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Development of Potent and Metabolically Stable APJ Ligands with High Therapeutic Potential

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The apelin ligand receptor system is an important target to develop treatment strategies for cardiovascular diseases. Although apelin exhibits strong inotropic effects, its pharmaceutical application is limited because no agonist with suitable properties is available. On the one hand, peptide ligands are too instable, and on the other hand, small-molecule agonists show only low potency. This study describes the development of apelin (APJ) receptor agonists with not only high activity but also metabolic stability. Several strategies including capping of termini, insertion of unnatural amino acids, cyclization, and lipidation were analyzed. Peptide activity was tested using a Ca^{2+}-mobilization assay and the degradation of selected analogues was analyzed in rat plasma. The best results were obtained by N-terminal lipidation of a 13-mer apelin derivative. This analogue displayed a half-life of 29 h in rat plasma, compared with 0.025 h for the wild-type peptide. Furthermore, in vivo pharmacokinetics revealed a clearance of 0.049 $L\,h^{-1}\,kg^{-1}$ and a half-life of 0.36 h. In summary, amino acid substitution and fatty acid modification resulted in a potent and 1000-fold more stable peptide that exhibits high pharmaceutical potential.

Introduction

The apelin (APJ) receptor consists of 377 amino acids and belongs to the class A rhodopsin-like G protein-coupled receptor (GPCR) family.[1] As this receptor is involved in many biological processes, the apelin system has high therapeutic potential for diseases such as cardiovascular disease, obesity, diabetes, inflammation, and neurological disorders.[2] These effects are mediated by the apelin ligand, which induces signal transduction via $G_{i/o}$ and G_q coupling, leading respectively to a decrease in cAMP levels, Ca^{2+} release, as well as activation of ERK1/2 and phospholipase C.[3]

With the exception of a newly discovered ELABELA (ELA) ligand, all other known apelin ligands derive from the 77-residue precursor preproapelin, which is cleaved at its N terminus.[4] All biologically active peptides differ in length with 13, 17 and 36 amino acids, but are strongly conserved within the 17-residue C-terminal fragment. Furthermore, apelin-13 appears in two forms with either N-terminal glutamine or pyroglutamate (pGlu).[5] The ligands are expressed in a wide variety of tissues such as stomach, heart, lung, kidney, liver, adipose tissue, gastrointestinal tract and brain.[6] The receptor is also broadly expressed and shows strong homology to angiotensin II receptor type 1.[1]

Notably, apelin is the most potent inotropic agent, which could be proven in several animal models.[7] Injection of apelin into rodents leads to a decrease in arterial blood pressure while glycemia is decreased and glucose uptake enhanced in insulin-resistant mice.[8] Apelin knockout mice were found to develop more severe pulmonary hypertension than wild-type mice upon exposure to chronic hypoxia, and injection of apelin attenuated myocardial hypertrophy in a rat model.[9] Infusion of apelin was also studied in healthy humans and revealed an increase in blood flow by releasing the vasodilator NO.[10] This vasodilative action was also proven in human isolated vessels.[11]

Due to its vasodilative and inotropic effects, apelin is a promising therapeutic target for treatment of heart failure and pulmonary arterial hypertension. At the beginning of heart failure, apelin levels increase, but decrease in later stages.[12] It was also shown that levels are decreased after early myocardial infarction and remain low for 24 weeks.[13]

Although apelin ligands bind the APJ receptor with high affinity and induce numerous biological effects, the therapeutic application is limited due to low metabolic stability. Apelin

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peptides are degraded within a few minutes. The bond between Pro[12] and Phe[13] was determined as the main cleavage site and is cleaved by the angiotensin converting enzyme related carboxypeptidase (ACE II).[14] One semipeptidic partial agonist E339-3D6 was found to activate the APJ receptor, but it was less active than the endogenous agonists.[15a] Although recent structure–activity relationship studies[15] have revealed more active compounds, single-digit nanomolar activity of a nonpeptidic ligand has only very recently been achieved.[16]

Several approaches are known to increase the metabolic stability of peptides. Besides the insertion of unnatural amino acids, the stabilization of structural motifs was shown to be highly suitable. However, no ordered structure could be observed for apelin with exception of the RPRL motif. This four-residue sequence forms a β-turn structure, as determined by 2D NMR in a micellar system.[17] Such turn structures were shown to be highly important for the biological activity of peptidic ligands.[18]

Hence, the aim of this study was to develop APJ receptor agonists with high activity and metabolic stability. As pGlu-apelin-13 is the most abundant and active ligand, this peptide sequence was chosen as the starting point.[7] By amino acid exchange and fatty acid modification, a potent and metabolically stable peptide with high pharmaceutical potential was designed.

Results

Peptide synthesis

All apelin analogues were synthesized based on the 13-mer pGlu-apelin for reasons described above.[7] Synthesis was carried out on Fmoc-Phe-Wang resin using Fmoc/*t*Bu chemistry. For C-terminally amidated peptides, Rink amide resin was used.

To ensure side specific palmitoylation, the lysine side chain was protected using 4-methoxytrityl (Mmt), which was orthogonally cleaved with 1% trifluoroacetic acid in dichloromethane. As the exchange of methionine to norleucine (Nle) did not affect peptide activity (see Table 1, **1**–**2**), further analogues were prepared with Nle at position 11 to avoid the potential oxidation of methionine.[19] All peptides were analyzed by LC–MS and purified by preparative HPLC.

Apelin drug development

Several strategies have been developed to transform peptides into drug leads by affecting the duration of action, selectivity, and bioavailability. To selectively modify the apelin sequence, the relevance of each amino acid for receptor signaling was analyzed by performing alanine and D-amino acid scans. All peptide analogues were tested for activity in a Ca^{2+}-mobilization assay and confirmed the results described in literature (data not shown).[3b,20] Briefly, high importance of amino acids Arg[2], Leu[5], Lys[8], Pro[10], Phe[13] and a minor influence of Arg[4] and Met[11] were determined by the alanine scan. Exchange of pGlu[1], Ser[6], Pro[3], His[7], Gly[9] and Pro[12] to Ala had no impact on the receptor activity. The D-amino acid scan revealed high activity for the peptide containing the D-Nle isomer and 10- to 20-fold decrease in activity for Gln[1], Arg[4], Leu[5], Lys[8] and Pro[12] analogues. An even greater loss of activity was detected for positions Arg[2], Pro[3], Ser[6], His[7], Pro[11] and Phe[13].

