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1   **Development of Potent and Proteolytically Stable Human Neuromedin U Receptor Agonists**

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50 **Abstract**

51 Neuromedin U (NMU) is a highly conserved endogenous peptide that is involved in a wide range of

52 physiological processes such as regulation of feeding behavior, the stress response and nociception.

53 The major limitation to use NMU as a therapeutic is its short half-life. Here, we describe the

54 development of a set of novel NMU-analogs based on NMU-8, by introducing unnatural amino acids

55 into the native sequence. This approach shows that it is possible to generate molecules with increased

56 potency and improved plasma stability without major changes of the peptidic nature or the

57 introduction of large conjugates. When compared to the native NMU-8 peptide, compounds **16**, **18** and

58 **20** have potent agonist activity and affinity for both NMU receptors. Selectivity towards NMUR1 was

59 observed when the Phe residue in position 4 was modified, whereas higher potencies at NMUR2 were

60 found when substitutions of the Pro residue in position 6 were executed. To study the effect of the

61 modifications on the proteolytic stability of the molecules, an *in vitro* stability assay in human plasma

62 at 37 °C was performed. All analyzed analogs possessed an increased resistance against enzymatic

63 degradation in human plasma resulting in half-lifes from 4 min for NMU-8, up to more than 23 h for

64 compound **42**.

65 **Graphical abstract**
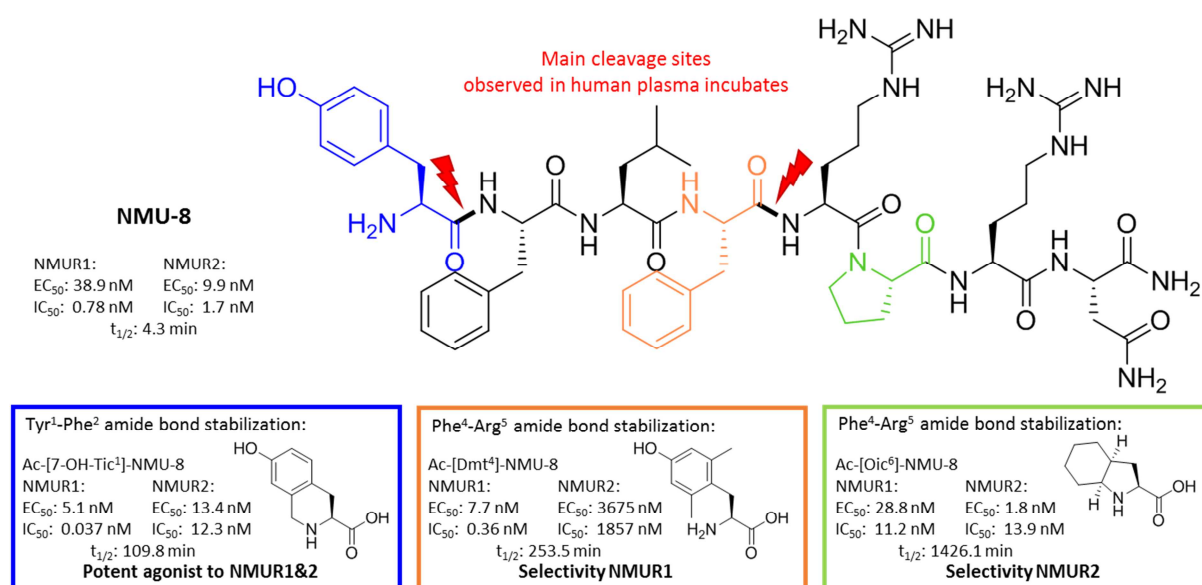
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Highlights

- Insertion of unnatural amino acids in NMU-8 leads to stable and selective ligands
- Potent NMUR ligands can be found by substitution of the Tyr[1] residue of NMU-8
- NMUR1 selectivity can be achieved by modification of Phe in position 4
- Replacement of the Pro[6] residue shifts the selectivity towards NMUR2
- Biodegradation profiles are studied in human plasma

Keywords

Neuromedin U (NMU), NMU-8, Neuromedin U receptor agonists, plasma stability

Abbreviations

1'Nal, 1'-naphtylalanine; 2'Nal, 2'-naphtylalanine; 7-OH-Tic, 7-hydroxy-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; ACN, acetonitrile; AUC, area under the curve; CNS, central nervous system; Dap, α,β-diaminopropionic acid; DCM, dichloromethane; DIC, diisopropyl carbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMEM, Dulbecco's Modified Eagle Medium; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Dmt, 2',6'-dimethyltyrosine; EC_{50}, half maximal effective concentration; E_{max}, maximal effect; FA, formic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; GPCR, G protein-coupled receptor; HBSS, Hanks Balanced Salt Solution; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; IP_3, inositol triphosphate; MS, mass spectrometry; NMU, neuromedin U; NMUR, neuromedin U receptor; Oic, octahydroindole carboxylic acid; PEG, polyethylene glycol; rt, room temperature; SAR, structure activity relationship; sc, subcutaneous; SPA, scintillation proximity assay; SPPS, solid phase peptide synthesis; TBTU, 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; TES, triethylsilane; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

91 **Introduction**

92 Neuromedin U (NMU) was initially discovered in porcine spine by Minamino *et al.* in 1985 and

93 named after its uterus stimulating activity [1]. Following its discovery, NMU was isolated from

94 different species and found to occur in two active forms, a 23 or 25 amino acid long peptide and a

95 truncated version of 8 or 9 amino acids, which is generated by cleavage at the *C*-terminus of the longer
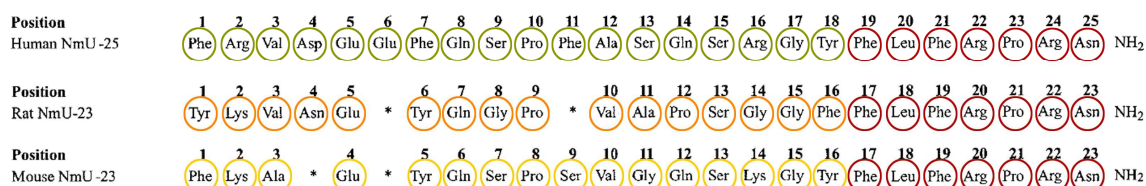
96 NMU sequence.

97

98 **Figure 1.** Human, rat and mouse NMU. The residues marked in red and the *C*-terminal amidation are

99 entirely conserved in mammalian species. The sequences are elongated with asterisks to align similar

100 amino acid residues. [Double column Figure]

101 A remarkable homology, in particular at the *C*-terminus, can be observed between the different

102 isoforms of NMU in different species (Figure 1). Across all mammals the *C*-terminal amidated

103 heptapeptide (Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$) is entirely conserved, which implies that this is an

104 important part for its biological functioning [2, 3]. The NMU peptide is widely distributed in the body,

105 both in the periphery and centrally, with the highest levels in the gastrointestinal tract and the

106 hypothalamus and anterior pituitary gland of the brain [4-6]. NMU plays a role in different

107 physiological processes such as smooth muscle contraction, blood pressure, regulation of the stress

108 response, feeding and energy homeostasis, nociception and immune regulation [3]. It exerts its effects

109 via two G protein-coupled receptors (GPCRs), NMUR1 and NMUR2. NMUR1 is particularly found in

110 the periphery, whereas NMUR2 is mainly expressed in the central nervous system (CNS) [2, 3]. Since

111 NMU interacts with the regulation of feeding behavior, exerting anorexigenic effects [7], there has

112 recently been a lot of interest in the development of NMUR agonists for the treatment of diabetes and

113 obesity, major health concerns for modern society [8, 9]. Moreover, NMU-deficient mice become

114 obese with age and these NMU$^{-/-}$ mice are characterized by an increased body weight and adiposity,

115 hyperphagia, and a decrease in locomotor activity and energy expenditure [10]. Conversely, mice

116 overexpressing NMU are leaner than wild type animals and are characterized by hypophagia and an

117 improved glucose tolerance [11].

