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Adrenomedullin 2.0 - Adjusting Key Levers for Metabolic Stability

Ria Schönauer, [a] *Sylvia Els-Heindl,* [a] *Jan-Patrick Fischer,* [a] *Johannes Köbberling,* [b] *Bernd Riedl,* [b] *and Annette G. Beck-Sickinger* [a*]

[a] Institut für Biochemie, Universität Leipzig, Brüderstraße 34, 04103 Leipzig, Germany

[b] Bayer Pharma AG, Aprather Weg 18A, 42113 Wuppertal, Germany

KEYWORDS

adrenomedullin (ADM), solid-phase peptide synthesis (SPPS), metabolic stability, plasma half-life, enzymatic cleavage, peptide drug, palmitoylation, lactamization, N-methylation

ABSTRACT

The 52 amino acid peptide hormone adrenomedullin (ADM) plays a major role in the development and regulation of the cardiovascular and lymphatic system and has therefore gained significant interest for clinical applications. As adrenomedullin exhibits low metabolic stability enhancement of the plasma half-life is essential for peptide-based drug design. Fluorescently labeled ADM analogues synthesized by Fmoc/*t*-Bu solid phase peptide synthesis were used to analyze their enzymatic degradation and specific fragmentation pattern in human blood plasma. The determination of important cleavage sites allowed the development of selectively modified peptides in a rational approach. By combining palmitoylation, lactam-bridging and N_{α}-methylation, ADM analogues protected from enzymatic cleavage in human blood were developed and revealed an explicitly elongated half-life of five days in comparison to the wild-type *in vitro*. Thus, this triple-modification did not alter the selectivity of the analogues at the AM_1 receptor, highlighting their potential for therapeutic applications.

Introduction

Adrenomedullin (ADM) is a 52 amino acid peptide hormone and member of the calcitonin family of peptides, which was initially discovered in 1993 by the isolation from human pheochromocytoma.[1] During the last 20 years, ADM was identified in multiple tissues and organs, for instance in the lung, the heart and in endothelial and vascular smooth muscle cells but also in the central nervous system.[2,3] Its biological effects are mainly mediated by binding to the calcitonin receptor-like receptor (CLR), which forms a receptor/co-receptor complex with the receptor activity-modifying protein 2 (RAMP2).[4] ADM participates in a variety of physiological and pathophysiological mechanisms, including the development and regulation of lymphatic and cardiovascular systems, tumor growth and metastasis, and exhibits antimicrobial properties.[2]

Its great vasodilatory potential proposes ADM as promising drug candidate for the treatment of cardiovascular diseases, since it exerts protective functions in hypertension, heart failure and myocardial infarction and has already been tested in clinical trials.[5,6] As a naturally occurring peptide, ADM provides explicit advantages for drug development, including high specificity and activity towards its target receptor. However, the low bioavailability and metabolic stability is a major drawback for the applicability of peptides as therapeutics, which is clearly illustrated by the short plasma half-life of ADM of only 22 min *in vivo*.[7-9] The lungs have been identified as the primary target and therefore as one of the major clearance sites for ADM. It is proposed that ADM disappears from the circulation probably after binding to its specific receptor, which was underlined by the finding that the peptide bound to its receptor/co-receptor-complex undergoes robust co-internalization and gets routed to the lysosomal pathway.[10-12] Furthermore, the instability of ADM against degradative enzymes in the plasma or cell membranes reduces the amount of bioactive peptide in the circulation and therefore limits its potential effectiveness. Although several studies already suggested that metalloproteases like MMP-2, thermolysin and NEP are involved in the cleavage of ADM,

the exact mechanisms underlying its degradation is not clear.[13-16] Knowledge about enzymatic target sites in the ADM sequence could therefore provide a base for the design of a rational modification strategy, which is balancing receptor activation and metabolic stability.

In this study the specific fragmentation pattern of ADM in human blood serum was elucidated, which provided implications for the synthesis of stabilized peptide variants using different chemical modifications. The combination of palmitoylation, lactam-cyclization and N-methylation led to the generation of a selective AM_1 receptor targeting ADM analogue, which revealed a remarkably improved *in-vitro* half-life, illustrating its prospects for potential therapeutic applications. Furthermore, this approach demonstrates how medium-large size biomolecules can be modified to gain higher biostability.

Results

Degradation of unmodified ADM in human blood plasma offers information for the stabilization of ADM

The stability of unmodified ADM was investigated by the incubation of a fluorescent 6-carboxytetramethylrhodamine-labeled analogue in human blood plasma. In earlier studies, it was already demonstrated that N-terminal truncation of ADM(1-52) (**1**) in the analogue [G^{14}]ADM(14-52) (**2**) and fluorescence labeling in Tam[G^{14}]ADM(14-52) (**3**) was tolerated in terms of receptor activation and internalization behavior at the AM_1 receptor.[10] Monitoring of the degradation process by RP-HPLC with fluorescence detection revealed a rapid reduction of the intact peptide within the first 72h of incubation (Figure 1C). MALDI-ToF-MS analysis was carried out to get insight into the structure of cleavage fragments in the time course of the degradation process (Figure 1A, B, D). Tam[G^{14}]ADM(14-50) was identified as a first major cleavage product, followed by the accumulation of Tam[G^{14}]ADM(14-45) and Tam[G^{14}]ADM(14-44). Peaks, which did not rise over time, contained small-sized fragments ranging from Tam[G^{14}]ADM(14-33) to Tam[G^{14}]ADM(14-23) that were rapidly degraded to the final cleavage product Tam[G^{14}]ADM(14-21). Additionally, a reduction of the disulfide bridge between Cys^{16} and Cys^{21} was observed, as both oxidized and reduced Tam[G^{14}]ADM(14-21) could be detected (Figure 1E).

Next, cleavage sites that had to be protected in order to preserve the agonistic properties of ADM at the AM_1 receptor were evaluated. Therefore, the synthesis of the cleavage products [G^{14}]ADM(14-50) (**4**), [G^{14}]ADM(14-45) (**5**) and [G^{14}]ADM(14-44) (**6**) that were found to be produced in the beginning of the degradation process as well as the final cleavage fragment [G^{14}]ADM(14-21) (**7**) was performed and their receptor activation potency was investigated. To additionally assess the influence of the intact cyclic structure, a linearized ADM variant [$G^{14,\ 16,\ 21}$]ADM(14-52) (**8**) with glycine residues instead of cysteines were generated at positions 16 and 21 as well as ADM(22-52) (**9**), which is known to be an antagonist.[17] None

of these analogues were able to activate the AM_1 receptor to any extent in a cyclic AMP readout (Figure S1). Thus, as not even the longest fragments resulting from C-terminal cleavage retained activity, it is necessary for the stabilization of ADM to prevent the enzymatic cleavage in the C- and the N-terminal peptide part, as well as the opening of the ring structure.