As apelin is degraded within minutes,[20] the main focus of this work was to increase the metabolic stability of the APJ ligand. To protect the peptide from degradation by exopeptidases, various N-terminal caps as well as C-terminal amidation were analyzed (Table 1). Since Gln/pGlu was not important for receptor activation, this amino acid was substituted by an acetyl group (**3**) and showed wild-type activity. Furthermore, peptide analogues were synthesized carrying five- and six-membered ring structures (Figure 1) at the N terminus (**4**–**7**). These studies revealed that the N terminus is highly flexible toward modifications, as these peptides were found to be

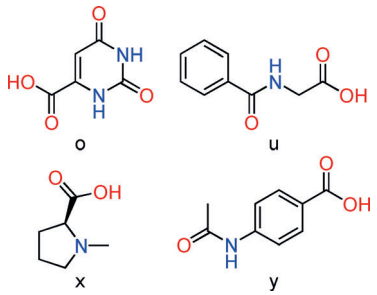

Figure 1. Five- and six-membered ring structures used as N-terminal caps: orotic acid (o), hippuric acid (u), N^{α}-methylproline (x), and 4-acetamidobenzoic acid (y).

Table 1. Sequences, analytics, and functional characterization of terminally modified apelin peptides.

Compd[a]	Sequence	M_r [Da]	$[M+H]^+$[b]	t_R [min][b]	EC_{50} [nM][c]	Efficacy [%][c]
1	pERPRLSHKGP**M**PF	1533.82	1533.82[d]	3.64[d]	2.6 ± 1.0	112 ± 1
2	pERPRLSHKGP-**Nle**-PF	1514.84	1515.86	7.96	2.0 ± 0.3	105 ± 2
3	**Ac**-RPRLSHKGP-**Nle**-PF	1445.82	1446.83	8.02	4.0 ± 0.3	113 ± 4
4	**o**RPRLSHKGP-**Nle**-PF	1541.82	1542.83	8.04	9.7 ± 1.3	107 ± 3
5	**u**RPRLSHKGP-**Nle**-PF	1564.86	1565.87	8.44	3.2 ± 0.3	105 ± 4
6	**x**RPRLSHKGP-**Nle**-PF	1514.88	1515.89	7.98	5.8 ± 0.7	106 ± 3
7	**y**RPRLSHKGP-**Nle**-PF	1564.86	1565.87	8.25	11.1 ± 1.2	102 ± 3
8	pERPRLSHKGP-**Nle**-PF-**NH_2**	1513.86	1514.87	7.79	170 ± 16.5	106 ± 4

[a] Apelin-13 was used as a positive control with an EC_{50} value of 1.2 ± 0.3 nM, with efficacy being set to 100%. [b] $[M+H]^+$ and retention time (t_R) determined by LC method 1. [c] Ca^{2+}-mobilization assay; E_{max} values are the means of at least two experiments ± SEM. [d] Determined by LC method 2.

active in the single-digit nanomolar range and could fully activate the receptor. In contrast, the C terminus was more restricted as amidation (**8**) led to an 85-fold decrease in activity.

To rigidify the apelin conformation, constrained amino acids such as N- or C^{α}-methylated amino acids were inserted (Table 2). These amino acids are suitable to mask protease cleavage sites; they also affect hydrophobicity as well as the formation of secondary structures. Therefore, Pro residues were replaced by aminoisobutyric acid (Aib) and N^{α}-Me-Ala-OH, respectively (**9**–**14**). These results revealed that Pro^{12} is not essential for receptor activation, as the Aib and N-Me-Ala substitutions (**11, 14**) yielded full agonists, active in the single-digit nanomolar range. Pro^{3} substitution revealed 98.2 nM for Aib (**9**) and 7.05 nM for N^{α}-Me-Ala-OH (**12**) exchange. In contrast, substitutions of Pro^{10} were not tolerated for Aib (**10**) and led to a ninefold loss of activity for N^{α}-Me-Ala-OH substitution (**13**).

Furthermore, all amino acids were exchanged with their N-methylated analogues. The obtained data revealed that N-methylation has no effect on receptor activation at position Arg^{2} (**15**), Arg^{4} (**16**), and only threefold loss of activity for Leu^{5} (**17**) and Phe^{13} (**23**) was observed. However, N-methylation of Ser (**18**), His (**19**), Lys (**20**), Gly (**21**), and Met (**22**) led to loss of function (Table 2).

Besides these local constraints, stabilization of secondary structures by cyclization can increase protease resistance and selectivity.[17b] Because the apelin ligand has a mainly unordered structure but can form a β-turn within the RPRL motif,[17] the idea was to stabilize this turn structure by inserting disulfide bridges (Table 3). Therefore, cysteine residues were introduced surrounding the RPRL motif. The Cys residue either replaced Ser^{6} (**24, 26**) or was inserted as an additional amino acid (**25**). However, these modifications led to a complete loss of function. Interestingly, an exchange of the terminal pGlu for Gln led to an almost complete restoration of function (Table 3). Another approach to effectively change the ADME properties of peptidic drugs is the modification by fatty acids.[21] Therefore, we analyzed whether palmitoylated (Palm) apelin analogues show increased stability but retain biological activity. Acylation was carried out at different positions. First, we decided to modify the endogenous apelin peptide at the N terminus (**27**) and the Lys^{8} (**28**) side chain (Table 3). Selective modifica-

Table 2. Sequences, analytics, and functional characterization of modified apelin peptides using N- or C-methylated amino acids.