6

H - Tyr[1] - Phe[2] - Leu[3] - Phe[4] - Arg[5] - Pro[6] - Arg[7] - Asn[8] - NH_2
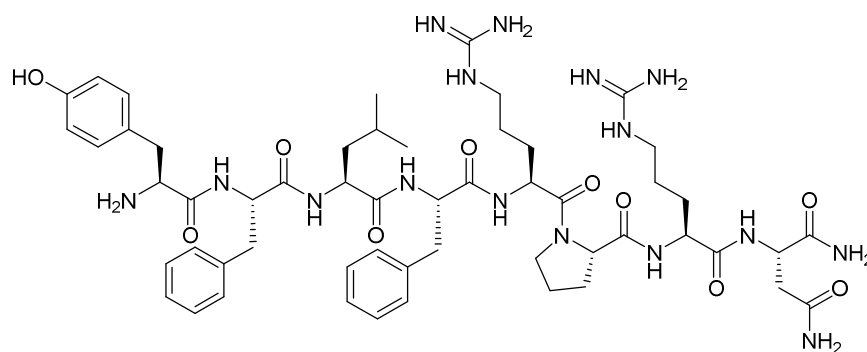

Figure 2. Structure and sequence of NMU-8 [single column Figure]

Several novel NMU-analogs have been synthesized over the past years. Like many other naturally occurring peptides, NMU has a typical short half-life of less than 5 min after subcutaneous (sc) injection [12]. To unveil critical amino acid residues and to develop potent peptidergic NMU-analogs with improved stability, Hashimoto *et al.* performed a first structure activity relationship (SAR) study on isolated chicken smooth muscle preparations with the NMU-8 peptide (Figure 2). D-amino acid substitutions revealed two non-competitive antagonists, namely [D-Pro[6]]-NMU-8 and [D-Leu[3],D-Pro[6]]-NMU-8 [13]. The introduction of acyl groups, such as an acetyl, benzoyl and propionyl moiety, to the *N*-terminus led to the discovery of peptides with an increased contractile activity [14]. Additional SAR studies on the peripheral avian NMU receptors [15-17] and the synthesis of selective hexapeptide agonists [18-20] revealed the importance of the *C*-terminal Pro-Arg-Asn-NH_2 segment for activation of both receptors.

Selectivity towards NMUR1 could be obtained by introducing Trp or 2'naphtylalanine (2'Nal) at position 3 (Leu in the natural sequence) and selectivity towards NMUR2 was reported when Arg[5] was replaced by an *α,β*-diaminopropionic acid (Dap) residue. Further elaboration of this SAR study resulted in a NMUR2 selective hexapeptide agonist, namely 3-cyclohexylpropionyl-Leu-Leu-Dap-Pro-Arg-Asn-NH_2 [18]. Although a selective agonist of NMUR1 was described by Takayama *et al.* as well, this analog (i.e. 2-thienylacetyl-Trp-Phe(4-F)-Arg-Pro-Arg-Asn-NH_2) still activated NMUR2 to an important extent [19]. A further finetuning of this molecule led to the discovery of a potent NMUR1 selective hexapeptide agonist by replacing Phe(4-F) with a (αMe)Trp [20].

Additionally, other novel analogs based on the truncated form of NMU, such as small lipidated NMU-analogs [21] and PEGylated NMU-8 [22], were already described to have potent anorectic and anti-obesity effects. Several molecules based on the long version of NMU were synthesized as well, including a NMU-25 conjugate with human serum albumin [23], a PEGylated NMU-25 derivative [24] and lipidated NMU-25 analogs [25]. These molecules were reported to display potent and long-lasting effects on food intake when peripherally administered in mice. Altogether, NMU holds a great therapeutic promise.

7

146 In this study, we describe the synthesis and *in vitro* evaluation of a series of peptidergic NMU ligands,

147 based on the naturally occurring NMU-8, as lead molecule. Although not naturally occurring in

148 human, NMU-8 was used as a lead since this peptide has the same *C*-terminal amino acid sequence as

149 human (h) NMU-25, and acts as a full agonist on the hNMURs without a loss in potency [26]. A first

150 set of analogs consisted of peptides with modifications described in literature on one hand and new

151 peptidergic ligands on the other hand. This approach allowed to verify if the previous reported activity

152 profiles on chicken crop smooth muscle preparations are similar when analyzed in the commonly used

153 human embryonic kidney 293 (HEK293) cell line. In a second set of NMU-analogs, steric bulk,

154 conformational constraints and 'peptoid' residues were introduced with the aim of developing peptides

155 with NMUR subtype selectivity, improved pharmacokinetics and enhanced proteolytic stability. The

156 effect of the introduced modifications on the affinity and activity at NMUR1 and NMUR2 was

157 evaluated on HEK293 cells transiently expressing one of these receptors. The effect of some key

158 modifications on the half-life of the analogs was determined in an *in vitro* stability assay with human

159 plasma.

160 **Material and Methods**

161 Chemicals

162 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU), *N,N*-
163 diisopropylethylamine (DIPEA), dimethylformamide (DMF), 4-methyl-piperidine, dichloromethane
164 (DCM), 1-hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC), bromoacetic acid, dimethyl
165 sulfoxide (DMSO), triethylsilane (TES), acetonitrile (ACN), fetal bovine serum and poly-D-lysine
166 were obtained from Sigma Aldrich (Saint Louis, Missouri, USA). Acetic anhydride, diethylether and
167 Pd/BaSO$_4$ were purchased from Millipore, Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA)
168 was obtained from Fluorochem Ltd (Hadfield, UK). All amino acids were purchased from Iris Biotech
169 GmbH (Marktredwitz, Germany). Tritium gas was from Technobexport (Moscow, Russia).
170 Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX^{TM}, Opti-MEM with GlutaMAX^{TM} and
171 Hanks Balanced Salt Solution (HBSS) were products from Gibco, Life Technologies (Carlsbad,
172 California, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, California, USA). Myo-[2-
173 ^{3}H(*N*)]inositol, Scintillation Proximity Assay (SPA)-yttrium silicate beads solution and Micro-Scint 20
174 were purchased from Perkin Elmer (Waltham, Massachusetts, USA). Human plasma was obtained
175 from the Belgian Red Cross (Leuven, Belgium). Formic acid (FA) was purchased from J.T. Baker
176 (Center Valley, Pennsylvania, USA).

177 General

178 Analytical high performance liquid chromatography (HPLC) was carried out on an Agilent 1100
179 HPLC system with ChemStation for LC 3D software (Discovery® BIO Wide Pore RP C18 column, 15
180 cm × 2.1 mm, 3 μm from Supelco, Bellefonte, Pennsylvania, USA). Gradient elution took place using
181 water with 0.1 % TFA and ACN with 0.1 % TFA as mobile phases. UV-detection was carried out at
182 215 nm. Peptides were purified using a preparative HPLC apparatus from Gilson with Unipoint
183 software package (Discovery® BIO Wide Pore C18 column, 25 cm × 21.2 mm, 5 μm from Supelco).
184 The same mobile phases were used as for analytical HPLC and UV detection was carried out at 215
185 nm as well. Mass spectrometry (MS) was performed on a Micromass Q-Tof micro spectrometer with
186 electrospray ionization. Data collection and spectrum analysis were executed with Masslynx software.

187 Peptide synthesis

188 All peptides were manually synthesized by the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid
189 phase peptide synthesis (SPPS) on Rink Amide AM resin (ChemImpex, 0.45 – 0.60 mmol g^{-1}). For
190 standard couplings, Fmoc-protected amino acids (3 equiv) were sequentially coupled with TBTU (3
191 equiv) and DIPEA (9 equiv) for 1 h at room temperature (rt) in DMF. Fmoc deprotection was carried
192 out in a two-step manner (5 min and 15 min) by treating the resin with a 20% (v/v) 4-methyl-
193 piperidine solution in DMF. After each coupling and Fmoc deprotection, the resin was washed

9

194 thoroughly with DMF (3x) and DCM (3x). Amino acids containing an unprotected phenol in their side

195 chain [7-hydroxy-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (7-OH-Tic) and 2',6'-

196 dimethyltyrosine (Dmt)] were coupled for 45 min at rt using a 2-fold excess of the Fmoc-protected

197 amino acid, HOBt (2 equiv) and DIC (2 equiv) in DMF. The same coupling conditions were in that

198 case used until completion of the peptide sequence, as well as when hydroxyl-containing 'peptoid'

199 residues were present in the sequence.

200 *N*-substituted glycines were prepared on the solid support using the following method: after Fmoc

201 deprotection, bromoacetylation of the amine was performed by adding 20 equiv of bromoacetic acid

202 and 25 equiv of DIC in DMF for 1 h. This mixture was filtered off and the resin was washed as

203 described above. Next, 20 equiv of the primary amine was added to the resin in DMSO and mixed for

204 1 h to introduce the side chain [27]. After insertion of the side chain, further peptide assembly was

205 continued as described above.