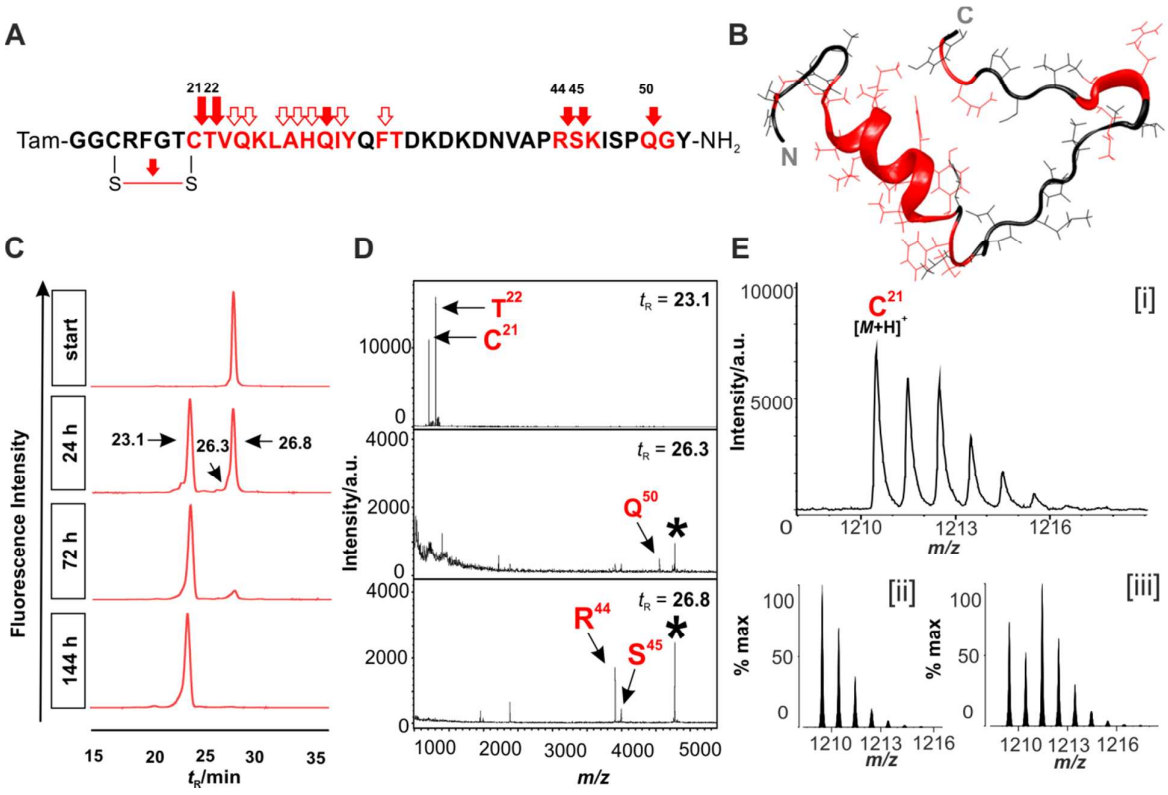


Figure 1. Degradation profile of ADM in human blood plasma.

A) Sequence of analogue **3** with cleavage sites in human blood plasma indicated by red colored letters. Filled and blank arrows show major and minor cleavage products, respectively, determined by fragment accumulation analyzed with RP-HPLC and MALDI-ToF mass spectrometry. B) Structure of ADM(15-52) indicating the position of cleavage sites in red, PDB: 2L7S.[18] C) RP-HPLC analysis of analogue **3** after 0, 24, 72 and 144 h of incubation in human blood plasma (fluorescence emission detected at λ = 573 nm). D) MALDI-ToF mass spectra of RP-HPLC peaks after 24 h (t_R = 23.1, 26.3 and 26.8 min). Signals corresponding to cleavage fragments are labeled with red letters. Asterisks indicate

signals of the intact peptide. E) MALDI-ToF-MS analysis of the oxidation state of the disulfide bridge for the cleavage fragment Tam[G^{14}]ADM(14-21). [i] Measured isotope pattern of Tam[G^{14}]ADM(14-21). [ii] Calculated isotope pattern for Tam[G^{14}]ADM(14-21) with completely oxidized disulfides. [iii] Calculated isotope pattern for a 1:1 ratio of Tam[G^{14}]ADM(14-21) with oxidized and reduced disulfides.

Palmitoylation and lactam-bonding increase the resistance against enzymatic degradation

As a first step towards the stabilization of ADM, the reduction-sensitive disulfide bridge of the native peptide was replaced by a structural element, which promised higher metabolic stability. By exchanging the cysteines at positions 16 and 21 with 2,3-diaminopropionic acid and glutamate, respectively, subsequent formation of a lactam bond between their sidechains, led to the cyclic peptide [G^{14}, ($Dpr^{16,}$ E^{21})]ADM(14-52) (**10**), which retained full agonistic behavior at the AM_1 receptor (EC_{50} = 2.8 nM, pEC_{50} = 8.56 ± 0.03; Figure S2A).

To additionally reduce the high number and broad distribution of cleavage sites within the ADM sequence, a modification with extensive protective properties in addition to lactam cyclization was required. The attachment of fatty acids, especially palmitic acid (Pam), has already been reported to effectively enhance the metabolic stability of different peptides.[19, 20] By side-chain palmitoylation of lysine either at position 14 (**11**), 33 (**12**), 45 (**13**) or 50 (**14**) or at the N-terminus (**15**), the optimal position for this modification in the ADM sequence could be identified. Whereas the C-terminally palmitoylated peptides **12**, **13** and **14** failed to activate the AM_1 receptor sufficiently, the side-chain palmitoylation of Lys^{14} in analogue **11** as well as N-terminal addition of palmitic acid in analogue **15** are well tolerated in terms of receptor activation (EC_{50} = 11.5 nM, pEC_{50} = 7.94 ± 0.09 and EC_{50} = 13.2 nM, pEC_{50} = 7.88 ± 0.06, respectively; Figure S2B).

Consequently, as only the combination of both modifications, palmitoylation and lactam bonding, was supposed to enhance the metabolic stability of ADM while keeping its activity, the double-modified analogue [K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**16**) was generated.

The synthesis of this peptide was highly efficient as the lactamization and the subsequent coupling of palmitic acid was nearly quantitative after 24 h of reaction time. Notably, including both modifications in one peptide influenced the AM_1 receptor activation only to a minor extent, as analogue **16** displayed agonistic behavior with an acceptable 7-fold decrease in potency compared to native ADM (EC_{50} = 22.3 nM, pEC_{50} = 7.65 ± 0.13; Figure 5A).

For the evaluation of the protective effects arising from the combination of the Lys^{14} side-chain palmitoylation and lactam-bonding, the fluorescently labeled analogue Tam[K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**17**) was prepared and incubated in human blood plasma (Figure 2A). In comparison to the unmodified ADM analogue **3**, the double-modified analogue **17** was clearly protected against enzymatic degradation, as the cleavage site at position Q^{50}/G^{51} was not present in this peptide and only the cleavage fragments Tam[K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$]ADM(14-45) and Tam[K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$]ADM(14-44) were accumulating over time. Degradation in the N-terminal part was reduced to a minimal amount and no cleavage was found within the ring-structure, which highlights the stabilization potential of the introduced lactam bridge. The generation of cleavage fragments was highly delayed in comparison to unmodified ADM, as the remarkable amount of 30 % intact peptide was still present after 144 h of incubation.

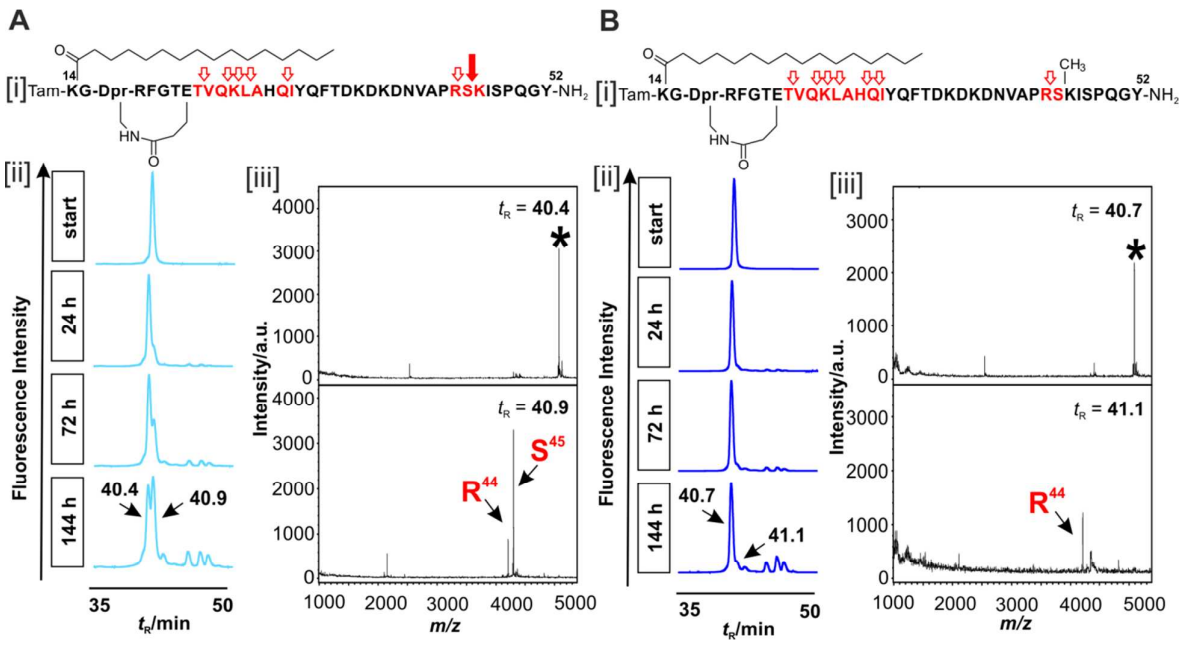


Figure 2. Degradation profile of stabilized ADM analogues in human blood plasma.