Compd[a]	Sequence	M_r [Da]	$[M+H]^+$[b]	t_R [min][b]	EC_{50} [nM][c]	Efficacy [%][c]
2	pERPRLSHKGP-**Nle**-PF	1514.84	1515.86	7.96	2.0 ± 0.3	105 ± 2
9	pER-**Aib**-RLSHKGP-**Nle**-PF	1502.84	1503.85	10.96	98.2 ± 25.3	101 ± 4
10	pERPRLSHKG-**Aib**-**Nle**-PF	1502.84	1503.86[d]	3.82[d]	> 1000	73 ± 2
11	pERPRLSHKGP-**Nle**-**Aib**-F	1502.84	1503.86[d]	3.82[d]	1.9 ± 0.6	100 ± 3
12	pER-***N*-Me-Ala**-RLSHKGP-**Nle**-PF	1502.84	1503.86	7.95	7.1 ± 1.6	96 ± 3
13	pERPRLSHKG-***N*-Me-Ala**-**Nle**-PF	1502.84	1503.86[d]	3.85[d]	18.8 ± 5.4	94 ± 3
14	pERPRLSHKGP-**Nle**-***N*-Me-Ala**-F	1502.84	1503.86[d]	3.83[d]	4.1 ± 0.6	92 ± 3
15	pE-***N*-Me-Arg**-PRLSHKGP-**Nle**-PF	1528.86	1529.88[d]	3.85[d]	4.7 ± 0.3	92 ± 3
16	pERP-***N*-Me-Arg**-LSHKGP-**Nle**-PF	1528.86	1529.88[d]	3.87[d]	2.4 ± 0.4	88 ± 4
17	pERPR-***N*-Me-Leu**-SHKGP-**Nle**-PF	1528.86	1529.87	7.70	7.4 ± 2.0	93 ± 1
18	pERPRL-***N*-Me-Ser**-HKGP-**Nle**-PF	1528.86	1529.88	3.85[d]	115 ± 10.4	92 ± 3
19	pERPRLS-***N*-Me-His**-KGP-**Nle**-PF	1528.86	1529.87	7.70	660 ± 172	90 ± 3
20	pERPRLSH-***N*-Me-Lys**-GP-**Nle**-PF	1528.86	1529.87	3.88[d]	437 ± 60.7	98 ± 9
21	pERPRLSHK-**Sar**-P-**Nle**-PF	1528.86	1529.88	7.82	167 ± 33.4	102 ± 3
22	pE-RPRLSHKGP-***N*-Me-Met**-PF	1548.81	1547.84	3.59[d]	170 ± 22.7	110 ± 6
23	pERPRLSHKGP-**Nle**-P-***N*-Me-Phe**	1528.86	1529.88	8.01	6.7 ± 0.8	104 ± 1

[a] Apelin-13 was used as a positive control with an EC_{50} value of 1.2 ± 0.3 nM, with efficacy being set to 100%. [b] $[M+H]^+$ and retention time (t_R) determined by LC method 1. [c] Ca^{2+}-mobilization assay; E_{max} values are the means of at least two experiments ± SEM. [d] Determined by LC method 2.

Table 3. Sequences, analytics, and functional characterization of cyclized and palmitoylated apelin peptides.

Compd[a]	Sequence[b]	M_r [Da]	$[M+H]^+$[c]	t_R [min][c]	EC_{50} [nM][d]	Efficacy [%][d]
2	pERPRLSHKGP-**Nle**-PF	1514.84	1515.86	7.96	2.0 ± 0.3	105 ± 2
24	QC*RPRLC*HKGP-**Nle**-PF	1648.84	1649.85	10.85	3.4 ± 0.7	93 ± 4
25	pEC*RPRLC*SHKGP-**Nle**-PF	1718.84	1719.86	7.85	> 1000	86 ± 9
26	pEC*RPRLC*HKGP-**Nle**-PF	1631.81	1632.83	7.87	> 1000	68 ± 4
27	**Palm**-QRPRLSHKGP-**Nle**-PF	1770.10	1771.12[e]	5.64[e]	12.3 ± 3.5	80 ± 4
28	pERPRLSHK(**Palm**)GP-**Nle**-PF	1753.07	1754.09[e]	6.27[e]	593 ± 47.5	68 ± 2
29	pERPRLSHK**K(Palm)**P-**Nle**-PF	1824.15	1825.16	12.64	38.4 ± 9.5	83 ± 3
30	pERPRLSHK**K(Palm)**P-**Nle**-**Aib**-F	1812.15	1813.16	12.60	23.1 ± 4.6	98 ± 2
31	**Palm**-QRPRLSHKGP-**Nle**-**Aib**-F	1758.10	1759.11	11.86	21.6 ± 4.5	93 ± 3

[a] Apelin-13 was used as a positive control with an EC_{50} value of 1.2 ± 0.3 nM, with efficacy being set to 100%. [b] '*' indicates point of side chain ring cyclization, in this case, formation of a disulfide bond. [c] $[M+H]^+$ and retention time (t_R) determined by LC method 1. [d] Ca^{2+}-mobilization assay; E_{max} values are then means of at least two experiments ± SEM. [e] Determined by LC method 2.

tion of the Lys side chain could be achieved using the Mmt protecting group. Additionally, Gly[9] was exchanged for Lys (**29**), as this residue was not important for ligand activity. These results revealed that acylation leads to a decrease in activity, which was only five- and twentyfold for the N-terminally and Lys[9]-modified peptides, respectively. For both agonists, the receptor activation was slightly decreased (**27**, **28**). The Lys[9] side chain modified analogue (**29**) could no longer activate the apelin receptor. To further increase the metabolic stability, apelin analogues were synthesized carrying palmitic acid at Lys[9] (**30**) or at the N terminus (**31**) as well as Aib at position Pro[12]. The analogue **30** showed a further decrease of activity. Insertion of Aib[12] and N-terminal palmitoylation (**31**) had no further impact on activity (Table 3).

Peptide stability

After analyzing the activity, we were interested in the effect of peptide modifications on the metabolic stability. Therefore, we determined the in vitro half-lives of the modified peptides during incubations in rat plasma at 37 °C. The endogenous agonist (**1**) was rapidly degraded with a half-life of 0.025 h. Insertion of Nle (**2**) as well as disulfide bridges (**26**) had only minor effects on the stability with $t_{1/2}$ = 0.13 h and 0.10 h, respectively (Table 4). The palmitoylated apelin analogue **31** was the most promising peptide with regard to stability and activity. These studies revealed a half-life during in vitro incubations in rat plasma of ~29 h for the palmitoylated derivative.

Table 4. In vitro half-lives of apelin analogues in rat plasma.