206 Acetylation of the *N*-terminus was performed with acetic anhydride (10 equiv) and DIPEA (5 equiv)

207 in DMF for 1 h. When unprotected reactive side chains were present, the acetylation was executed for

208 30 min with half of the standard amounts, more precisely 5 equiv of acetic anhydride and 2.5 equiv of

209 DIPEA. Cleavage from the resin and side chain deprotection were performed with TFA/TES/water

210 (95/2.5/2.5 v/v/v) at rt for 3 h. The resulting cleavage mixture was evaporated to remove the TFA. The

211 residue was next added to cold diethylether and centrifuged for 3 min at 3000 rpm. The precipitate was

212 redissolved in a mixture of water and ACN (1/1 v/v) and lyophilized. Finally, the crude peptide was

213 purified by preparative reversed phase HPLC (RP-HPLC) and lyophilized again. The structure of the

214 pure product was confirmed by high-resolution MS (HRMS). The purity of all peptides was more than

215 95% according to HPLC analysis.

216 Tritium labeling of NMU-8

217 Iodogen (0.92 µmol) was dissolved in 200 µL CH_2Cl_2 and the solution was evaporated to dryness

218 under nitrogen in a reaction vial. NMU-8 (0.69 µmol) was dissolved in 1 mL of 50 mM Na_2HPO_4

219 buffer pH 7.0 and the solution was added to the freshly prepared iodogen film. 2 µmol of NaI in 50

220 mM Na_2HPO_4 buffer pH 7.0 was added and the mixture was stirred at rt for 1 min. Next, the solution

221 was removed and mixed with 125 µL of 2 mg/mL $Na_2S_2O_5$ in 50 mM Na_2HPO_4 buffer pH 7.0. The

222 resulting solution was purified by RP-HPLC (Vydac 218TP54 column (250 x 4.6 mm, 5 µm) with a

223 yield of 0.8 mg (73%)). For the tritium labeling, $[Tyr^1(3'-I)]$-NMU-8 was dissolved in 200 µL DMF

224 containing 1 µL of trimethylamine and the solution was reacted with $^3H_2$ gas in the presence of

225 PdO/BaSO4 (10% Pd) catalyst for 2 h at room temperature. The catalyst was filtered off and the labile

226 tritium atoms were removed with repeated evaporation from EtOH/water (1/1 v/v) solution. Finally,

227 the tritium labeled peptide was purified by HPLC (Phenomenex Luna C18(2) column, 5 µm, Torrance,

228 California, USA). The molar activity was determined with HPLC area under the curve (AUC)

229 measurement using a calibration curve of NMU-8, and was found to be 1.09 Tbq/mmol (29.4

230 Ci/mmol).

231 *In vitro* functional assay on NMUR1 and NMUR2

232 An inositol triphosphate (IP_3) SPA analysis was performed to study the functional activity of the novel

233 NMU-analogs at NMUR1 and NMUR2, since both receptors are described to signal mainly through

234 the $G\alpha_{q/11}$ pathway [25, 28]. HEK293 cell cultures were maintained in DMEM with GlutaMAX™,

235 supplemented with 10 % fetal bovine serum, 180 units/mL of penicillin and 45 µg/mL streptomycin.

236 These cultures were incubated at 37 °C with 10 % CO_2 and a relative humidity of 95 %. The day prior

237 to the transfection with NMUR1, HEK293 cells were seeded in transparent 96-well plates coated with

238 poly-D-lysine with a density of 30 000 cells/well. Transfection was carried out by incubating the

239 plates for 5 h with 10 ng of hNMUR1 plasmid and 0.15 µL of lipofectamine 2000 in Opti-MEM with

240 GlutaMAX™. An empty vector (pCMV) transfection was carried out as a negative control. One day

241 after transfection, the HEK293 cells were incubated for 24 h with 5 µCi of myo-[2-^{3}H(N)]inositol per

242 well in DMEM with GlutaMAX™. For the preparation of HEK293 cells expressing NMUR2, the

243 calcium phosphate transfection method was used. The day prior to the transfection, HEK293 cells

244 were inoculated in T175 flasks with a density of 6 000 000 cells in 20 mL of culture medium. The

245 transfection mixture was prepared by dripping a solution of the hNMUR2 plasmid and calcium

246 chloride (2 M) in tris-EDTA-buffer into 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

247 buffered saline (2x). This mixture was slowly added onto the cells while holding the flask vertically.

248 The HEK293 cells were incubated for 5 h at 37 °C. As a negative control, an empty vector (pcDNA)

249 transfection was carried out. The day after the transfection, cells were seeded out in transparent 96-

250 well plates coated with poly-D-lysine with a density of 35 000 cells/well using culture medium

251 supplemented with 5 µL myo-[2-^{3}H(N)]inositol per mL. On the assay day, the plates were washed

252 twice with HBSS and incubated during 30 min at 37 °C with 100 µL/well of 10 mM lithium chloride

253 in HBSS. To each well, 5 µCi of a peptide solution was added and incubated for 60 min at 37 °C.

254 After peptide stimulation, the cells were lysed with 50 µL/well of 10 mM FA on ice for 30 min. 35 µL

255 of the lysates was transferred into white 96-well plates and 80 µL of SPA-yttrium silicate beads

256 solution was added to each well. The plates were sealed and shaken for 30 min. After spinning the

257 plates for 5 min at 1500 rpm, the decay was measured, with a delay of 8 h, during 2 min/well with the

258 Topcount (Packard Instrument Company, Meriden, Connecticut, USA). Each NMU analog was tested

259 in a concentration range of 10^{-5} to 10^{-11} M in at least 3 independent experiments using triplicates.

260 Competitive binding assay at NMUR1 and NMUR2

261 To evaluate the affinity of the novel NMU-analogs for the NMURs, a competitive binding study was

262 performed with [^{3}H]-NMU-8 as radioligand. Transfection of the HEK293 cells with hNMUR1 or

263 hNMUR2 was carried out in the same ways as describe above for the functional assay with the only

264   difference that myo-[2-^{3}H(N)]inositol was not added to the medium. On the assay day, the plates were

265   washed twice with binding buffer (50 mM HEPES buffer at pH 7.5 supplemented with 5 g/L bovine

266   serum albumin). 50 µL per well of binding buffer was added and incubated at 4 °C during 15 min. To

267   each well, 5 µL of the peptide dilution series was added, followed by 50 µL of [^{3}H]-NMU-8 (250 cpm,

268   0.23 nM). The plates were incubated for 3 h at 4 °C. Afterwards, the plates were washed twice with

269   binding buffer and 100 µL of Micro-Scint 20 was added to each well. The plates were sealed and

270   gently shaken for 30 min. The decay was measured with the Topcount instrument. Each NMU analog

271   was tested in a concentration range of 10^{-5} to 10^{-11} M in at least 3 independent experiments using

272   triplicates.

273   *In vitro* plasma stability

274   An *in vitro* stability assay in human plasma was performed to study the effect of the introduced

275   modifications on enzymatic stability. Prior to the stability test, selectivity of the assay, stability of the

276   compound in the injection solvent, linearity, accuracy and precision of the method were investigated.

277   The NMU analogs were dissolved in water and consecutive dilutions were prepared with the same

278   solvent. An aqueous peptide solution was spiked in human plasma (10/90 v/v), which was incubated at

279   37 °C, with a final plasma concentration of 112 µM. Samples were taken at selected time points,

280   depending on the analyte (Supporting Information, Table S1), by transferring 100 µL of the spiked

281   plasma into a test tube and stopping the degradation process by adding 300 µL of methanol with 0.1%

282   (v/v) FA (4 °C). Suspensions were vortexed for 15 s and kept at 4 °C for 30 min. After centrifugation

283   at 14 000 rpm for 15 min, 100 µL of the supernatant was diluted with 100 µL of water in the injection

284   vial. Samples were stored in the autosampler (4 °C) prior to injection. Analysis was performed on a

285   Dionex Ultimate 3000 gradient RP-HPLC system using water/ACN/FA (95/5/0.1 v/v/v) and

286   ACN/water/FA (95/5/0.1 v/v/v) for elution (CORTECS C18+, 2.1 x 150 mm, 2.7 µm column from

287   Waters, Milford, Massachusetts, USA). For the calculation of the peptide half-life, only points with an

288   AUC higher than the AUC of the lowest standard were used. The concentrations were calculated by

289   use of the calibration curve and transferred to a semi-log chart presenting the log concentrations as a

290   function of time. To study the fragmentation profile, the samples were transferred to the LC-MS

291   system and the degradation products were identified by analyzing the m/z ratio of all peaks of the

292   spectrum.