A) Degradation of double-modified analogue **17** and B) of triple-modified analogue **20**. [i] Sequences and cleavage sites in human blood plasma indicated by red colored letters. Filled and blank arrows show major and minor cleavage fragments, respectively. [ii] RP-HPLC analysis of the degradation progress after 0, 24, 72 and 144 h of incubation in human blood plasma (fluorescence emission detected at λ = 573 nm). [iii] MALDI-ToF mass spectra of selected peaks after 144 h (analogue **17**: t_R = 40.4 min and 40.9 min; analogue **20**: t_R = 40.7 min and 41.1 min). Signals corresponding to cleavage fragments are labeled with red letters. Asterisks indicate signals of the intact peptide.

Selective N-methylation of ADM completes the set of protective modifications

Although the double-modified analogue **17** exhibited a superior stability in human blood plasma compared to the unmodified ADM **3**, a significant loss of intact peptide by the cleavage at positions S^{45}/K^{46} and R^{44}/S^{45} was observed after 144 h. Thus, these peptide bonds had to be additionally protected from enzymatic attacks. The method of choice was the selective introduction of N_α-methylated lysine at position 46. To combine this feature with

the beneficial effects of palmitoylation and lactam-bridging, we synthesized the triple-modified ADM analogue [K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$, N_α-Me-K^{46}]ADM(14-52) (**18**). N_α-methylated lysine and the following serine were introduced into the peptide sequence prior to the lactamization and palmitoylation steps (Figure 3). Whereas the addition of Fmoc-N_α-Me-Lys(Boc)-OH to ADM(47-52) appeared to be facile, the coupling reaction of the next amino acid Fmoc-Ser(*t*-Bu)-OH with standard coupling conditions showed up to be virtually impossible. By applying a high excess of coupling reagents in a solvent mixture of DMF, DCM and NMP under heating and rapid shaking we achieved to generate appropriate yields, as the subsequent lactamization and palmitoylation efficiencies were comparable to those of the double-modified analog **16**.

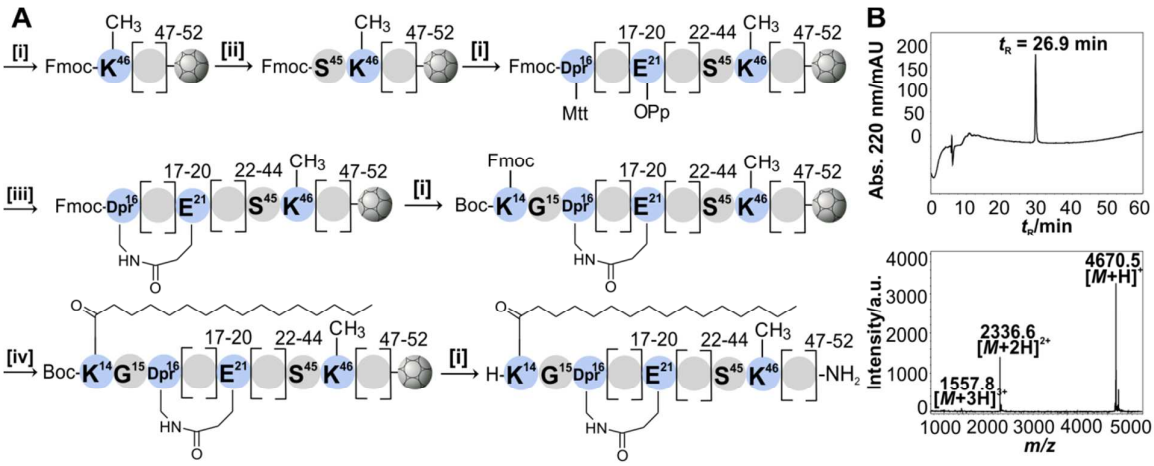


Figure 3. Synthesis and characterization of stabilized ADM.

A) Synthesis of the triple-modified ADM analogue **18**. [i] Solid-phase peptide synthesis (SPPS): 8 equiv amino acid/Oxyma/*N,N'*-diisopropylcarbodiimide (DIC) in DMF. [ii] Coupling of Fmoc-Ser(*t*-Bu)-OH to N_α-Me-Lys(Boc)-ADM(46-52) on TGR R resin: 5 equiv Fmoc-Ser(*t*-Bu)-OH/ 10 equiv HOBt/ 10 equiv DIC in DMF/DCM/NMP (1:1:1, *v/v/v*); 50°C; 1300 rpm; 24 h. [iii] Selective cleavage of *N*-β-4-methyltrityl/γ-2-phenylisopropyl (Mtt/OPp) protection groups: DCM/TIS/TFA (93:5:2, *v/v/v*); 12 x 2 min. Lactamization: 6 equiv *N*-hydroxybenzotriazole (HOBt)/DIC in DMF; 24 h. [iv] Palmitoylation: 5 equiv palmitic

acid/HOBt/DIC in DMF; 24 h. [iv] Full cleavage and deprotection: TFA/thioanisole (TA)/ethane-1,2-dithiol (EDT) (90:7:3, *v/v/v*). B) Analytical RP-HPLC of the purified analogue **18** (Jupiter 5µ C_{18} 300 Å column, linear gradient of 20-70% (*v/v*) eluent B in eluent A over 40 min) and MALDI-ToF mass spectrum displaying $[M+H]^+$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ signals (M_{calc} = 4669.54 Da).

To evaluate the influence of the additional methyl group on AM_1 receptor activation, cAMP assays were conducted with [G^{14}, N_α-Me-K^{46}]ADM(14-52) (**19**), which only contained the single modification and the triple-modified analogue **18**. Interestingly, the single-modified analogue **19** showed a noticeable loss of potency (EC_{50} = 186.5 nM , pEC_{50} = 6.77 ± 0.07; SI Figure S2A) that was, however, compensated in the triple-modified ADM analogue **18**. Indeed, analogue **18** exhibited only a two-fold loss of potency in comparison to the double-modified analogue **16** (EC_{50} = 51.5 nM, pEC_{50} = 7.29 ± 0.05; Figure 5A).

For the investigation of the stabilizing effects arising from this additional modification, the analogue was labeled with the fluorescence dye Tam at the N-terminus, incubated in human blood plasma and analyzed under the same conditions as the unmodified and the double-modified ADM peptides (Figure 2B). Using MALDI-MS, it could be demonstrated that the additional incorporation of N_α-Me-Lys^{46} led to the complete inhibition of the cleavage at position R^{44}/S^{45} and a significant reduction of the cleavage at position S^{45}/K^{44}. The protection of these peptide bonds clearly enhanced the stability of ADM by reducing the generation of cleavage fragments to a minimal amount, hence allowing 50 % of intact peptide to be present after 144 h of incubation in human blood plasma.

Stabilized ADM enhances plasma half-life and prolongs AM_1 receptor activation

The fast and exhaustive degradation of the unmodified short ADM analogue **3** in human blood plasma was represented by a half-life of only 9.7 h, according to RP-HPLC (Figure 4A). N-terminal palmitoylation and lactam-bridging of the double-modified analogue **17** and the additional beneficial effect of N_α-methylation of the triple-modified analogue

Tam[K^{14}(Pam), (Dpr^{16}, E^{21})$_{lac}$, N_α-Me-K^{46}]ADM(14-52) (**20**) greatly improved the peptides resistance against proteolytic cleavage and resulted in a half-life of 84.6 h and 126.2 h, respectively. Whereas palmitoylation in the single-modified analogue Tam[K^{14}(Pam)]ADM(14-52) (**21)** enhanced the overall stability of ADM, the single modified analogues Tam[G^{14}, (Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**22**) and Tam[G^{14}, N_α-Me-K^{46}]ADM(14-52) (**23**) in contrast displayed only minor or no impact on the plasma half-life (Figure S2B), which demonstrates that the stabilization effect of this set of modifications is not simply additive but arises from their combination.