Compd	Sequence	$t_{1/2}$ [h]
1	pERPRLSHKGP**M**PF	0.025
2	pERPRLSHKGP-**Nle**-PF	0.13
26	pEC*RPRLC*HKGP-Nle-PF	0.10
31	**Palm**-QRPRLSHKGP-**Nle**-**Aib**-F	29

To further investigate the metabolism of compound **31** the peptide was labeled with 5,6-carboxytetramethylrhodamine (TAMRA). To accomplish this, Gly[9] was exchanged with Lys, which allowed site-specific TAMRA introduction through use of the Mmt protecting group. The peptide was then incubated in human plasma for 120 h, and the peptide content determined by HPLC using a fluorescence detector. These studies also revealed that the peptide is metabolically stable, as 90% of the original compound could be detected after 120 h (Figure 2A). Furthermore, the peptide was incubated in porcine liver homogenate and revealed a half-life of ~4 h under these conditions. These studies demonstrated that the bonds between Lys[8], Lys[9], Pro[10], and Nle[11] are cleaved first (Figure 2B). However, no cleavage of the ACE II cleavage site between Pro[12] and Phe[13] was observed, as Pro[12] was substituted with Aib, which is not recognized by this enzyme.[22] It should be noted that cleavage between Lys[8], Lys[9] and Pro[10] is not relevant, as it was only introduced for the fluorescence label. Additionally, preliminary pharmacokinetic (PK) parameters were determined and revealed a low apparent clearance of 0.049 $L\,h^{-1}\,kg^{-1}$, but a short half-life of 0.36 h in vivo (male Wistar rat, Figure 2C). Hence, all data confirm that active apelin agonists with high metabolic stability were synthesized.

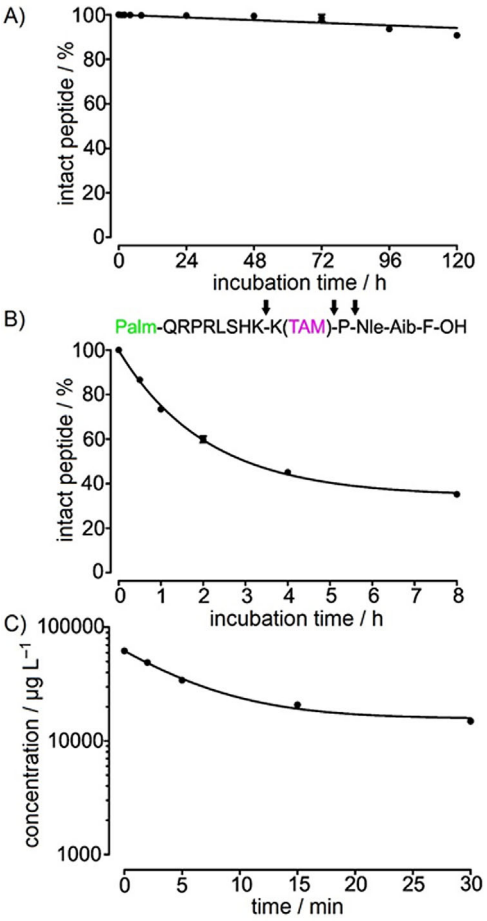

Figure 2. Stability data of palmitoylated apelin analogue **31**: A) analysis of degradation in human plasma, B) porcine liver homogenate, and C) in vivo pharmacokinetics.

Discussion

The aim of this study was to develop potent and metabolically stable apelin analogues. So far, all APJ ligands described show half-lives of only minutes in plasma.[10b,15,23] Peptides as pharmaceuticals are highly important given their high potency, selectivity, and low toxicity. Furthermore, a main advantage is that they can address undruggable targets, as they are able to disrupt protein–protein interactions, which can be difficult to achieve with small molecules. However, there are several drawbacks such as low proteolytic stability, resulting in rapid clearance, low hydrophilicity, poor membrane permeability, and thus a low oral bioavailability, which have to be overcome.[18]

Therefore, numerous modifications were described within the last decades to improve the ADME properties of peptides. This includes the substitution of amino acids by unnatural or constrained amino acids, capping of termini and amide bond

surrogates, as well as cyclization or modifications like PEGylation, lipidation or PASylation.[24]

First, the Met residue was exchanged to Nle (**2**) to decrease oxidation, as this peptide showed wild-type-like potency. Secondly, the termini were capped to prevent degradation by exopeptidases. However, C-terminal amidation (**8**) led to loss of function, which was also shown by Medhurst et al.[3b] Capping of the N terminus (**3**–**7**) had no impact on receptor activation as we could also insert different moieties at the N terminus. Only very bulky six-membered rings like *N*-acetamidobenzoic acid or orotic acid led to a fivefold decrease in activity.

Because the cleavage of the bond between Pro^{12} and Phe^{13} by ACE II is one of the main reasons for the short half-life,[14] modifying this part of the peptide sequence seems to be highly suitable. Therefore, substitution of these amino acids by constraints will make this peptide bond poorly accessible for proteases. Pro^{12} exchange by Aib (**11**) led to a still active analogue ($EC_{50}=1.9$ nM) while substitution of Pro^{3} (**9**) and Pro^{10} (**10**) was not possible ($EC_{50}=98.2\pm25.3$ and $EC_{50}>1$ μM, respectively). Instead of Aib, the N^{α}-methylated Ala was introduced and led to only a slight decrease in function, probably due to a higher structural similarity to Pro (**12**–**14**).

Murza et al.[20] analyzed several Pro^{12} and Phe^{13} substitutions and could show high potency and partially improved plasma stability. A different approach was performed by Zhang et al., in which the cleavage site was removed.[23] In this study, truncated 10-mers were synthesized (apelin 2–11) and further modified. These peptides showed reasonable activities, but still degradation within minutes ($t_{1/2}\sim2$ min) in rat plasma, although ACE II cleavage could not occur, as the Pro–Phe recognition site was not present.

To further protect all bonds from cleavage, an N-Me scan was performed (**15**–**23**). Substitution of Gly, Met, Lys, His and Ser showed a high impact on receptor activation due to the steric hindrance or conformational changes due to the methyl group. All other N-Me analogues led to only weak decreases in potency.