293   Data analysis and statistical evaluation

294   Graphical representations and statistical evaluation were carried out with GraphPad Prism software

295   (San Diego, California, USA). EC_{50} values were determined by nonlinear regression using sigmoidal

296   dose-response curve fit. Calculation of the IC_{50} values was performed as well with sigmoidal dose-

297   response curve fitting (variable slopes). Microsoft® Office Professional 2010 Excel was used for

298   calculations of the peptide half-life.

Results and Discussion

Peptide synthesis

The truncated NMU-8 was taken as lead structure for the synthesis of all analogs, since it is a naturally occurring form of the neuropeptide, of which the *C*-terminus is considered to be the critical part of the peptide for receptor activation [3]. All targeted peptides were synthesized by Fmoc-based SPPS on Rink Amide AM resin (*vide supra*) using TBTU as coupling reagent. The *N*-substituted glycines (the so-called 'peptoid' residues) in peptides **31** - **38** were prepared using the solid phase submonomer method [27]. Amino acids with an unprotected phenol in their side chain (7-OH-Tic, Dmt) were coupled using DIC/HOBt in order to prevent O-acylation. For these analogs, as well as for the hydroxyl-containing peptoids, the remaining peptide sequence was assembled using the DIC/HOBt mediated couplings. No difficulties were observed to couple Fmoc-Leu-OH to the secondary amine of the peptoid residues. All peptides were purified to greater than 95% purity by RP-HPLC, and their structure was confirmed by HRMS (Table S2, supporting information). The tritium labeled NMU-8 which was required for the radioligand displacement experiments, was obtained by first iodination of NMU-8 [29], to Tyr(3'-I)-NMU-8, followed by catalytic reduction with an excess of tritium gas. The crude [^{3}H]-NMU-8 was purified by RP-HPLC resulting in the radioligand with a radiochemical purity of more than 98% and with a specific activity of 1.09 TBq/mmol (29.4 Ci/mmol).

316 **Table 1.** Sequences and *in vitro* activity and affinity of the literature based NMU-8 analogs (first generation) on hNMUR1 and hNMUR2. [double column]

	Sequence	hNMUR1			hNMUR2			Selectivity
		Potency		Affinity	Potency		Affinity	IC_{50} NMUR2/
		EC_{50} (nM)	Emax (%)	IC_{50} (nM)	EC_{50} (nM)	Emax (%)	IC_{50} (nM)	IC_{50} NMUR1
1	H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	38.9	100.0	0.78	30.8	100.0	1.7	2
2	**Ac**-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	0.51	111.3	0.29	0.71	100.0	2.0	7
3	H-Tyr-Phe-Leu-Phe-**Ala**-Pro-Arg-Asn-NH$_2$	709.4	57.1	-	391.0	68.1	-	-
4	**Ac**-Tyr-Phe-Leu-Phe-**Ala**-Pro-Arg-Asn-NH$_2$	274.6	61.0	-	242.2	75.5	-	-
5	H-**D-Tyr**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	18.6	71.3	-	34.3	103.7	-	-
6	**Ac-D-Tyr**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	8.1	57.4	-	6.2	99.0	-	-
7	H-Tyr-Phe-Leu-Phe-Arg-**D-Pro**-Arg-Asn-NH$_2$	3171	73.1	1167	1079	110.7	802.9	0.7
8	**Ac**-Tyr-Phe-Leu-Phe-Arg-**D-Pro**-Arg-Asn-NH$_2$	2271	71.0	844.4	2002	80.9	1365	2
9	H-Tyr-Phe-**D-Leu**-Phe-Arg-**D-Pro**-Arg-Asn-NH$_2$	NA	NA	775.5	2495	98.4	6132	8
10	**Ac**-Tyr-Phe-**D-Leu**-Phe-Arg-**D-Pro**-Arg-Asn-NH$_2$	NA	NA	3755	NA	NA	1089	0.3
11	H-Tyr-Phe-Leu-Phe-Arg-Pro-**D-Arg**-Asn-NH$_2$	683.7	56.6	-	616.1	131.1	-	-
12	**Ac**-Tyr-Phe-Leu-Phe-Arg-Pro-**D-Arg**-Asn-NH$_2$	343.4	64.1	-	382.3	138.8	-	-
13	**3-cyclohexylpropionyl**-Leu-**Leu-Dap**-Pro-Arg-Asn-NH$_2$	NA	NA	4286	3.8	91.9	43.9	0.01
14	**3-cyclohexylpropionyl**-Leu-**Leu-Dap-D-Pro**-Arg-Asn-NH$_2$	NA	NA	18320	926.3	100.4	10700	0.6

317

318 Modifications compared to the native NMU-8 sequence (**1**) are marked in bold. EC_{50} values are calculated based on the IP$_3$ SPA data. E$_{max}$ is the percentage of the maximum
319 response at 10^{-5} M compared with the NMU-8 response at the same concentration. IC_{50} values are calculated based on the competitive binding assay. Receptor selectivity is
320 expressed as the ratio of the IC_{50} value for NMUR2 over the IC_{50} value for NMUR1 of each NMU-analog. NA: not applicable, no agonist activity up to 10^{-6} M. -: not
321 determined.

14

322 **Biological evaluation of the first set of NMU-analogs**

323 Evaluation of the agonistic activities at the NMURs of the first set of NMU-analogs was performed in

324 the present study on HEK293 cells transiently expressing hNMUR1 or hNMUR2 using an inositol

325 triphosphate (IP_3) SPA assay. The affinity of a selection of analogs for the NMURs was determined in

326 these cells using competitive displacement with [3H]-NMU-8 as radioligand. Table 1 shows the results

327 for the modified peptides (Supporting information, Table S3, for results presented as mean ± SEM).

328 *N-terminal acetylation*

329 It is reported that enzymatic degradation of the NMU-8 peptide mainly occurs via the *N*-terminus [14].

330 With the aim to develop NMU-analogs possessing an increased stability, *N*-terminal modifications

331 were introduced in the peptide sequence. Sakura *et al.* described that acetylation of the *N*-terminal Tyr[1]

332 leads to an increased resistance against proteolysis. Moreover, it has previously been shown that this

333 modification led to an increase in contractile activity on chicken crop smooth muscle preparations

334 [14]. In the present study, we evaluated Ac-NMU-8 (**2**) in an IP_3 accumulation assay on HEK293 cells,

335 an immediate read-out for receptor activation. The introduction of an acetyl group at the *N*-terminus

336 (**2**) led to a more than 40-fold increase in potency on both NMURs (with EC_{50} values of 0.51 nM and

337 0.71 nM for NMUR1 and NMUR2, respectively) when compared to the native NMU-8 (**1**) (EC_{50} =

338 38.9 nM for NMUR1 and 30.8 nM for NMUR2). *N*-terminal acetylation of **1** did not alter the affinity

339 for the NMURs. Acetylation of the *N*-terminus was systematically carried out in the current study, to

340 present both the acetylated and non-acetylated form of all analogs, and led in general to an increase of

341 the relative activity on both NMURs, when compared to the non-acetylated peptide.

342 *[Ala[5]]-NMU-8*

343 A previous study by Funes *et al.* reported a tool for shifting selectivity towards NMUR2 when

344 replacing Arg[5] by an Ala residue [30]. However, in our hands this modification (**3**) led only to a

345 slightly increased selectivity for NMUR2 (EC_{50} for NMUR1= 709.4 nM and EC_{50} for NMUR2 =

346 391.0 nM) instead of the 15-fold shift reported by Funes *et al*. The loss in potency, compared to

347 NMU-8, came with partial agonism on both NMURs. *N*-terminal acetylation of this analog (**4**)

348 increased the potency for both receptors, although activity remained low, as compared to NMU-8.

349 However, partial agonists can be of therapeutic importance since they could be regarded as molecules

350 exerting both agonistic, when low levels of the endogenous ligand is present, and antagonist effects,

351 when present together with a full agonist competing for the same binding site [31].