Subsequently, it was investigated whether the increase in stability also effectively led to a prolonged AM_1 receptor activation. Therefore, the cAMP accumulation in AM_1 receptor expressing HEK293 cells of the unmodified short ADM analogue **2** and the triple-modified analogue **18** was compared. Both peptides were incubated in human blood plasma prior to stimulation (Figure 4C). Plasma solutions with peptide concentrations of 10^{-8} M were used to determine the receptor responses depending on the incubation time. Samples that were taken at the starting point of the incubation defined the maximal receptor activation as well as the maximal amount of intact peptide. Additional dilutions of these samples to concentrations of 10^{-9} M and 10^{-10} M were prepared to evaluate the receptor responses, which corresponded to 10% or 1% of intact peptides, respectively. As expected, analogue **2** showed a dramatic reduction in activation potency at the AM_1 receptor after 144 h of incubation in human blood plasma, displaying almost a 100-fold loss of intact peptide. The triple-modified analogue **18**, in contrast, still possessed 50% receptor activation potency, which accounts for less than a 10-fold reduction of the intact ligand after 144 h of incubation.

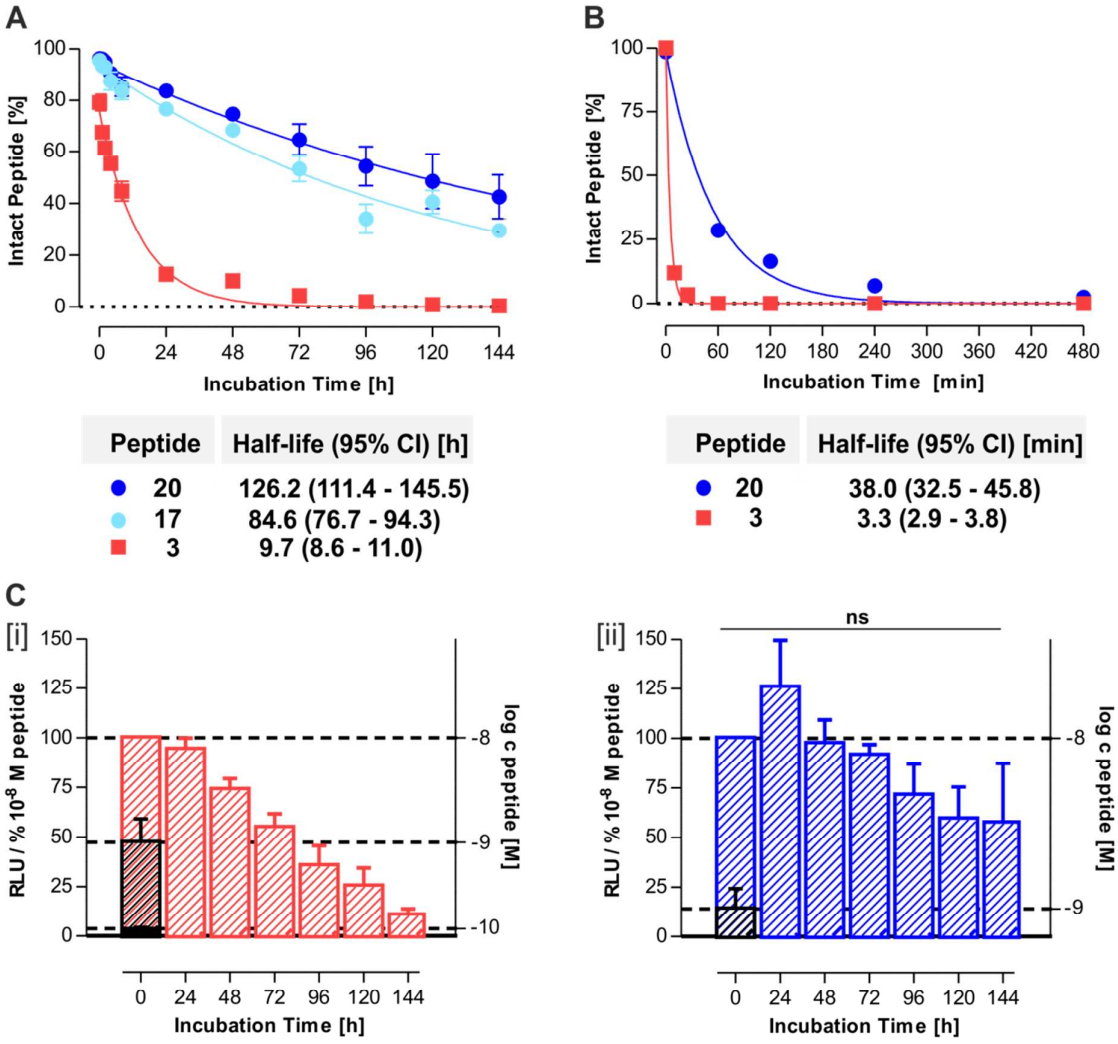


Figure 4. Half-life and AM_1 receptor activation of unmodified and stabilized ADM analogues.

Determination of half-lifes A) in human blood plasma (analogues **3**, **16** and **20**) and B) in porcine liver homogenate (analogues **3** and **20**) using one phase decay analysis of RP-HPLC peak integrals containing intact peptide (fluorescence emission detected at λ = 573 nm). Data points represent mean ± SEM of ≥ 2 independent experiments. B) Investigation of AM_1 receptor activation potency for [i] analogue **3** and [ii] analogue **20**. Ligands were incubated in human blood plasma for 0, 24, 48, 72, 96, 120 and 144 h and cAMP accumulation of 10^{-8} M ligand solution was detected using HEK 293 cells transiently transfected with the AM_1

receptor. Maximal receptor activation (100%) was determined using 10^{-8} M peptide (t = 0) and minimal activation (0%) using serum without peptide (t = 0). Dotted lines indicate concentrations of 10^{-9} (t = 0; black dashed bars) and 10^{-10} M peptide dilutions (t = 0; black filled bar) representing 10- and 100-fold loss of peptide, respectively. Bar graphs represent mean ± SEM of two independent experiments.

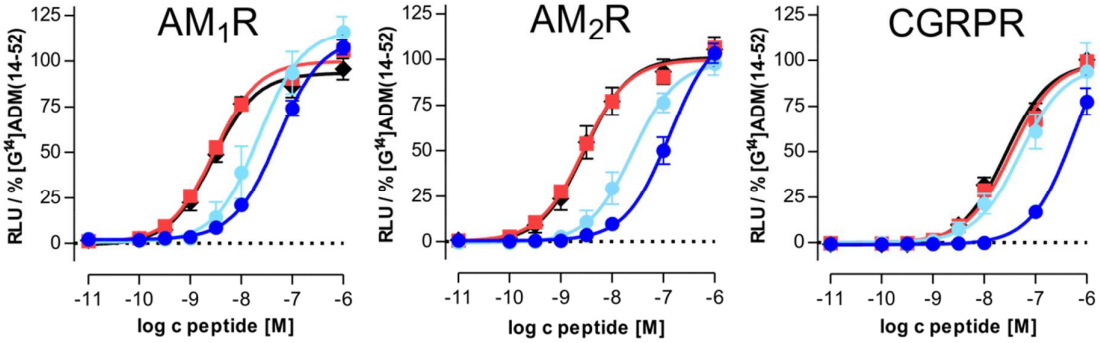
Chemical modifications improve the stability of ADM in porcine liver homogenate

To further validate the stabilizing effects of the introduced modifications, the unmodified analogue **3** and the triple-modified ADM **20** were additionally incubated in porcine liver homogenate. Notably, the unmodified peptide **3** was rapidly degraded with a half-life of 3.3 min, whereas the triple-modified analogue **20** revealed a considerably elongated half-life of 38 min, which represents an 11-fold increase in stability (Figure 4B). Thus, the stability of the triple-modified peptide **20** showed an improvement in stability that was comparable to the effects observed in human blood plasma, which strongly supports the effectivity of the applied chemical modification strategy.

Stabilized ADM analogues are full agonists at the AM_1 receptor

For a prospective use in clinical applications, the selectivity of the modified ADM analogues at the AM_1 receptor is of great importance. Therefore, the receptor activation of the unmodified short ADM analogue **2** as well as the stabilized ADM analogues **16** and **18** was evaluated not only at the AM_1 but also at the related AM_2 and CGRP receptors. The analogues **16** and **18** both showed agonistic behavior at the AM_1 receptor with an expectable loss of activation potency in comparison to analogue **2** (7-fold and 17-fold, respectively) regarding the substantial modifications. Analogue **16** comprised a selectivity that was similar to the unmodified ADM **1** and **2** at the AM_2 receptor and showed a 5-fold loss in selectivity at the CGRP receptor. Importantly, the triple-modified analogue **18** displayed wildtype-like

selectivity at both the AM_2 receptor and the CGRP receptor, demonstrating that the introduced modifications do not influence the peptide selectivity.