Another successful approach to design stable and active peptide analogues is to strengthen secondary structures. This can be achieved by insertion of constraint amino acids like Aib as described above, which can induce α-helix formation. Additionally, rigidification can be achieved by cyclization.[17] Structural analysis of the apelin peptide revealed that the RPRL motif forms a β-turn structure in a micellar system,[17a] although apelin peptides have also been shown to be random coils in aqueous solutions.[17b] In this study, the four-residue motif was stabilized by disulfide bridges (**24**–**26**). Thereby, Cys residues were introduced and revealed one active analogue. However, only a minor impact on stability was determined with a half-life of ~6 min in plasma. Recently, a similar analogue was published by Brame et al.[10b] and was shown to act as a biased apelin agonist with activation of the G protein signaling cascade, but decreased receptor internalization. This activity was also proven in human as it causes vasodilation at higher doses. A higher in vitro stability in rat plasma with a half-life of 17.4 min relative to the wild-type with a half-life of 2.3 min was also determined.[10b]

Recently, the impact of polyethylene groups on the cardiovascular function of apelin was investigated.[25] N-Terminally PEGylated apelin 36 was synthesized and showed similar biological effects in vivo, but a longer in vivo exposure time than the wild-type, suggesting higher plasma stability.[25] Another important modification to improve the ADME properties of apelin is the lipidation of the peptide sequence. Thereby, the peptide is shielded due to formation of micelles, and further protection is achieved by binding to serum proteins.[26] However, this most often leads to decreased aqueous solubility but can be reversed by the introduction of hydrophilic residues such as glutamate.[21,27] In the case of apelin, the solubility was only weakly affected, as the peptide sequence consists of several charged and polar amino acids, and thus no linker was necessary.

The apelin sequence was modified by palmitic acid at the N-terminus (**27**) and the Lys^{8} (**28**) side chain. Furthermore, Gly^{9} was exchanged to Lys and also acylated (**29**). The obtained results revealed that acylation led to a decrease in activity, which was 300-fold lower for the Lys^{8} analogue and modest for the N-terminally and Lys^{9} side chain modified peptide. These peptides were further altered by insertion of Aib at position Pro^{12} that had no additional impact on potency (**30**, **31**).

Currently several lipidated peptides are approved for use in humans, such as the palmitoylated GLP-1 analogue liraglutide to treat type 2 diabetes and obesity. Endogenous GLP-1 has a plasma half-life of 1.5–2 min, whereas palmitoylation leads to a plasma half-life of 13 h.[28] A recent report in the patent literature describes palmitoylated apelin peptides being evaluated for a diabetic indication with improved half-lives.[2f]

After synthesis and determination of receptor activation, preliminary PK parameters of derivative **31** were analyzed. The modifications revealed a strong protection against proteases, demonstrated by a peptide in vitro half-life of 29 h in rat plasma. To our knowledge, this peptide is the most stable apelin analogue described so far with the exception of some nonpeptidic agonists. These were shown to have plasma stabilities of >10 h but were less active.[15b] Besides the high in vitro metabolic stability, a low in vivo clearance was also determined due to prolonged blood retention by serum albumin binding.

Conclusions

In summary, by the introduction of unnatural amino acids as well as lipidation of the 13-mer apelin, a very potent and stable peptide was synthesized. As next steps, the biodistribution and in vivo effects in significant disease models will be determined. So far, the potential clinical use of apelin peptides has been limited due to a very short half-life. The stable analogue presented herein will facilitate in vivo investigation of apelin effects and progress in drug development.

Experimental Section

Peptide synthesis: Apelin-13 was obtained from Bachem. Automatic peptide synthesis was performed on the microwave peptide synthesizer Liberty (CEM) in scale ranges of 0.10 to 0.25 mmol.

Fmoc-protected amino acids were purchased from Novabiochem, Bachem or Protein Technologies. Solid-phase resins were purchased from Novabiochem or Bachem. The resin loading was 0.37–0.4 mmol g^{-1}. Peptides were synthesized on Wang or Rink amide resin depending on the desired C-terminus. Cleavage of the fluorenylmethoxycarbonyl (Fmoc) protecting group was achieved using 20% piperidine in *N*,*N*-dimethylformamide (DMF; Liberty, CEM) or using 30% piperidine in DMF (Symphony X, Protein Technologies). Amino acids were coupled using 4 equiv amino acid, 4 equiv 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 4 equiv 1-hydroxybenzotriazole hydrate (HOBt·H_2O) in DMF (0.5 M), and 12 equiv *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methyl-2-pyrrolidone (NMP). On the Symphony X, amino acids were coupled using 8 equiv amino acid, 8 equiv diisopropylcarbodiimide (DIC), and 8 equiv Oxyma in DMF (0.5 M). Coupling and Fmoc cleavage were carried out on the Liberty at 75 °C or at room temperature on the Symphony X. Histidine and cysteine were coupled at 50 °C on the Liberty. Manual coupling was performed in DMF using 5 equiv of the respective acid (0.5 M), 5 equiv HOBt·H_2O and 5 equiv DIC. The coupling of TAMRA was performed using 2 equiv TAMRA, 2 equiv HATU and 2 equiv DIPEA in DMF overnight.

Peptides were completely deprotected using trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (94:3:3) and TFA/thioanisole (TA)/1,2-ethanedithiol (EDT) (90:7:3) for peptides containing Cys and Met. Oxidized methionine was reduced using 1.6% EDT and 1.2% trimethylsilylbromide in TFA for 2 h at RT. Disulfide bridges were formed by shaking peptides in 0.1 M phosphate buffer (pH 7.6) at concentrations of 0.5 mg mL^{-1}. N-terminal acetylation was performed using 10 equiv acetic anhydride in CH_2Cl_2 (1:10) and 10 equiv DIPEA at RT for 1 h.