352 *Chirality switches*

353 A D-amino acid scan of NMU-8 was performed by Hashimoto *et al.* and tested on isolated chicken

354 crop smooth muscle preparations [13]. Inspired by the SAR studies which were performed in 'the

355 early days of NMU research', we decided to synthesize some of these analogs and evaluate them on

15

356 cell cultures expressing the NMURs. [D-Tyr[1]]-NMU-8 was reported to enhance the contractile activity

357 in the smooth muscle preparation, presumably due to an increased resistance to aminopeptidase-like

358 degradation [13]. In our hands, replacing Tyr[1] by D-Tyr led to peptide **5**, and its acetylated form **6**,

359 with comparable potencies to NMU-8 for NMUR1 and NMUR2, although a decrease of its maximal

360 effect (E_{max}, effect at 10^{-5} M compared with the response of the same concentration of NMU-8) on

361 NMUR1 was observed (e.g., 57.4 % for **6**). However, rather than an increase in potency compared to

362 the parent peptide, a decreased potency was observed especially when comparing activities of the

363 acetylated forms **6** versus **2**. In the literature, [D-Pro[6]]-NMU-8 and [D-Leu[3],D-Pro[6]]-NMU-8 were

364 found to be weak non-competitive antagonists in chicken crop preparations [13]. We included both

365 molecules and their *N*-terminal acetylated forms (compounds **7-10**) in our study. These ligands indeed

366 did not show potent agonistic activity in the *in vitro* cell-based assays. To further investigate if these

367 molecules could act as antagonists on the NMURs, their ability to compete with radiolabeled agonist,

368 [^{3}H]-NMU-8, was evaluated. These compounds (**7 – 10**) all showed a 1000-fold, or even higher,

369 decrease in affinity for NMUR1. On NMUR2, binding studies revealed a major loss in affinity,

370 ranging from a 400 to more than 3000-fold decrease. These results demonstrate that these

371 modifications lead to a loss in affinity and potency of the molecules. To our knowledge, no peptidergic

372 antagonists for NMUR1 or NMUR2 have been proven to exert activity *in vivo* until now. This

373 correlates with the observation that [D-Pro[6]]-NMU-8 failed to exert antagonistic activity *in vivo* [32].

374 However, a small molecule antagonist for NMUR2, R-PSOP, was reported by Liu *et al* [33].

375 Replacing Arg[7] by D-Arg (**11, 12**) resulted in full agonists with an even higher intrinsic activity than

376 NMU-8 on NMUR2 (E_{max} values of 131.1 % and 138.8 % for **11** and **12**, respectively), but these

377 analogs gave way to a decrease in potency on both receptors. However, these molecules still possessed

378 similar potencies on NMUR1, as compared to the activity on NMUR2, they were only able to partially

379 activate this receptor. Finally, Takayama *et al* described a selective NMUR2 receptor agonist, namely

380 3-cyclohexylpropionyl-Leu-Leu-Dap-Pro-Arg-Asn-NH$_2$ [18], which was resynthesized as compound

381 **13** together with the analog where Pro was replaced by D-Pro (**14**), in order to verify whether this

382 could lead to selective NMUR2 receptor antagonism. Also in our hands, analog **13** acted as a selective

383 NMUR2 agonist with a lower affinity but conserved potency on NMUR2 as compared to NMU-8. The

384 additional D-amino acid substitution did not give rise to a NMUR2 selective antagonists as compound

385 **14** possessed a weak affinity and in consequence a low activity on both NMURs.

386 **Table 2.** Sequences and *in vitro* activity and affinity of the novel NMU-8 analogs (second set) for hNMUR1 and hNMUR2. [double column]

		hNMUR1			hNMUR2			Selectivity
		Potency		**Affinity**	**Potency**		**Affinity**	IC$_{50}$ NMUR2/
	Sequence	EC$_{50}$ (nM)	Emax (%)	IC$_{50}$ (nM)	EC$_{50}$ (nM)	Emax (%)	IC$_{50}$ (nM)	IC$_{50}$ NMUR1
1	H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	38.9	100.0	0.78	30.8	100.0	1.7	2
15	H-**7-OH-Tic**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	5.5	103.5	0.086	3.5	115.3	21.4	249
16	**Ac-7-OH-Tic**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	5.1	99.3	0.037	13.4	115.8	12.3	332
17	H-**2'Nal**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	65.8	95.1	0.97	4.8	106.1	9.9	10
18	**Ac-2'Nal**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	1.6	90.9	0.88	1.6	83.9	5.6	6
19	H-**Dmt**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	10.4	95.0	0.34	0.83	100.7	4.3	13
20	**Ac-Dmt**-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH$_2$	4.0	109.1	0.15	0.70	91.5	2.4	16
21	H-Tyr-Phe-Leu-**7-OH-Tic**-Arg-Pro-Arg-Asn-NH$_2$	94.5	94.6	8.6	624.3	109.1	1010	117
22	**Ac**-Tyr-Phe-Leu-**7-OH-Tic**-Arg-Pro-Arg-Asn-NH$_2$	12.3	88.1	1.2	192.6	86.1	330.8	276
23	H-Tyr-Phe-Leu-**1'Nal**-Arg-Pro-Arg-Asn-NH$_2$	41.4	101.1	0.31	225.7	109.8	370.5	1195
24	**Ac**-Tyr-Phe-Leu-**1'Nal**-Arg-Pro-Arg-Asn-NH$_2$	4.3	105.3	0.41	246.5	86.4	39.1	95
25	H-Tyr-Phe-Leu-**2'Nal**-Arg-Pro-Arg-Asn-NH$_2$	51.0	103.0	0.51	378.0	104.7	424.4	832
26	**Ac**-Tyr-Phe-Leu-**2'Nal**-Arg-Pro-Arg-Asn-NH$_2$	4.2	109.0	0.32	304.2	96.2	195.3	610
27	H-Tyr-Phe-Leu-**Dmt**-Arg-Pro-Arg-Asn-NH$_2$	77.9	96.1	1.6	1286	103.4	1649	1031
28	**Ac**-Tyr-Phe-Leu-**Dmt**-Arg-Pro-Arg-Asn-NH$_2$	7.7	104.4	0.36	3675	96.7	1857	5158
29	H-Tyr-Phe-Leu-**Oic**-Arg-Pro-Arg-Asn-NH$_2$	184.9	72.2	66.2	472.1	92.0	1546	23
30	**Ac**-Tyr-Phe-Leu-**Oic**-Arg-Pro-Arg-Asn-NH$_2$	93.0	83.1	68.6	1830	92.5	253.4	4
31	H-Tyr-Phe-Leu-**N(Benzyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	1053	73.7	46.3	1103	103.1	1403	30
32	**Ac**-Tyr-Phe-Leu-**N(Benzyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	172.2	73.6	83.1	1278	85.4	1019	12
33	H-Tyr-Phe-Leu-**N(4-OH-Benzyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	593.7	54.6	173.8	1407	69.4	1961	11
34	**Ac**-Tyr-Phe-Leu-**N(4-OH-Benzyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	191.7	60.1	27.9	2686	75.4	1813	65
35	H-Tyr-Phe-Leu-**N(Phenethyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	822.7	75.5	30.2	355.7	121.8	261.6	9
36	**Ac**-Tyr-Phe-Leu-**N(Phenethyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	138.9	83.1	62.9	250.0	99.9	316.1	5
37	H-Tyr-Phe-Leu-**N(4-OH-Phenethyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	574.4	57.6	100.1	382.2	94.3	428.9	4
38	**Ac-Tyr-Phe-Leu-N(4-OH-Phenethyl)Gly**-Arg-Pro-Arg-Asn-NH$_2$	216.6	60.6	3.9	339.2	80.7	206.9	53
39	H-Tyr-Phe-Leu-Phe-Arg-**Tic**-Arg-Asn-NH$_2$	5496	79.0	162.3	45.5	99.3	13.0	0.08
40	**Ac**-Tyr-Phe-Leu-Phe-Arg-**Tic**-Arg-Asn-NH$_2$	230.0	72.9	54.4	5.4	91.8	11.2	0.2
41	H-Tyr-Phe-Leu-Phe-Arg-**Oic**-Arg-Asn-NH$_2$	273.2	106.3	24.3	10.2	90.4	12.7	0.5
42	**Ac**-Tyr-Phe-Leu-Phe-Arg-**Oic**-Arg-Asn-NH$_2$	28.8	95.0	11.2	1.8	84.3	13.9	1