Peptide	pEC_{50} AM_1	pEC_{50} AM_2	pEC_{50} CGRPR
1	8.56 ± 0.03(#)	8.54 ± 0.10	7.59 ± 0.07
2	8.53 ± 0.07(#)	8.56 ± 0.04	7.48 ± 0.06
16	7.65 ± 0.13	7.60 ± 0.10	7.31 ± 0.15
18	7.29 ± 0.05	6.88 ± 0.06	6.22 ± 0.14

Figure 5. Receptor activation of unmodified and stabilized ADM analogues.

Accumulation of cAMP was determined using HEK 293 cells transiently transfected with the AM_1, AM_2 or CGRP receptor. The analogues **16** and **18** prefer the AM_1 receptor over AM_2 and CGRPR. ((#) previously published. [10])

Discussion

As a potent vasodilatory peptide hormone, ADM plays a major role in the regulation of blood pressure and, moreover, has been investigated for its cardio-protective effects in animal models and human patients. Recently, numerous clinical trials and patents highlighted its outstanding potential as diagnostic tool and therapeutic target.[2, 21] However, the low metabolic stability of ADM, which exhibits a circulating half-life of only 22 min *in vivo*[7], leads to limitations in its applicability. Therefore, there is a strong interest to identify and stabilize the weak points in the peptide sequence of ADM in order to improve the circulation half-life for a more efficient use in clinical applications.

The N-terminally truncated ADM analogue **2** was used as a lead peptide for the introduction of stabilizing modifications, as the first 15 amino acids of the full-length peptide were neither required for receptor binding nor activation or internalization.[10, 17, 22, 23] First, optimization of the peptide was carried out in human blood plasma by a rational approach to strengthen stability at positions that are prone to degradation. Fluorescence labeling with 6-carboxytetramethylrhodamine (Tam) was a convenient method to monitor the degradation process by specific tracking of labeled cleavage fragments using RP-HPLC and MALDI-MS analysis.[24, 25] As expected, analogue **3** exhibited a low *in vitro* half-life of 9.7 h, as it was rapidly degraded into a multitude of smaller fragments. While the flexible middle part of ADM (position 34–43) was not targeted by enzymatic attacks, cleavage sites were mainly located in the helix-forming region following the disulfide-bridged ring-structure (position 21-33), which has already been reported to be a target for enzymatic degradation of ADM.[13,14] The N-terminal ring-structure containing fragment Tam[G^{14}]ADM(14-21) was identified to be the final accumulating product that, notably, was found to be partially reduced. Interestingly, cleavages sites were also found in the very C-terminal part of the sequence between positions 44/45, 45/46 and 50/51, which were not discovered so far. In order to establish a stabilization strategy with priority on the most relevant cleavage sites, it was elucidated whether any of the

major degradation products retained the potency to activate the AM_1 receptor. Consistent with previous findings, none of the tested fragments provoked AM_1 signaling to any extent.[13, 14, 26] These effects could be attributed to the lack of the C-terminally amidated Tyr^{52} residue, which has been reported to be crucial for AM_1 receptor binding and signaling.[17, 27] Linearization abolished receptor signaling comparable to the antagonistic peptide **8**, which confirms the finding that not solely the presence of the amino acids 16-21 but also their correct arrangement is a the key activating motif of ADM.[17,23] Therefore, a successful stabilization strategy for ADM had to meet two requirements to preserve the agonistic properties of the peptide at the AM_1 receptor: First, the bioactive ring-structure between Cys^{16} and Cys^{21} had to be intact, and second, the N-terminal and the C-terminal part of the ADM sequence had to be protected from enzymatic cleavage.

As the interest in the application of peptides in clinical approaches is continuously rising, various synthetic methods for enhancing their therapeutic potential were established during the last years.[28-30] Essentially, the disulfide-bonded ring structure of ADM exhibits an inherent stabilizing effect, as cyclic amino acid chains are able to adopt conformations, which may be unfavorable for fitting into the catalytic pockets of proteolytic enzymes.[31] However, the detected partial reduction of the disulfide-bridge after incubation in human blood serum abolishes the protective influence and furthermore, disrupts the key activation motive of ADM. Therefore, exchanging the reduction sensitive Cys-Cys interaction by a bond with higher resistibility against the conditions, which predominate in the circulation, was assumed to intensify this intrinsic stabilization feature of the native peptide. The disulfide bonded ring structures of the related peptides calcitonin and CGRP have already been replaced by ethylene or lactam bonds in earlier studies.[32-34] For the stabilization of the ring-structure of ADM, lactam cyclization by diaminopropionic acid and glutamate was found to be a convenient method, as orthogonal deprotection of acid labile protection groups allowed the selective interaction of the desired residues using standard coupling conditions. Remarkably, although

this substitution conferred significant changes into the major activation site of ADM, the generated ADM analogue displayed similar AM_1 receptor activation potency as the unmodified peptide.

Besides the stabilization of the ring structure, a further modification was required to obtain substantial protection of the peptide backbone from proteolytic attacks. The covalent attachment of fatty acids has emerged as an impressive tool to improve the circulation half-life of peptides and has already led to the development of potent long-acting peptide drugs, like the anti-diabetics.[35] The stabilizing potential of peptide lipidation can be attributed to reversible interactions with the plasma carrier protein serum albumin, which shields its cargo from enzymatic attacks during the transport through the blood stream.[36] The size of the resulting peptide-albumin complex additionally exceeds the cut-off for glomerular ultrafiltration as it was also found for peptides linked to large-sized polyethylene glycol moieties.[37] Recently, N-terminal PEGylated human full-length ADM was found to exhibit an increased stability in rat plasma in comparison to native ADM, which was, however, not reflected by a prolonged duration of action in this study.[38]

Addition of long alkyl chains, such as palmitic acid, was suggested to be suitable for the generation of long-acting and highly active peptide analogues.[20] Here, side-chain palmitoylation of the N-terminal Lys^{14} was identified as a position in the ADM sequence, which tolerates this capacious modification, as it appeared to be superior over the C-terminally located positions 33, 45 and 50 with respect to AM_1 receptor activation.

Interestingly, incubation of single-modified ADM analogues **22** and **21** in human blood plasma, containing the lactam-cyclized ring-structure or the palmitoylation, revealed a 1.6-fold and 6-fold elongation of plasma half-life in comparison to the unmodified ADM, respectively. However, it could be assumed that a partial linearization of the analogue **21** following the reduction of the disulfide-bridge, which is certainly not prevented by palmitoylation, alters the overall structure of the peptide and thereby improves the

accessibility for enzymatic attacks. For CGRP that is closely related to ADM und exhibits a similar structure, the disulfide-bonded ring is assumed to confer a helix-stabilizing effect.[39] The increased stability of the lactam-cyclized analogue **22** in comparison to unmodified ADM, suggests a strengthening in the overall peptide conformation, which also points to this direction. The combination of both protective modifications, lactam cyclization and palmitoylation, can therefore be considered to lead to a cooperative effect for the stabilization of ADM. In fact, incubation of the double-modified ADM analogue **17** in human blood plasma resulted in a significantly delayed production of N-terminal cleavage fragments and the complete absence of cleavage after position 50, leading to a remarkable 8.7-fold extended half-life of 84.6 h in comparison to unmodified ADM.

Besides the substantial benefits arising from the effects of palmitoylation and lactam-cyclization, the C-terminal cleavage after positions 44 and 45 was not slowed down, thus reducing the amount of intact peptide considerably. To additionally protect these remaining cleavage sites, N_α-methylation was carried out, which was shown to effectively protect neighbored peptide bonds and was used to enhance the proteolytic resistance for several bioactive peptides, like angiotensin, endothelin or neurotensin.[40, 41] Indeed, the incorporation of N_α-methylated Lys^{46} into the sequence of ADM in combination with lactam-cyclization and palmitoylation completely inhibited the cleavage at position 45 and additionally reduced the cleavage at position 44 to a minimal amount. Consequently, the triple-modified ADM analogue **20** showed a striking improvement in stability with a half-life of 126.2 h in human blood plasma. This represents a 13-fold elongation in comparison to the unmodified ADM. Notably, the single-modified analogue **23** revealed no enhancement in plasma half-life compared to analogue **3**, which clearly demonstrates that N-terminal cleavage is not a requirement but rather independent from C-terminal processing of the peptide sequence. Thus, the powerful protective effect of N_α-Me-K^{46} arises only in combination with palmitoylation and lactam-cyclization, which suppress the degradation of the N-terminal part of ADM.