Purification and analysis: Peptides were purified by preparative reversed-phase high-performance liquid chromatography (HPLC: Agilent 1260 Infinity; Column: XBridge BEH C_{18} OBD Prep Column, 130 Å, 10 μm, 19 mm × 250 mm; eluent A: H_2O + 0.05% TFA; eluent B: MeCN + 0.05% TFA). Peptides were characterized by LC–MS (ThermoFisher Scientific LTQ-Orbitrap-XL; HPLC: Agilent 1200SL; column: Agilent, POROSHELL 120, 3 × 150 mm, SB-C_{18} 2.7 μm). Peptides were characterized by LC–MS using two methods. *Method 1*: Waters Acquity I UPLC equipped with a Waters Synapt G2S mass spectrometer. Conditions: Waters BEH300 2.1 × 150 mm, C_{18} 1.7 μm column; eluent A: H_2O + 0.01% formic acid; eluent B: MeCN + 0.01% formic acid; gradient: 0.0 min 2% B → 1.5 min 2% B → 8.5 min 95% B → 10.0 min 95% B; oven temp: 50 °C; flow rate: 0.50 mL min^{-1}; UV detection: 220 nm. *Method 2*: Agilent 1200SL HPLC equipped with a ThermoFisher Scientific LTQ-Orbitrap-XL mass spectrometer. Conditions: Agilent POROSHELL 120.3 × 150 mm, SB-C_{18} 2.7 μm column; eluent A: H_2O + 0.1% TFA; eluent B: MeCN + 0.1% TFA; gradient: 0.0 min 2% B → 3.0 min 2% B → 15.0 min 80% B; oven temp: 40 °C; flow rate: 0.75 mL min^{-1}; UV-detection: 210 nm.

Ca^{2+}-mobilization assay: APJ receptor agonists are tested on a stable, recombinant human APJ-Gα16 receptor cell line. In the reporter cell line APJ receptor activation is coupled to intracellular calcium mobilization, which is monitored with a fluorescent calcium indicator protein (GCaMP3) as previously described.[29] Cells were cultured at 37 °C and 5% CO_2. Intracellular calcium mobilization was monitored for 90 s after ligand addition using a FLIPR Tetra (Molecular Devices). Peptides were tested in serial dilutions. EC_{50} values were calculated from the concentration–response curves. Efficacies were calculated in relation to the natural peptide apelin-13 which was set to 100%.

Stability and pharmacokinetics: In vitro metabolic stability studies of 5,6-TAMRA-modified analogues were carried out in human plasma and porcine liver homogenate.[30] The peptide was incubated at a concentration of 10^{-5} M in human plasma and liver homogenate (50 mg mL^{-1} in PBS) at 37 °C and 300 rpm. Samples were taken after distinct times and mixed with EtOH/MeCN (1:1 *v*/*v*). After incubation at −20 °C, the supernatant was transferred into 0.22 μm Spin-X tubes and centrifuged; 50 μL of the flow-through was analyzed on the HPLC column VariTide RPC (Varian: 250 × 4.6 mm; 6 μm; 200 Å) using linear gradients of eluent B in A (eluent A = 0.1% TFA in H_2O, eluent B = 0.08% TFA in MeCN). The chromatogram was recorded at λ_{ex} = 525 nm and λ_{em} = 573 nm. Degradation products were identified by MALDI-TOF MS (Ultraflex III MALDI-TOF/TOF, Bruker Daltonics). Half-life was calculated by one-phase decay analysis with GraphPad Prism 5. Data points represent mean ± SEM of two independent experiments. Samples were taken and analyzed once after 120 h of incubation in human blood plasma.

For stability analysis, plasma from male Wistar rats was used. Each peptide was spiked into 1 mL plasma at a concentration of 1 μg mL^{-1}. The mixture was constantly shaken at 37 °C. At each of the following time points (0, 0.17, 0.5, 1, 2, 4, 6, 24 and 27 h) a probe was taken and diluted with a fourfold excess of MeCN. After shaking for 5 min at 4 °C, the probe was centrifuged at 16 000 *g*. The supernatant was evaporated to dryness and re-solubilized in H_2O + 0.1% FA/MeCN + 0.1% FA (95:5%) using ultrasound. A ThermoFisher Accela 1250 analytical LC (flow rate: 400 μL min^{-1}) equipped with an ACE 3 C_{18}–300 150 × 2.1 mm column was used for chromatography. A 9 min gradient using the eluents A) H_2O + 0.1% FA and B) MeCN + 0.1% FA was applied. Quantification of the peptides was performed by using peak area from extracted ion chromatograms of specific high-resolution ESI-MS experiments with a ThermoFisher Q-Exactive mass spectrometer. The half-life was calculated by using relative signal intensities and a line of best fit.

The PK samples of apelin were analyzed with multiple reaction monitoring on an AB SCIEX Qtrap 6500 System coupled to an Agilent 1290 UHPLC System with a Waters xBridge BEH300 C4 4.5 μm 2.1 × 50 mm analytical column. Plasma samples were stabilized with 10 μL 10% FA upon sample removal. For sample preparation 100 μL of stabilized plasma were precipitated with 500 μL 3.5% trichloroacetic acid.

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Keywords: apelin · G protein-coupled receptors · lipidation · peptides · structure–activity relationships

[1] B. F. O'Dowd, M. Heiber, A. Chan, H. H. Heng, L. C. Tsui, J. L. Kennedy, X. Shi, A. Petronis, S. R. George, T. Nguyen, *Gene* **1993**, *136*, 355–360.

[2] a) L. Yang, L. X. Chen, *Int. J. Path. Clin. Med.* **2011**, *31*, 42–48; b) B. Cheng, J. Chen, B. Bai, Q. Xin, *Peptides* **2012**, *37*, 171–173; c) D. Wu, L. He, L. Chen, *Mol. Biol. Rep.* **2014**, *41*, 6691–6703; d) B. Chandrasekaran, O. Dar, T. McDonagh, *Eur. J. Heart Failure* **2008**, *10*, 725–732; e) S. M. R.

Kazemi-Bajestani, V. B. Patel, W. Wang, G. Y. Oudit, *Cardiology Res. Practice* **2012**, *2012*, 823193; f) F. P. M. O'Harte, P. R. Flatt (University of Ulster), Int. PCT Pub. No. WO2015165936 A1, **2015**.

[3] a) A. Pauli, M. L. Norris, E. Valen, G. L. Chew, J. A. Gagnon, S. Zimmerman, A. Mitchell, J. Ma, J. Dubrulle, D. Reyon, S. Q. Tsai, J. K. Joung, A. Saghatelian, A. F. Schier, *Science* **2014**, *343*, 1248636; b) A. D. Medhurst, C. A. Jennings, M. J. Robbins, R. P. Davis, C. Ellis, K. Y. Winborn, K. W. Lawrie, G. Hervieu, G. Riley, J. E. Bolaky, N. C. Herrity, P. Murdock, J. G. Darker, *J. Neurochem.* **2003**, *84*, 1162–1172; c) N. A. Chapman, D. J. Dupré, J. K. Rainey, *Biochem. Cell Biol.* **2014**, *92*, 431–440.