Modifications compared to the native NMU-8 sequence (**1**) are marked in bold. EC$_{50}$ values are calculated based on the IP$_3$ SPA data. E$_{max}$ is the percentage of the maximum response at 10^{-5} M
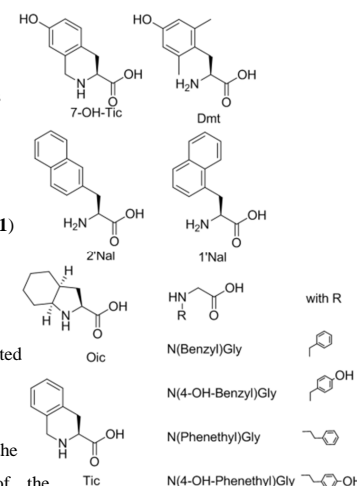
414 compared with the NMU-8 response at the same concentration. IC$_{50}$ values are calculated based on the competitive binding assay. Receptor selectivity is expressed as the ratio of the IC$_{50}$ value for NMUR2 over the IC$_{50}$ value for NMUR1 of each
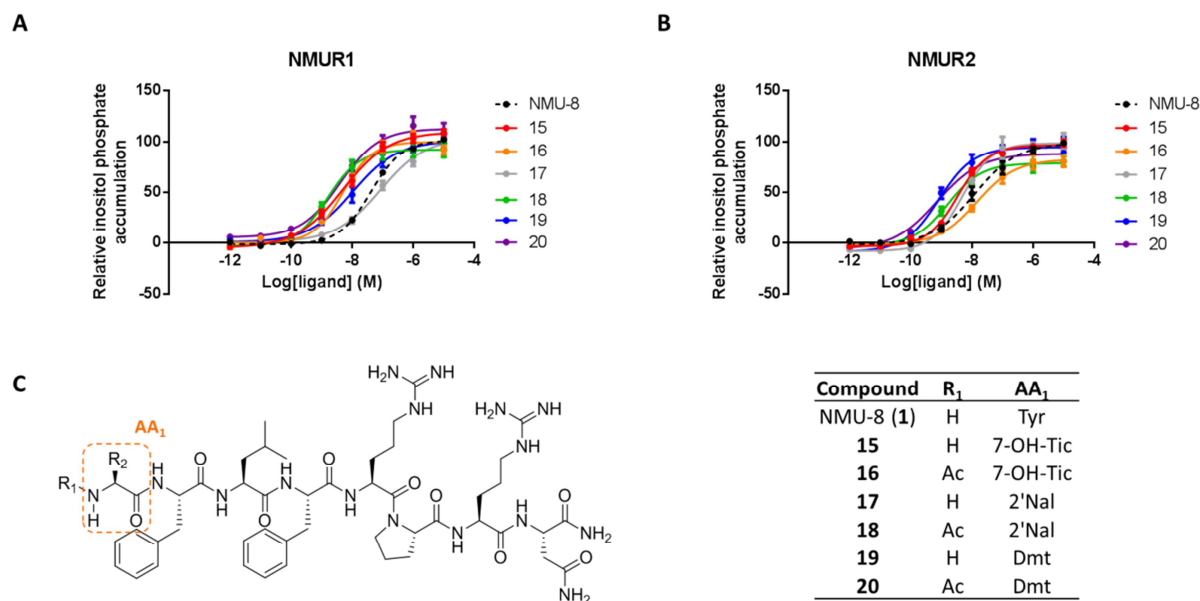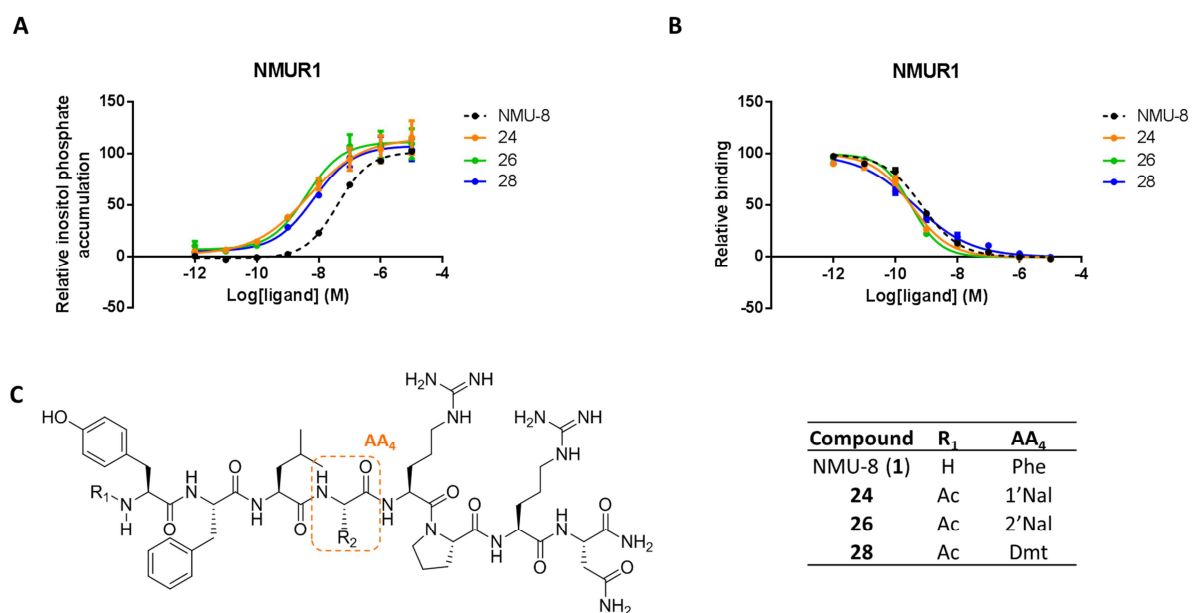415 NMU- analog.

416

Figure 3. *In vitro* evaluation of the effect of Tyr[1] substitution on receptor activation. IP$_3$ accumulation assay performed on HEK293 cells, transiently expressing A) NMUR1 or B) NMUR2. C) Structures of the novel NMU-analogs **15, 16**, **17**, **18**, **19** and **20**. Data are shown as mean ± SEM (n=3). [double column]

Biological evaluation of the second set of NMU-analogs

Substitution of the N-terminal tyrosine residue

To protect the peptides from cleavage by aminopeptidases, the *N*-terminus of NMU-8 was modified. Figure 3 gives an overview of the NMU-analogs with modification at the Tyr[1] residue and their activity at NMUR1 and NMUR2. An analog wherein Tyr[1] was replaced by 7-OH-Tic was synthesized together with the *N*-terminally acetylated peptide, to give compounds **15** and **16**. Both molecules showed a very high, picomolar affinity for NMUR1, which is about ten times better than those of the parent compounds **1** and **2**, whereas their affinity for NMUR2 decreased. In the activity assay, they were however non-selective, full agonists with a 15 % increase of the maximal effect at NMUR2 (Table 2). When Tyr[1] was replaced by 2'-naphtylalanine (2'Nal), a non-selective agonist for the NMURs (**17**) with similar affinity and potency as compared to compound **1** was found. The acetylated analog (**18**) however, is more potent than NMU-8 at NMUR1 and NMUR2, with a similar activity on both receptors and a 6-fold higher affinity for NMUR1 (Table 2 and Figure 3). Finally, a substitution by Dmt resulted in potent, but non-selective, NMUR agonists **19** and **20**. They possessed a subnanomolar affinity for NMUR1, yet an increased potency on NMUR2 was observed.

These modifications of the Tyr residue, namely replacement by 7-OH-Tic, 2'Nal or Dmt, led to the discovery of potent NMU-analogs. It indicates that the side chain hydroxyl function in this position is not important for receptor activation. However, an aromatic moiety should be present. Consequently,

18

when the *N*-terminus was acetylated, these analogs became even more potent on both receptors, presenting all an increased potency compared to NMU-8, with compound **16** as the only exception. It has an approximately 4-fold lower potency on NMUR2 than the analog with the free amine at the *N*-terminus (**15**). However, it was equipotent on this receptor as compared with the native NMU-8 peptide. All compounds (**15** – **20**) possessed higher affinity for NMUR1 although most molecules lead to an increased activity on NMUR2.

