21}$ 610.3560, found (FTICR-ESI-MS) 610.3585 $(M + 4H)^{4+}$.

4.1.6. PEG-NMe17A2 (**9**)

Compound **5** was extended by SPPS, introducing amino acids in the following order: Fmoc-Arg(Pbf)–OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)–OH, Fmoc-$(PEG)_6$ propionic acid. A portion (0.05 mmol) of resin-bound peptide was cleaved as previously described and puri-fied using a BiPhe RP-HPLC analytical column (method B), eluting at 11.2 min. The desired peptide was isolated as a white solid after lyophilization (6.5 mg, 8%). Monoisotopic MW calculated for $C_{127}H_{199}BrN_{35}O_{29}$ 690.1142, found (FTICR-ESI-MS) 690.1151 $(M + 4H)^{4+}$.

4.2. Ca^{2+}-mobilization assay

Chem-5 cells (Millipore, USA), stably expressing APJ-Gα15 were seeded in 100 μL medium (HBSS, 20 mM HEPES, pH 7.4) into 96-well plates (20.000 cells/well) and incubated for 24 h at 37°C and 5% CO_2. After that time the buffer was removed and exchanged for fresh medium (HBSS, 20 mM HEPES, probenecid 2.5 mM, pH 7.4). The fluorescence dye was added (Calcium 6-QF, Molecular Devices, USA) and incubated for 2 h at 37°C and 5% CO_2. Test compounds were diluted in HBSS buffer at pH 7.4 and a respective aliquot added to each well according to the dilution series. Fluorescence signals were measured on a Spectramax i3x plate-reader station (Molecular Devices, USA). Experiments were done in triplicate and analyzed using Prism 5.01 (GraphPad, USA, Fig. S8).

4.3. Blood pressure assay

Mice were anesthetized with 1.5% isoflurane/oxygen, and body temperature was monitored and maintained at 36 °C by a heating pad. The aorta was cannulated via the right carotid artery using a PV loop catheter (model 1.2F from Scisense, Transonic) in order to continuously record arterial blood pressure and heart rate (LabScribe 2.0, Scisense). Peptides **3**–**9** (1.4 μM/kg body weight) or the same volume of saline was injected via the right jugular vein. Results are reported as systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP), and heart rate (HR).

4.4. APJ radioligand binding experiments

Crude membrane preparation from CHO cells stably expressing the wild-type rat apelin receptor tagged at its C-terminal part with EGFP (enhanced green fluorescent protein; clone B70; apelin receptor-EGFP), were prepared as previously described [32]. Membrane preparations (1 μg total mass of membranes/assay) were incubated for 3 h at 20 °C with 0.1 nM of the radioligand, [^{125}I]-(Glp [1], Nle [11], Tyr [13]) apelin-13 (Perkin Elmer) in binding buffer (50 mM Hepes pH 7.5, 5 mM $MgCl_2$, 1% BSA) alone or in the presence of apelin-17 or apelin analogs at various concentrations (from 1 pM to 1 μM). The reaction was stopped with cold binding buffer and filtered on Whatman GF/C filters. After washing the filters, radioactivity was counted.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.01.040.

Abbreviations

ACE2	angiotensin-converting enzyme 2
Aib	aminoisobutyric acid
APJ	apelin receptor
BP	blood pressure
BiPhe	Biphenyl
DIPEA	N,N-diisopropylethylamine
EIC	extracted-ion chromatogram
HOAt	1-hydroxy-7-azabenzotriazole
KLKB1	plasma kallikrein
LVDP	left ventricle developed pressure
MABP	mean arterial blood pressure
max dP/dt	maximum derivative of change in systolic pressure over time
min dP/dt	minimum derivative of change in diastolic pressure over time
NEP	neprilysin
Nle	norleucine
Orn	ornithine
p-TsOH	para-toluenesulfonic acid
Pmc	2,2,5,7,8-pentamethylchroman-6- sulfonyl
Rh	recombinant human
RP	reversed phase
SPPS	solid phase peptide synthesis
Trt	trityl

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