[4] a) S. C. Chng, L. Ho, J. Tian, B. Reversade, *Dev. Cell* **2013**, *27*, 672–680; b) K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M. X. Zou, Y. Kawamata, S. Fukusumi, S. Hinuma, C. Kitada, T. Kurokawa, H. Onda, M. Fujino, *Biochem. Biophys. Res. Commun.* **1998**, *251*, 471–476; c) C. J. Charles, *Cardiovasc. Hematol. Agents Med. Chem.* **2007**, *5*, 1–10.

[5] a) Y. Habata, R. Fujii, M. Hosoya, S. Fukusumi, Y. Kawamata, S. Hinuma, C. Kitada, N. Nishizawa, S. Murosaki, T. Kurokawa, H. Onda, K. Tatemoto, M. Fujino, *Biochim. Biophys. Acta Mol. Cell Res.* **1999**, *1452*, 25–35; b) G. Barnes, A. G. Japp, D. E. Newby, *Heart* **2010**, *96*, 1011–1016; c) C. Galanth, A. Hus-Citharel, B. Li, C. Llorens-Cortès, *Curr. Pharm. Des.* **2012**, *18*, 789–798.

[6] a) C. J. Charles, M. T. Rademaker, A. M. Richards, *J. Endocrinol.* **2006**, *189*, 701–710; b) A. M. Tycinska, A. Lisowska, W. J. Musial, B. Sobkowicz, *Clin. Chim. Acta* **2012**, *413*, 406–410.

[7] a) I. Szokodi, P. Tavi, G. Földes, S. Voutilainen-Myllylä, M. Ilves, H. Tokola, S. Pikkarainen, J. Piuhola, J. Rysä, M. Tóth, H. Ruskoaho, *Circ. Res.* **2002**, *91*, 434–440; b) E. A. Ashley, J. Powers, M. Chen, R. Kundu, T. Finsterbach, A. Caffarelli, A. Deng, J. Eichhorn, R. Mahajan, R. Agrawal, J. Greve, R. Robbins, A. J. Patterson, D. Bernstein, T. Quertermous, *Cardiovasc. Res.* **2005**, *65*, 73–82; c) J. C. Simpkin, D. M. Yellon, S. M. Davidson, S. Y. Lim, A. M. Wynne, C. C. T. Smith, *Basic Res. Cardiol.* **2007**, *102*, 518–528.

[8] J. Boucher, B. Masri, D. Daviaud, S. Gesta, C. Guigne, A. Mazzucotelli, I. Castan-Laurell, I. Tack, B. Knibiehler, C. Carpene, Y. Audigier, J. S. Saulnier-Blache, P. Valet, *Endocrinology* **2005**, *146*, 1764–1771.

[9] a) S. M. Chandra, H. Razavi, J. Kim, R. Agrawal, R. K. Kundu, V. de Jesus Perez, R. T. Zamanian, T. Quertermous, H. J. Chun, *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*, 814–820; b) I. Falcao-Pires, N. Goncalves, T. Henriques-Coelho, D. Moreira-Goncalves, R. Roncon-Albuquerque, Jr., A. F. Leite-Moreira, *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296*, H2007–2014.

[10] a) A. G. Japp, N. L. Cruden, D. A. Amer, V. K. Li, E. B. Goudie, N. R. Johnston, S. Sharma, I. Neilson, D. J. Webb, I. L. Megson, A. D. Flapan, D. E. Newby, *J. Am. Coll. Cardiol.* **2008**, *52*, 908–913; b) A. L. Brame, J. J. Maguire, P. Yang, A. Dyson, R. Torella, J. Cheriyan, M. Singer, R. C. Glen, I. B. Wilkinson, A. P. Davenport, *Hypertension* **2015**, *65*, 834–840.

[11] a) J. J. Maguire, M. J. Kleinz, S. L. Pitkin, A. P. Davenport, *Hypertension* **2009**, *54*, 598–604; b) A. Salcedo, J. Garijo, L. Monge, N. Fernandez, A. Luis Garcia-Villalon, V. Sanchez Turrion, V. Cuervas-Mons, G. Dieguez, *Regul. Pept.* **2007**, *144*, 50–55.

[12] a) K. S. Chong, R. S. Gardner, J. J. Morton, E. A. Ashley, T. A. McDonagh, *Eur. J. Heart Failure* **2006**, *8*, 355–360; b) B. Chandrasekaran, P. R. Kalra, J. Donovan, J. Hooper, J. R. Clague, T. A. McDonagh, *J. Card. Failure* **2010**, *16*, 556–561.

[13] R. A. Weir, K. S. Chong, J. R. Dalzell, C. J. Petrie, C. A. Murphy, T. Steedman, P. B. Mark, T. A. McDonagh, H. J. Dargie, J. J. McMurray, *Eur. J. Heart Failure* **2009**, *11*, 551–558.

[14] a) C. Vickers, P. Hales, V. Kaushik, L. Dick, J. Gavin, J. Tang, K. Godbout, T. Parsons, E. Baronas, F. Hsieh, S. Acton, M. Patane, A. Nichols, P. Tummino, *J. Biol. Chem.* **2002**, *277*, 14838–14843; b) A. Murza, K. Belleville, J. M. Longpré, P. Sarret, É. Marsault, *Biopolymers* **2014**, *102*, 297–303.