Figure 4. *In vitro* evaluation of the effect of Phe[4] substitution on affinity and receptor activation. A) IP$_3$ accumulation assay performed on HEK293 cells, transiently expressing NMUR1. B) Competitive binding assay with [^{3}H]-NMU-8 carried out on HEK293 cells, transiently expressing NMUR1. C) Structures of the novel NMU-analogs **24**, **26** and **28**. Data are shown as mean ± SEM (n = 3). [double column]

Introduction of unnatural amino acids in position 4

Modifications of the Phe residue in position 4 were introduced to prevent the cleavage of the peptide at the biodegradation site Phe-Arg, which was proposed by Takayama *et al.* after *in vitro* stability assays in rat serum [19]. By replacing Phe[4] by 7-OH-Tic, both the analogs with a free amine *N*-terminus (**21**) and the acetylated form (**22**) were agonists on NMUR1. Compound **21** resulted in an agonist with a minor loss in affinity and potency on this receptor. On the other hand, its acetylated form (**22**) possessed an increased affinity and potency, as compared to NMU-8, although a loss of more than 10 % of the maximal agonist activity was observed (Table 2). On NMUR2, this modification led to a drop in affinity, and consequently the activity. Moreover, the maximal effect on NMUR2 decreased with more than 10 % for analog **22**. When a bulky aromatic residue, 1'Nal (**23**) or 2'Nal (**25**), was introduced in the sequence, full agonists were obtained for NMUR1 with similar potencies as NMU-8. When these analogs were acetylated at their *N*-terminus, to give compounds **24**

463 and **26** respectively, potent agonists were found with a 10-fold higher activity at NMUR1 and an

464 increased affinity. For these compounds (**23 – 26**), a loss in affinity and activity for NMUR2 was

465 found. All potent NMUR1 selective NMU-analogs, obtained by modification of Phe[4], are presented in

466 Figure 4. When a bulky Tyr-analog was introduced in the position of Phe[4] (i.e. by replacing Phe with

467 Dmt to give **27**), a NMUR1-selective agonist with a slightly diminished potency (EC_{50}= 77.9 nM), as

468 compared to NMU-8, was again obtained. The acetylated analog (**28**) showed a higher potency and

469 affinity for NMUR1 (Figure 4). A last modification which was introduced in this position consisted of

470 the replacement of Phe by octahydroindolecarboxylic acid (Oic), a residue that can be regarded as a

471 saturated and constrained Phe surrogate. Unfortunately, both compounds **29** and **30**, were

472 characterized by a lower potency and affinity on both NMURs as compared to NMU-8.

473 Importantly, these results reveal that the modification of Phe in position 4 can serve as a tool to obtain

474 NMU-analogs with an increased selectivity for NMUR1. Moreover, it shows that a hydroxyl function

475 in the side chain of Phe is tolerated since the NMUR1 activation is unaltered when Dmt was

476 introduced. However, when Phe is replaced by 7-OH-Tic, a decrease in affinity for NMUR1 was

477 found. This could be explained by the fact that an unfavorable conformational constraint is imposed,

478 and hence that the side chain is forced in a less favorable position for binding to the NMURs [34].

479 Remarkably, this loss in affinity could be restored by the introduction of an acetyl group at the *N*-

480 terminus. Moreover, when Phe is replaced by Oic, the potency diminished even more due to a loss in

481 affinity for the NMURs.

482 *N-substituted glycines in position 4*

483 A series of *N*-substituted glycines, the so-called 'peptoid' residues, was introduced in the NMU-8

484 sequence to mimic the aromatic character of Phe, while aiming to protect the degradation site Phe-Arg

485 by using another strategy than for the analogs of the previous section. Moreover, a shift of the side

486 chain from the α-carbon to the nitrogen in neurotensin(8-13) has been shown to shift the receptor

487 selectivity from NTSR1 to NTSR2 [35]. Hence, benzyl, 4-hydroxy-benzyl, phenethyl and 4-hydroxy-

488 phenethyl moieties were inserted instead of Phe[4] in order to verify whether selectivity could be

489 impacted. However, all NMU-analogs with a *N*-substituted Gly instead of Phe at position 4

490 (compounds **31 – 38**) resulted in weak agonists with a loss in potency and a lower E_{max} compared to

491 our endogenous lead peptide NMU-8. Moreover, a decreased affinity for both NMURs was observed

492 as well. It can be speculated that the backbone amide is necessary for receptor binding and activation.

493 Another explanation for the loss of affinity and activity could be that the side chain is not aligned into

494 the right position to favor binding and, subsequently, to exert an effect on the NMURs. Of note, to

495 compensate for such an unfavorable shift of the side chains, the homologated phenethyl and 4-

496 hydroxy-phenethyl 'side chains' were applied, even though unsuccessfully.

497 *Substitution of the proline residue*

498 The naturally constrained Pro[6] residue was replaced by the conformationally constrained 1,2,3,4-

499 tetrahydroisoquinoline-3-carboxylic acid (Tic) residue. This bridged residue led to poor partial

500 NMUR1 agonists (**39** and **40**), which possessed a decreased receptor affinity as compared to NMU-8.

501 Although these analogs had a lower affinity for NMUR2 than compound **1**, the non-acetylated peptide

502 was able to fully activate NMUR2 with only a 4.5 fold higher EC_{50} value. Moreover, compound **40**

503 was found to have a 2-fold higher potency at NMUR2, but it was not able to fully activate the receptor

504 (E_{max} = 91.8 %). Similarly to the insertion of Tic at this position, replacement of Pro by Oic led to a

505 potent agonist (**41**) on NMUR2, even though it was already stated in literature that the Pro-Arg-Asn-

506 NH_2 segment is critical for receptor activation [18]. Moreover, when this peptide was *N*-terminally

507 acetylated (**42**), the NMU-8 potency was exceeded. On NMUR1, the substitution of Pro by Oic led to

508 a full agonist with a 7-fold decrease in potency. However, when an acetylation was introduced at the

509 *N*-terminus, the NMU-8 potency was restored. Both compounds **41** and **42** possessed a decreased

510 affinity for the NMURs. Interestingly, modifications in this, previously described, critical part of the

511 NMU-8 sequence show that other cyclic amino acids are tolerated in this region of the peptide.

512 Substitution of Pro gave rise to molecules with an increased NMUR2 selectivity and high potency at

513 this receptor subtype, which makes it an attractive tool to further develop receptor subtype selective

514 analogs.

In vitro **plasma stability**

516 To evaluate the effect of the introduced modifications on the proteolytic stability of the NMU-analogs,

517 *in vitro* degradation studies in human plasma at 37 °C were performed on a selection of the novel

518 peptides. The percentage of the intact NMU-analog was measured over time by HPLC-UV analysis

519 (Figure 5). Afterwards the samples were analyzed by means of MS and degradation profiles were

520 studied (Figure 6). Depending on the stability of the peptide, samples were taken at different time

521 points (Table S1).

522 NMU-8 (**1**) was characterized by a short half-life of only 4.3 ± 0.2 min. It was rapidly degraded at its

523 unprotected *N*-terminus where Tyr[1] was cleaved from the sequence. Since acetylation of the *N*-

524 terminal amine was systematically performed on all NMU-analogs described in this study, the

525 resistance to biological degradation of compound **2** was evaluated next. When an acetyl group was

526 introduced at the *N*-terminus of the NMU-8 sequence, a more than 25-fold increase of the half-life

527 (117.8 ± 0.7 min) was observed. The prior cleavage site Tyr[1]-Phe[2] was protected by this *N*-terminal

528 modification and degradation took place by cleavage of the peptide between the Phe[2] and Leu[3]

529 residues. Substitutions of the Tyr residue did not lead to a further increase of the enzymatic stability

530 with calculated half-lifes of 109.8 ± 3.1 min, 128.8 ± 1.7 min and 154.5 ± 10.2 min for compounds **16**,

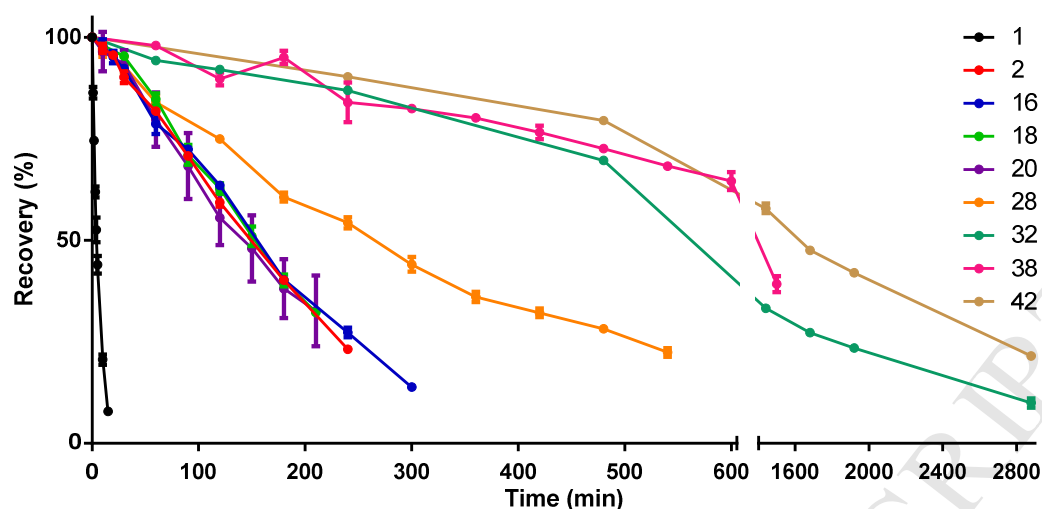531 **18** and **20**, respectively.