Hence, selective stabilization of ADM is not the sum of separate effects, but depending on a synergistic combination of modifications.

The effectivity of the modification strategy was supported by an 11-fold elongated half-life of the triple-modified analogue **20** compared to the unmodified ADM **3** in porcine liver homogenate. Thus, the introduced modifications exert their stabilizing effects under different degrading environments, since they inhibit the cleavage of ADM by plasmatic enzymes and also resist the degradation machinery of the liver.

Importantly, the stabilized ADM peptides were proven to behave as agonistic ligands for their target receptor AM_1, albeit profound synthetic modifications were introduced. Whereas lactam-cyclization did not affect receptor activation, palmitoylation, either separate or in combination with lactam-cyclization, contributed to a slight reduction of agonist activity, with a 4- and 7-fold loss of agonistic potency. The N_α-methylated single modified analogue, in contrast, showed a significant 50-fold loss of function at the AM_1 receptor. Recently, structural insights into the binding of ADM to the CLR/RAMP2 complex revealed a contribution of Lys^{46} and thus, modifications in this part of the peptide can have an impact on receptor activation. This undesired effect, however, was assumed to be counterbalanced by the combination with palmitic acid and lactam cyclization. Palmitoylation can increase the effective concentration in the proximity of the receptor due to interactions with the cell membrane or stabilize helical peptide parts, which leads to a pre-oriented structure that could lead to an enhanced receptor activation.[42, 43] Similarly, the finding that the lactam cyclization improves the stability of the whole peptide while keeping wild-type like activity suggests that the rigidified ring-structure influences the arrangement of the overall peptide conformation to a favorable orientation. Albeit not reaching the potency of unmodified ADM, combining N_α-methylation with palmitoylation and lactam cyclization led at least to a 3-fold improvement of the AM_1 activation properties of the triple modified ADM analogue **18** in comparison to analogue **19**. In order to reduce the risk of side-effects in therapeutic treatment, it is of great

importance that the stabilized ADM analogues are selective for their target receptor AM_1 against the related CGRP and AM_2 receptors.[4] Remarkably, the double modified analogue **16** as well as the triple-modified **18** exhibited a selectivity profile, which was comparable to that of the unmodified ADM further expanding their suitability for clinical applications.

To ensure that the improved plasma-half life was transferred into a prolonged stimulation of the AM_1 receptor, cAMP signaling was tested with solutions of the unmodified analogue **2** and the triple-modified analogue **18** after incubation in human blood plasma. While the cAMP signal induced by analogue **2** was rapidly descending with proceeding incubation time, the triple-modified ADM analogue **18** possessed a stable receptor activation profile for 6 days of incubation. The remaining AM_1 receptor activity at day 3 and 6 of the incubation reflected approximately 10% and 1% of intact unmodified ADM, respectively, but revealed less than 10% degradation of the stabilized ADM analogue. These studies were in excellent agreement with the results obtained from the HPLC-analysis by confirming an improvement in half-life for the stabilized ADM analogue by a factor of 10. The assignment of an enhanced metabolic stability with a prolonged mode of action *it vitro* generates promising perspectives for *in vivo* applications.

As the probability for enzymatic attacks naturally increases with the length of the peptide sequence, the majority of the contemporarily produced peptide drugs are of low or medium size, thus prohibiting the clinical application of many therapeutically relevant peptides.[28] Additionally, the introduction of stabilizing modifications often requires difficult synthesis strategies[44], which can tremendously reduce yields and purities and only a low number of multiple stabilized peptides were reported. N_α-methylation combined with cyclization could greatly improve the half-life of carbetocin.[45] Another example is a conjugate of GLP-1 and human serum albumin that exhibited enhanced proteolytic stability by introduction of an D-amino acid.[46] Peptide analogues containing three modifications, like the small integrin inhibitor cilengitide[41,47], indeed display exceptional cases, presumably due to the increased

synthetic complexity. Here we could show that double-modified palmitoylated and lactam-cyclized ADM exhibited a considerably increased plasma half-life, which was impressively further enhanced by additional N_α-methylation. Thus, the synthesis of the triple-modified ADM raises new perspectives for the drugability of long and difficult peptides.

Conclusion

ADM is as a large peptide hormone with two separate bioactive regions that are located at the two opposite termini of its sequence. The high number and the broad distribution of cleavage sites lead to numerous inactive metabolites, which require a combination of several collaborative modifications to obtain a powerful protection against enzymatic degradation. The synthetic triple-modified ADM analogue combines palmitoylation, lactam-cyclization and N_α-methylation, which impressively increases the half-life in blood plasma and liver homogenate and leads to a prolonged duration of activity *in vitro*. However, further experiments have to prove, if these stabilizing effects can be translated to the *in vivo* system. Concerning its remarkable metabolic stability, receptor activation potential and selectivity, stabilized ADM represents a highly promising candidate for prospective clinical applications.

Experimental section

General synthesis methods

Synthesis method 1: solid-phase peptide synthesis

ADM analogues were synthesized by by solid-phase peptide synthesis (SPPS) using the Fmoc/*t*-Bu strategy at a 15 µmol scale on NovaSyn®TGR R resin (Merck KGaA, Darmstadt, Germany) to generate C-terminally amidated peptides. NovaSyn®TGR HMP resin (Merck KGaA, Darmstadt, Germany) or 2-Chlorotrityl resin (Iris Biotech, Marktredwitz, Germany) was used for the preparation of peptide acids. Automated SPPS was performed with a peptide synthesizer (SYRO I, MultiSynTech, Bochum, Germany) using 8-fold molar excess of *N*-α-Fmoc- or *N*-α-Boc-protected amino acids (Orpegen, Heidelberg, Germany; Iris Biotech, Marktredwitz, Germany; Merck KGaA, Darmstadt, Germany; Sigma-Aldrich, St. Louis, USA;), ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) and *N,N′*-diisopropylcarbodiimide (DIC) in DMF. All automated coupling steps were carried out twice with a reaction time of 40 min. Fmoc protecting groups were cleaved using 40 % piperidine in DMF (*v*/*v*) for 3 min and 20 % piperidine in DMF (*v*/*v*) for 10 min.

Manual couplings were performed with a 5-fold molar excess of amino acid and the reagents 1-hydroxybenzotriazole (HOBt) and DIC in DMF at room temperature for 24 h. The coupling of Fmoc-Ser(*t*-Bu)-OH to [N_α-Me-Lys(Boc)^{46}]ADM(46-52) on resin was achieved by the application of 5 equiv amino acid and 10 equiv of HOBt and DIC in a solvent mixture of DMF, DCM and NMP (1:1:1, *v/v/v*) under constant shaking at 1300 rpm and 50°C for 24 h. Manual Fmoc-removal was performed using 30 % piperidine in DMF (*v*/*v*) for twice 10 min.

Synthesis method 2: lactam-cyclization

For the on-resin lactam-cyclization between the side chains of diaminopropionic acid at position 16 and glutamate at 21, respectively, the orthogonal protected amino acids Fmoc-Dpr(*N*-β-4-methyltrityl)-OH and Fmoc-Glu(γ-2-phenylisopropyl)-OH were introduced into the peptide sequence. The *N*-β-4-methyltrityl (Mtt) and γ-2-phenylisopropyl (OPp) protection

groups were removed simultaneously by incubation of the resin 12 times for 2 min in a cleavage cocktail of DCM/triisopropylsilane (TIS)/trifluoroacetic acid TFA (93:5:2, *v/v/v*). The free amino acid side-chains were subsequently connected by amide bond formation using 6 equiv of HOBt and DIC in DMF for 24 h.

Synthesis method 3: palmitoylation

Selective palmitoylation of the peptide N-terminus or lysine side-chains was achieved with 5 equiv palmitic acid (Pam), HOBt and DIC in DMF for 24 h after orthogonal cleavage of lysine protecting groups. Therefore, either the N-terminal amine or the side chain of lysine was deprotected from Fmoc or 4,4-dimethyl-2,6-dioxocyclohex-1-ylidenethyl (Dde) protection groups, respectively. Dde cleavage was carried out with 3% hydrazine in DMF 12 times for 10 min.