[15] a) X. Iturrioz, R. Alvear-Perez, N. De Mota, C. Franchet, F. Guillier, V. Leroux, H. Dabire, M. Le Jouan, H. Chabane, R. Gerbier, D. Bonnet, A. Berdeaux, B. Maigret, J. L. Galzi, M. Hibert, C. Llorens-Cortes, *FASEB J.* **2010**, *24*, 1506–1517; b) J. F. Margathe, X. Iturrioz, R. Alvear-Perez, C. Marsol, S. Riche, H. Chabane, N. Tounsi, M. Kuhry, D. Heissler, M. Hibert, C. Llorens-Cortes, D. Bonnet, *J. Med. Chem.* **2014**, *57*, 2908–2919; c) S. Hachtel, P. Wohlfart, J. Weston, M. Mueller, E. Defossa, K. Mertsch, J.-H. Weng, R. A. Binnie, F. Abdul-Latif, W. J. Bock, A. Walser (Sanofi), US Pat. No. US20140094450 A1, **2014**; d) S. Narayanan, R. Maitra, J. R. Deschamps, K. Bortoff, J. B. Thomas, Y. Zhang, K. Warner, V. Vasukuttan, A. Decker, S. P. Runyon, *Bioorg. Med. Chem.* **2016**, *24*, 3758–3770; e) A. Murza, É. Besserer-Offroy, J. Coté, P. Bérubé, J.-M. Longpré, R. Dumaine, O. Lesur, M. Auger-Messier, R. Leduc, P. Sarret, É. Marsault, *J. Med. Chem.* **2015**, *58*, 2431–2440.

[16] A. B. Pinkerton, L. H. Smith, L. A. Haile (Sanford-Burnham Medical Research Institute), Int. PCT Pub. No. WO2015184011 A2, **2015**.

[17] a) D. N. Langelaan, E. M. Bebbington, T. Reddy, J. K. Rainey, *Biochemistry* **2009**, *48*, 537–548; b) N. J. M. Macaluso, R. C. Glen, *ChemMedChem* **2010**, *5*, 1247–1253; c) X. Fan, N. Zhou, X. Zhang, M. Mukhtar, Z. Lu, J. Fang, G. C. DuBois, R. J. Pomerantz, *Biochemistry* **2003**, *42*, 10163–10168; d) D. N. Langelaan, T. Reddy, A. W. Banks, G. Dellaire, D. J. Dupré, J. K. Rainey, *Biochim. Biophys. Acta* **2013**, *1828*, 1471–1483; e) J. Hamada, J. Kimura, J. Ishida, T. Kohda, S. Morashita, S. Ichihara, A. Fukamizu, *Int. J. Mol. Med.* **2008**, *22*, 547–552.

[18] a) D. J. Craik, D. P. Fairlie, S. Liras, D. Price, *Chem. Biol. Drug Des.* **2013**, *81*, 136–147; b) G. Ruiz-Gómez, J. D. A. Tyndall, B. Pfeiffer, G. Abbenante, D. P. Fairlie, *Chem. Rev.* **2010**, *110*, PR1–PR41.

[19] E. R. Stadtman, J. Moskovitz, R. L. Levine, *Antioxid. Redox Signaling* **2004**, *5*, 577–582.

[20] A. Murza, A. Parent, E. Besserer-Offroy, H. Tremblay, F. Karadereye, N. Beaudet, R. Leduc, P. Sarret, E. Marsault, *ChemMedChem* **2012**, *7*, 318–325.

[21] V. Mäde, S. Babilon, N. Jolly, L. Wanka, K. Bellmann-Sickert, L. E. Diaz Gimenez, K. Mörl, H. M. Cox, V. V. Gurevich, A. G. Beck-Sickinger, *Angew. Chem. Int. Ed.* **2014**, *53*, 10067–10071; *Angew. Chem.* **2014**, *126*, 10231–10235.

[22] a) C. Guanga, R. D. Phillips, B. Jianga, F. Milani, *Arch. Cardiovasc. Dis.* **2012**, *105*, 373–385; b) M. Iwata, R. T. Cowling, S. J. Yeo, B. Greenberg, *J. Mol. Cell. Cardiol.* **2011**, *51*, 542–547.

[23] Y. Zhang, R. Maitra, D. L. Harris, S. Dhungana, R. Snyder, S. P. Runyon, *Bioorg. Med. Chem.* **2014**, *22*, 2992–2997.

[24] V. M. Ahrens, K. Bellmann-Sickert, A. G. Beck-Sickinger, *Future Med. Chem.* **2012**, *4*, 1567–1586.

[25] Z. Q. Jia, L. Hou, A. Leger, I. Wu, A. B. Kudej, J. Stefano, C. Jiang, C. Q. Pan, G. Y. Akita, *Peptides* **2012**, *38*, 181–188.

[26] P. Kurtzhals, S. Havelund, I. Jonassen, B. Kiehr, U. D. Larsen, U. Ribel, J. Markussen, *Biochem. J.* **1995**, *312*, 725–731.

[27] a) K. Bellmann-Sickert, A. G. Beck-Sickinger, *ChemMedChem* **2011**, *6*, 193–200; b) V. Mäde, K. Bellmann-Sickert, A. Kaiser, J. Meiler, A. G. Beck-Sickinger, *ChemMedChem* **2014**, *9*, 2463–2474; c) L. B. Knudsen, P. F. Nielsen, P. O. Huusfeldt, N. L. Johansen, K. Madsen, F. Z. Pedersen, H. Thogersen, M. Wilken, H. Agerso, *J. Med. Chem.* **2000**, *43*, 1664–1669.

[28] E. E. Ladenheim, *Drug Des. Dev. Ther.* **2015**, *9*, 1867–1875.

[29] J. Nakai, M. Ohkura, K. Imoto, *Nat. Biotechnol.* **2001**, *19*, 137–141.

[30] K. B. Kostelnik, S. Els-Heindl, N. Kloting, S. Baumann, M. von Bergen, A. G. Beck-Sickinger, *Bioorg. Med. Chem.* **2015**, *23*, 3925–3932.

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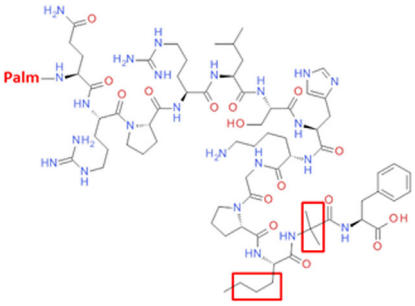
Development of Potent and Metabolically Stable APJ Ligands with High Therapeutic Potential



Tough as nails: The shortest known endogenous apelin receptor ligand was modified by unnatural amino acids, cyclization, and palmitoylation to increase the metabolic stability. In vitro and in vivo stability tests revealed Palm-QRPRLSHKGP-Nle-Aib-F as a very active and stable apelin analogue.