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Figure 5. Percentage of recovery of NMU-analogs in human plasma incubated at 37 °C. The experiments were performed in triplicate and the results are presented as mean ± SEM (n = 3). [single column Figure]

Although introduction of a modified amino acid in position 1 gave rise to molecules with a similar half-life compared to Ac-NMU-8 (**2**), it was observed that the major cleavage site of compound **16** was situated in the middle of the sequence, between Phe^4 and Arg^5. This position was proposed earlier to be an important biodegradation site [19]. Next, the peptide was cleaved further at the same position as described for compound **2**, namely after Phe^2. The NMU-analog **18** was characterized by the same major cleavage site as for **16**, but the introduction of 2'Nal in the sequence led to stabilization of the labile Phe^2-Leu^3 segment. In a smaller extent the peptide was cleaved between Pro^6 and Arg^7. When an unnatural amino acid was introduced in position 4, e.g., Dmt instead of Phe for compound **28**, a 2-fold increase of the half-life in human plasma was found, as compared to Ac-NMU-8 **2** and an almost 60-fold increase when it was compared to the native peptide **1**. The major biodegradation site remained in the middle of the sequence, namely at Dmt^4-Arg^5, but the cleavage was significantly hampered via the replacement of Phe by Dmt at this position. As expected, the introduction of the 'peptoid' residues at Phe^4 resulted into NMU-analogs with an increased resistance against proteolytic degradation. The stability assay revealed that compound **32** had a half-life of 837.4 ± 27.8 min (or 14.6 ± 0.8 h) in human plasma. When Phe^4 was replaced by *N*(4-OH-phenethyl)Gly (**38**), it was even higher, namely 1096.1 ± 56.1 min (18.3 ± 0.9 h). Moreover, the switch of Phe^4 by *N*-substituted glycines changed the biodegradation profile. The major cleavage site moved towards the *C*-terminus of the peptide, more precisely between Arg^7 and Asn^8. A last subset of modifications which was used in this study, is the substitution of the Pro residue. When it was replaced by Oic to give **42**, a molecule with a high resistance to enzymatic degradation was found. The half-life of analog **42** in human plasma was 1426.1 ± 67.4 min or 23.8 ± 1.1h. The major degradation site for this analog was as well located between Arg^7-Asn^8. When considering the activity profile of compound **42**, and taking its proteolytic

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stability into account, this analog can be considered as a good lead for the development of potent and stable NMUR2 agonists.

	$t_{1/2}$ (min)	Sequence and cleavage sites								
1	4.3 ± 0.2	H-	Tyr -¹	Phe -	Leu -	Phe -	Arg -	Pro -	Arg -	Asn - NH₂
2	117.8 ± 0.7	Ac-	Tyr -	Phe -¹ Leu -		Phe -	Arg -	Pro -	Arg -	Asn - NH₂
16	109.8 ± 3.1	Ac-	7-OH-Tic -	Phe -² Leu -		Phe - ¹	Arg -	Pro -	Arg -	Asn - NH₂
18	128.8 ± 1.7	Ac-	2'Nal -	Phe - Leu -		Phe - ¹	Arg -	Pro -²	Arg -	Asn - NH₂
28	253.5 ± 6.0	Ac-	Tyr -	Phe -² Leu -		Dmt - ¹	Arg -	Pro -	Arg -	Asn - NH₂
32	837.4 ± 27.8	Ac-	Tyr -	Phe -² Leu -		N(Benzyl)Gly -	Arg -	Pro -	Arg -¹	Asn - NH₂
38	1096.1 ± 56.1	Ac-	Tyr -	Phe -² Leu -		N(4-OH-phenethyl)Gly -	Arg -	Pro -	Arg -¹	Asn - NH₂
42	1426.1 ± 67.4	Ac-	Tyr -	Phe -² Leu -		Phe -	Arg -	Oic -	Arg -¹	Asn - NH₂

Figure 6. Cleavage sites of the NMU analogs after incubation in human plasma at 37 °C. The major biodegradation sites are indicated with dotted lines of which the numbers represent the order of cleavage.

Previously, the stability of some NMU-based molecules was already reported in literature. Lipidation of NMU-8 markedly increased its half-life in mouse plasma as compared to hNMU [21]. Moreover, when the effect on proteolytic stability was studied of molecules without high molecular carriers, improved resistance towards degradation was observed as well. The selective NMUR1 agonist, 2-thienylacetyl-Trp-(αMe)Trp-Arg-Pro-Arg-Asn-NH₂, which was recently described by Takayama *et al*, possessed an improved *in vitro* serum stability with a half-life of approximately 180 min [20]. Here, we discovered molecules with plasma half-lifes up to 23 h. The potent NMUR1 agonist, compound **28**, possessed a half-life of 253.5 ± 6.0 min. In the present study, human plasma was used for the determination of the biodegradation profiles. This does not allow direct comparison with the metabolic stability of 2-thienylacetyl-Trp-(αMe)Trp-Arg-Pro-Arg-Asn-NH₂, which was studied in serum. Moreover, it was reported that the preferential biodegradation site of this NMUR1 selective agonist was located between the Arg and Asn residues and that the key enzyme, responsible for this cleavage, was found to be thrombin [20, 36]. The present study is giving more accurate information when compared to studies where experiments were performed with 25% rat or human serum [19, 20], since in plasma the inactive prothrombin is present, giving a closer representation of physiological conditions.

581

582 **Conclusions**

583 A novel set of NMU-analogs was developed and characterized *in vitro*. The core sequence of the

584 NMU-peptide, more precisely NMU-8, was taken as lead molecule for the SAR-study. Potent agonists

585 (compounds **15 – 20**), with equal activity on both NMURs, were obtained when the Tyr[1] residue was

586 modified, with affinities and activities in the nM range. Moreover, these peptides turned out to have an

587 elevated resistance against proteolytic degradation, resulting in molecules with an increased half-life in

588 human plasma. We demonstrated that substitution of Phe[4] could serve as a tool for NMUR1 selectivity

589 and improved plasma stability, which resulted in the discovery of potent NMU-analogs with increased

590 NMUR1 selectivity and enhanced stability in human plasma, more precisely analog **24**, **26** and **28**. By

591 synthesizing peptides with a modified Pro[6] residue, an increased potency on NMUR2 was observed.

592 Moreover, a remarkable effect on the enzymatic stability was found, going from 4 min for NMU-8 to

593 approximately 24 h for **42**. By evaluating the effect of D-amino acid substitution on receptor affinity,

594 we demonstrated that the previously reported tool for antagonism led to a loss in affinity rather than

595 antagonism. We believe that this novel set of NMU-analogs can have potential in the development of

596 receptor selective NMU-based peptides with improved proteolytic stability.

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Author contributions

ADP synthesized the ligands, performed the experiments and wrote the paper. CM supported the peptide synthesis and purification as well as the identification of the metabolites. YVW optimized and contributed to the plasma stability assay. LJS contributed to the functional assay. CT provided the [^{3}H]-NMU-8 peptide. VC and AVE are co-supervisors and helped revising the manuscript. AVE supervised the *in vitro* stability study. DT contributed to confine the synthesis strategy. BH and MMR supervised and designed the *in vitro* activity and binding assays. IS and SB are promotors and supervisors of the practical work and the design of the research study. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Highlights

- Insertion of unnatural amino acids in NMU-8 leads to stable and selective ligands

- Potent NMUR ligands can be found by substitution of the Tyr[1] residue of NMU-8

- NMUR1 selectivity can be achieved by modification of Phe in position 4

- Replacement of the Pro[6] residue shifts the selectivity towards NMUR2

- Biodegradation profiles are studied in human plasma