Synthesis method 4: fluorescence labeling

Fluorescence labeling of ADM analogues using 6-carboxytetramethylrhodamine (Tam) was performed as described in previous studies.[10]

Synthesis method 5: cleavage of the peptides from the solid support and deprotection

The peptides were cleaved from the resin and simultaneously deprotected by incubation in TFA/thioanisole (TA)/ethane-1,2-dithiol (EDT) (90:7:3, *v/v/v*) for 3 h, precipitated and washed with ice-cold diethyl ether.

Synthesis method 6: peptide purification

Purification of the crude peptides was carried out by reversed phase high performance liquid chromatography (RP-HPLC) (Shimadzu, Columbia, USA). Linear gradients of eluent B in A (Eluent A = 0.1 % TFA in water; Eluent B = 0.08 % TFA in acetonitrile (ACN)) were applied on C_{18}-columns (Kinetex 5µm XB-C18 100Å: 250 mm × 21.2 mm, 5 µm, 100 Å, Phenomenex, Torrance, USA; XBridge BEH C18 OBD 130Å: 250 mm × 19 mm, 10µm, 130

Å, Waters, Milford, USA) or a C_{12}-column (Jupiter 10µm Proteo 90 Å: 250 mm × 21.2 mm, 10 µm, 90 Å) and UV absorption mas detected at λ = 220 nm.

Synthesis method 7: disulfide bond formation

For peptides containing cysteines at position 16 and 21 the disulfide bond formation was achieved in solution as described.[10]

Synthesis method 8: peptide characterization

The identity of the peptides was confirmed using MALDI-ToF-MS (Ultraflex III MALDI-ToF/ToF, Bruker Daltonics, Billerica, USA) as well as ESI-MS (High-capacity ion trap ESI-MS, Bruker Daltonics) and the purity of the peptides was analyzed with a LaChrome Elite RP-HPLC system (VWR, Darmstadt, Germany) on different columns (Phenomenex Jupiter 5µ C12, 90 Å, Phenomenex Jupiter 4 µ C18, 300 Å, Phenomenex Kinetex 5 µ XB-C18, 100 Å, Varian VariTide RPC, 200 Å.).

*Synthesis of ADM(1-52) (**1**), [G^{14}]ADM(14-52) (**2**) and Tam[G^{14}]ADM(14-52) (**3**)*

The synthesis of the analogues **1, 2** and **3** was carried out as previously described.[10]

*Synthesis of [G^{14}]ADM(14-50) (**4**), [G^{14}]ADM(14-45) (**5**), [G^{14}]ADM(14-44) (**6**) and [G^{14}]ADM(14-21) (**7**)*

The analogues **4, 5, 6** and **7** were synthesized on NovaSyn®TGR HMP resin and 2-Chlorotrityl resin at a 15 µmol scale as described in synthesis method 1. The crude peptides were cleaved from the resin and simultaneously deprotected according to synthesis method 5. Purification was achieved using synthesis method 6. The disulfide bridge between the cysteines at position 16 and 21 was formed as previously described.[10] The peptides were characterized using MALDI-ToF-MS and RP-HPLC according to synthesis method 8.

*Synthesis of [$G^{14, 16, 21}$]ADM(14-50) (**8**) and ADM(22-52) (**9**)*

The analogues **8** and **9** were synthesized on NovaSyn®TGR R resin at a 15 µmol scale as described in synthesis method 1. The peptides were cleaved from the resin and simultaneously deprotected using synthesis method 5 and subsequently purified according to synthesis method 6. The peptides were characterized using MALDI-ToF-MS and RP-HPLC according to synthesis method 8.

*Synthesis of [G^{14},(Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**10**) and Tam[G^{14},(Dpr^{16}, E^{21})$_{lac}$]ADM(14-52)(**22**)*

The analogues **10** and **22** were synthesized on NovaSyn®TGR R resin at a 15 µmol scale as described in synthesis method 1. For lactam-cyclization, cysteines at positions 16 and 21 were replaced by Fmoc-Dpr(Mtt)-OH and Fmoc-Glu(OPp)-OH. Selective deprotection and lactam-cyclization was achieved according to synthesis method 2. Additional N-terminal labeling of analogue **22** with the fluorophore 6-carboxytetramethylrhodamine was performed as previously described.[10] Afterwards, the peptides were cleaved from the resin and completely deprotected using synthesis method 5 and purified according to synthesis method 6. The peptides were characterized using MALDI-ToF-MS and RP-HPLC according to synthesis method 8.

*Synthesis of [K^{14}(Pam)]ADM(14-52) (**11**), [G^{14}, K^{33}(Pam)]ADM(14-52) (**12**), [G^{14}, K^{45}(Pam)]ADM(14-52) (**13**), [G^{14}, K^{50}(Pam)]ADM(14-52) (**14**), Pam[K^{14}]ADM(14-52) (**15**) and Tam[K^{14}(Pam)]ADM(14-52)*

The analogues **11, 12, 13, 14**, **15** and **21** were synthesized on NovaSyn®TGR R resin at a 15 µmol scale as described in synthesis method 1. Dde-protected lysine was introduced to allow selective palmitoylation of analogue **11** and **21** at position 14, of analogue **12** at position 33, of analogue **13** at position 45 and of analogue **14** at position 50. Orthogonal deprotection with hydrazine and subsequent coupling of palmitic acid was achieved as described in synthesis method 3. N-terminal palmitoylation of analogue **15** was carried out after Fmoc-deprotection

analogously to synthesis method 1. Additional N-terminal labeling of analogue **21** with the fluorophore 6-carboxytetramethylrhodamine was performed as previously described.[10] The peptides were cleaved from the resin and completely deprotected according to synthesis method 5 and purified as described in synthesis method 6. The peptides were characterized using MALDI-ToF-MS and RP-HPLC according to synthesis method 8.

*Synthesis of [K^{14}(Pam),(Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**16**) and Tam[K^{14}(Pam),(Dpr^{16}, E^{21})$_{lac}$]ADM(14-52) (**17**)*

The analogues **16** and **17** were synthesized on NovaSyn®TGR R resin at a 15 µmol scale as described in synthesis method 1. For lactam-cyclization, cysteines at positions 16 and 21 were exchanged by Fmoc-Dpr(Mtt)-OH and Fmoc-Glu(OPp)-OH. Selective deprotection and lactam-cyclization was achieved according to synthesis method 2. Dde-protected lysine was introduced at position 14 to allow selective palmitoylation. Orthogonal deprotection with hydrazine and subsequent coupling of palmitic acid was achieved as described in synthesis method 3. Additional N-terminal labeling of analogue **17** with the fluorophore Tam was performed as previously described.[10] The peptides were cleaved from the resin and completely deprotected according to synthesis method 5 and purified according to synthesis method 6. The peptides were characterized using MALDI-ToF-MS and RP-HPLC as described in synthesis method 8.

*Synthesis of [K^{14}(Pam),(Dpr^{16}, E^{21})$_{lac}$, N_α-Me-K^{46}]ADM(14-52) (**18**), [G^{14}, N_α-Me-K^{46}]ADM(14-52) (**19**), Tam[K^{14}(Pam),(Dpr^{16}, E^{21})$_{lac}$, N_α-Me-K^{46}]ADM(14-52) (**20**) and Tam[G^{14}, N_α-Me-K^{46}]ADM(14-52) (**23**)*

The analogues **18, 19, 20** and **23** were synthesized on NovaSyn®TGR R resin at a 15 µmol scale until position 47 according to synthesis method 1. Next, N_α-methylated lysine was manually coupled at position 46 with the standard coupling protocol described in synthesis method 1 using 5-fold molar excess of amino acid and the reagents HOBt and DIC in DMF

for 24h at room temperature. The following residue serine 45 was coupled manually with a special coupling protocol using 5 equiv amino acid and 10 equiv of HOBt and DIC in a solvent mixture of DMF, DCM and NMP (1:1:1, *v/v/v*) under constant shaking at 1300 rpm and 50°C for 24h. Afterwards the peptide chain was elongated with SPPS as described in synthesis method 1. For lactam-cyclization of the analogues **18** and **20**, cysteines at positions 16 and 21 were exchanged by Fmoc-Dpr(Mtt)-OH and Fmoc-Glu(OPp)-OH. Selective deprotection and lactam-cyclization was achieved according to synthesis method 2. Dde-protected lysine was introduced at position 14 to allow selective palmitoylation of analogues **18** and **20**. Orthogonal deprotection with hydrazine and subsequent coupling of palmitic acid was achieved as described in synthesis method 3. N-terminal labeling of analogue **20** and **23** with the fluorophore Tam was performed as previously described.[10] The peptides were cleaved from the resin and completely deprotected according to synthesis method 5 and purified according to synthesis method 6. The peptides were characterized using MALDI-ToF-MS and RP-HPLC as described in synthesis method 8.

