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Research paper

Development of glycine-α-methyl-proline-containing tripeptides with neuroprotective properties

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ABSTRACT

Herein is described the synthesis of novel glycine-α-methyl-proline-containing tripeptides (**GP^{Me}X** tripeptides namely **GP^{Me}R**, **GP^{Me}K**, and **GP^{Me}H**) with the aim of obtaining derivatives highly stable in human plasma and able to counteract neuroinflammatory processes that are distinctive of neurodegenerative pathologies. The syntheses of **GP^{Me}R**, **GP^{Me}K**, and **GP^{Me}H** were all achieved both by introducing the Pro^{Me} residue into the Gly-Pro-Arg (**GPR**) sequence in place of the native Pro in P2 position and replacing the basic amino acid Arg in P3 position by Lys or His.

Results showed that all novel **GP^{Me}X** tripeptides are stable in human plasma ($t_{1/2}$ > 51 h) and that **GP^{Me}H** – generating stable intramolecular H-bond in a C_{11}-turn by interaction of His imidazole ring and Gly carbonyl group – restored physiological levels of nitric oxide deriving from neuronal NOS (nNOS) activity, thus preventing the inflammatory response by suppression of the NF-kB activity and, consequently, the expression of inflammatory genes such as inducibile NOS (iNOS). Therefore, **GP^{Me}H** could be a lead compound for further development of peptidomimetics able to contrast neuroinflammatory processes.

1. Introduction

It is now clearly established the relationship between a class of brain pathologies, including Parkinson's (PD) and Alzheimer's (AD) diseases, and neuroinflammation [1–3]. Indeed, inflammatory mediators (TNF-α, interleukin-1β, IFNγ, growth factors) and enzymes (iNOS) are released in response to glial cells activation, with consequent deposition of amyloid beta (Aβ) plaques or formation of Lewy bodies, respectively in AD- or PD-affected neurons [4,5]. Notably, such neuroinflammatory processes are also connected to oxidative stress through a mechanism involving glial activation mediated by reactive oxygen and nitrogen species (ROS and RNS), which is accountable for damaging neurons [6].

Medicinal chemistry- and technology-based approaches were recently employed to realize molecules able to hamper or interrupt

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; Bop-Cl, bis(2-oxo-3-oxazolidinyl)phosphonic chloride; CD, circular dichroism; cNOS, constitutive NOS; CNS, central nervous system; CSF, cerebrospinal fluid; Dex, Dexamethasone; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; FCS, fetal calf serum; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; GPE, Gly-Pro-Glu; GPR, Gly-Pro-Arg; IFN-γ, interferon gamma; IL-1β, Interleukin-1 beta; iNOS, inducibile NOS; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; nNOS, neuronal NOS; PD, Parkinson's disease; Pro^{Me}, α-methyl-proline; ROS, reactive oxygen species; RNS, reactive nitrogen species; PI, propidium iodide; TEA, triethylamine; TFE, tetrafluoroethylene.

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the oxidative stress-neuroinflammation that is typical of neurodegeneration [7–11]. Small peptides and peptidomimetics, such as Gly-Pro-Glu (**GPE**) or its analog Gly-Pro^{Me}-Glu (NNZ-2566), Ac-Pro-Gly-Pro (Ac-PGP), and Gly-Pro-Arg (**GPR**), were explored as neuroprotective agents both in ischemic brain injuries and neurodegenerative diseases [12–15]. Especially, NNZ-2566, deriving from the introduction of α-methyl-proline (Pro^{Me}) into the GPE sequence – conferring it a half-life equal to 74 min in brain – reduced the cognitive deficit in rat models of brain damage due to ischemic injuries [16]. The role of acidic amino acids, such as Glu in P3 position of GPE, was extensively studied; biological studies showed that the replacement of Glu with Arg resulted effective in protecting neurons from Aβ toxicity. In fact, such **GPR** peptide was noticeable for its aptitude of powerfully preventing Aβ-induced apoptosis in rat hippocampal neurons by interfering with caspase-3 and p53 expression [17]. Although, **GPR** is a promising compound for the management of AD, its use is hindered by the peptidic nature that makes it exposed to the proteases digestion.