Stability assay in human blood plasma and porcine liver homogenate

Fluorescently labeled peptides were incubated in a concentration of 10^{-5} M in human blood plasma or porcine liver homogenate (50 mg/ml in PBS) under constant shaking at 37°C. Samples were taken at the beginning and at additional time-points during the incubation and precipitated for at least 1 h at -20°C with EtOH/ACN (1:1). After a first centrifugation step for 30 sec at 14000 rpm, the supernatant was centrifuged again for 1 h at 12000 rpm using Costar® Spin-X® Centrifuge Tube Filters (0.22 μm). The fluorescence emission of the peptide solutions was detected by RP-HPLC at λ = 573 nm using linear gradients of eluent B in A (Eluent A = 0.1 % TFA in water; Eluent B = 0.08 % TFA in ACN) on a Varian VariTide RPC column (250 mm × 4.6 mm, 6 μm, 200 Å, Agilent, Santa Clara, USA). MALDI-ToF-MS analysis was used to identify the peaks containing intact peptide and integration of the RP-HPLC chromatograms enabled the determination of the proportion of intact peptide in each

sample. Peaks that contained intact peptide (intensity > 10%) and additional fragments were corrected by comparison of MALDI-ToF mass spectrometry signals. The plasma half-life of the peptides were calculated by one-phase decay analysis with GraphPad Prism 5 (GraphPad Software, La Jolla, USA). The data points represent mean ± SEM of at least two independent experiments.

Cell culture

Human embryonic kidney cells (HEK293) were cultured in Ham´s F-12 with L-glutamine (1/1; *v*/*v*) (Lonza, Basel, Switzerland) and Dulbecco's Modified Eagle's Medium with 4.5 g l^{-1} glucose and L-glutamine (DMEM) (Lonza, Basel, Switzerland) supplemented with 15% heat inactivated fetal calf serum (FCS) (Biochrom GmbH, Berlin, Germany) under humidified atmosphere at 37°C and 5% CO_2.

Generation of AM_1, AM_2 and CGRP receptor expressing cells

Cells expressing the AM_1 receptor were generated by transient co-transfection of the plasmids CLR_eYFP_pVITRO2 (containing the calcitonin receptor-like receptor (CLR) fused to the enhanced yellow fluorescent protein (eYFP)), RAMP2_eCFP_pVITRO2 (containing the receptor activity-modifying protein 2 (RAMP2) fused to the enhanced cyan fluorescent protein (eCFP)) and the pGL4.29[*luc2P*/CRE/Hygro] plasmid (Promega, Madison, USA) (containing a luciferase reporter gene (luc2P) under the control of cAMP response element (CRE)) as previously described in detail.[10] To create cells expressing the AM_2 and CGRP receptor, the same protocol was used for the co-transfection of CLR_eYFP_pVITRO in combination with plasmids containing RAMP3 and RAMP1, respectively, which were generated similarly as described for RAMP2_eCFP_pVITRO2. Briefly, for RAMP3_eCFP_pVITRO2 the cDNA of RAMP3 (amplified from RAMP3_pcDNA1.1: Dr. Ingo Flamme, Bayer HealthCare AG, Wuppertal) and the C-terminal enhanced cyan fluorescent protein (eCFP) (amplified from eCFP_C1: Clontech,

Saint-Germain-en-Laye, France) were fused by a linker sequence using the PCR overlap extension method and subsequently ligated into a pVITRO2-blasti-mcs vector (Invivogen, Toulouse, France). Similarly, RAMP1_eCFP_pVITRO2 was created by inserting the cDNA of RAMP1 (amplified from RAMP1_pcDNA1.1: Dr. Ingo Flamme, Bayer HealthCare AG, Wuppertal) fused to eCFP (amplified from RAMP2_eCFP_pVITRO2) into a pVITRO2-blasti-mcs vector.

cAMP assay

The transfected cells were seeded in 96-well plates overnight and starved for 1 h with DMEM/Ham´s F-12 (1/1; *v*/*v*) without FCS prior to stimulation. After 3h of incubation with ligand solutions, the addition of ONE-Glo™ substrate (Promega, Madison, USA) allowed luminescence measurement with an Infinite M200 (Tecan, Maennedorf, Switzerland). For the evaluation of EC_{50} and pEC_{50} values, the detected luminescence values of peptide solutions with concentrations ranging from 10^{-6} to 10^{-11} M were fitted to dose-response curves with a standard slope (n_H = 1) after normalization to [G^{14}]ADM(14-52) using GraphPad Prism 5 (GraphPad Software, La Jolla, USA). The AM_1 receptor activation potency of peptides, which were incubated in human blood plasma prior to stimulation, was investigated with plasma solutions diluted to 10^{-8} M with DMEM/Ham´s F-12 (1/1; *v*/*v*) containing 15% FCS. The luminescence value of the sample taken at the starting point of plasma incubation was used for normalization of the following samples for each peptide and diluted serum without peptide defined the lower limit. To estimate the remaining amount of intact peptide in the incubated samples, the luminescence values of 10^{-9} and 10^{-10} M dilutions of the starting sample were normalized to the 10^{-8} M concentration of the same ample and served as indicators for 10- and 100-fold loss of peptide, respectively. All data points display mean ± SEM of at least two independent experiments, performed in triplicates.

ASSOCIATED CONTENT

Supporting Information.

Chromatographic and mass spectrometric characterization of the peptides, AM_1 receptor activation of cleavage fragments, palmitoylated and single-modified analogues, stability in human blood plasma of single-modified analogues.

AUTHOR INFORMATION

Corresponding Author

* Phone, 0049 341 97-36900; Fax, 0049 341 97-36909; eMail, abeck-sickinger@uni-leipzig.de.

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ABBREVIATIONS

ACN, acetonitrile; ADM, adrenomedullin; AM_1R, adrenomedullin receptor 1; AM_2R, adrenomedullin receptor 2; CGRPR, calcitonin gene-related peptide receptor; CLR, calcitonin receptor-like receptor; CRE, cAMP response element; Dde, 4,4-dimethyl-2,6-dioxocyclohex-1-ylidenethyl; DIC, *N,N*′-diisopropylcarbodiimide; DMEM, Dulbecco's Modified Eagle's Medium; eCFP, enhanced cyan fluorescent protein; EDT, ethane-1,2-dithiol; eYFP, enhanced yellow fluorescent protein; FCS, fetal calf serum; HOBt, *N*-hydroxybenzotriazole; MMP-2,

matrix metalloproteinase-2; Mtt, *N*-β-4-methyltrityl; NEP, neutral endopeptidase, OPp, γ-2-phenylisopropyl; Oxyma, 2-cyano-2-(hydroxyimino)acetate; Pam, palmitic acid; RAMP, receptor activity-modifying protein; RP-HPLC, reversed phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; TA, thioanisole; Tam, 6-carboxytetramethylrhodamine; TIS, triisopropylsilane.

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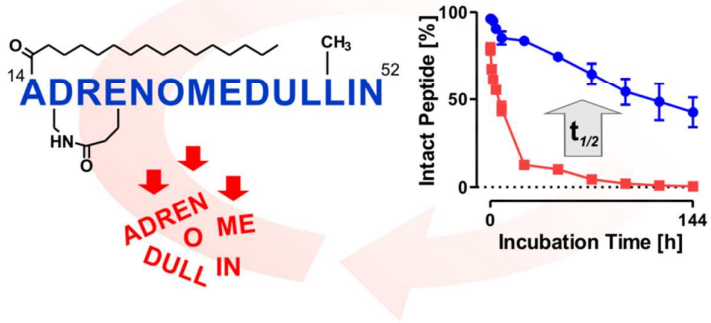
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