In this work, using the peptidomimetic strategy, we focused on the replacement and/or modification of the amino acids composition of the **GPR** sequence with the aim of improving its proteases resistance, still retaining neuroprotective activity. Based on these data, we synthesized **GP^{Me}X** tripeptides where the native proline was replaced with Pro^{Me} to insert a local conformational restriction at the prolyl peptide bond. Pro^{Me} residue, such as other alkylated amino acids, can balance α-helical structures, β-turns, or extended conformations permitting the trans peptide bond conformation [18]. Moreover, the introduction of Pro^{Me} renders tripeptides more resistant to proteases activity by enhancing plasma stability [19].

Subsequently, we explored the role of arginine by substituting it with other basic amino acids – such as Lys and His – to investigate and verify whether arginine is essential or not for the preservation of the biological activity. The new **GP^{Me}X** tripeptides H-Gly-Pro^{Me}-Arg (**GP^{Me}R**), H-Gly-Pro^{Me}-Lys (**GP^{Me}K**), and H-Gly-Pro^{Me}-His (**GP^{Me}H**) were synthesized and their conformations in different solutions [tetrafluoroethylene (TFE), MeOH, and H_2O] were studied by circular dichroism (CD) spectroscopy. The **GP^{Me}X** tripeptides were also subjected to stability evaluation in human plasma and *in vitro* studies on human neuroblastoma SH-SY5Y cells to assess their efficacy in counteracting the toxicity of inflammatory molecules secreted by activated glial cells.

2. Experimental section

Materials. All the reagents, unless otherwise stated, were from Sigma Aldrich Co. (St. Louis, MO, USA). All other chemicals used were of the highest purity commercially available. GPR was prepared as reported by Kawasaki et al. [20].

General Procedures. Chromatographic purifications were performed on silica gel using column chromatography (Merck 60, 70–230 mesh ASTM silica gel), and compounds were detected with UV light (λ = 254 nm). Before performing biological studies, chemical structures and purities (>98%) of **GP^{Me}R**, **GP^{Me}K**, and **GP^{Me}H** were confirmed by ^{1}H,^{13}C NMR, HR-MS spectra, and HPLC analysis. NMR spectra were recorded with a Varian VXR-300 spectrometer (300 MHz). HR-MS spectra were recorded using TOF/Q-TOF Mass Spectrometer (AGILENT Technologies 6540 UHD Accurate Mass Q-TOF LC/MS), Agilent Dual Jet Stream Column as ion source, and a Supelco Ashentis C8 2.1 × 3 mm 2.7 μm column. The capillary temperature was set at 300 °C.

Optical rotations were recorded at 20 °C with a Perkin–Elmer 241 polarimeter. The purity of the **GP^{Me}X** tripeptides was determined by analytical HPLC using a Waters 600 HPLC equipped with a X-Bridge BEH130 C-18, 5 μm, 4.6 × 250 mm column with Waters 2996 PDA detector, and H_2O/CH_3CN (0.1% TFA) as solvent system in the form of a linear gradient from 10 to 90% of CH_3CN over 70 min and a flow rate of 1 mL/min. Microanalyses were carried out on a 1106 Carlo Erba CHN analyzer. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values.

2.1. Z-Gly-Pro^{Me}-OMe (*4*)

To a mixture of **2** (2.8 g, 15.58 mmol) and Z-Gly-OH (**3**) (3.42 g, 16.36 mmol) in dry DCM (106 mL) was added TEA (2.99 mL, 21.5 mmol); the reaction mixture was left under stirring for 20 min at −20 °C and then added with DCC (3.73 g, 18.07 mmol) in dry DCM (53 mL). Subsequently, the solution was allowed to stir for 20 h at 20 °C. The resulting white mixture was filtered, extracted with DCM/HCl 1 N, $NaHCO_3$ and evaporated. The crude was purified on silica gel using AcOEt/n-hexane (9:1) to give **4**. Yield: 74%; R_f = 0.65 (AcOEt); $[\alpha]_D$ = - 98.4° (c = 1, MeOH); HPLC purity: 96%; ^{1}H NMR ($CDCl_3$) δ: 1.38 (3H, s, α-CH_3), 1.67–1.95 (4H, m, β- and γ-Pro), 3.16–3.36 (2H, t, J = 6.7 Hz, δ-Pro), 3.50 (3H, s, OMe), 3.74–3.78 (2H, m, α-Gly), 4.93 (2H, s, Ph-CH_2), 5.83–5.86 (1H, J = 4.3 Hz, t, NH), 7.11–7.18 (5H, m, Ar); ^{13}C NMR ($CDCl_3$) δ: 21.25 (α-CH_3), 23.79 (CH_2 γ-Pro), 38.10 (CH_2 β-Pro), 43.44 (CH_2 α-Gly), 46.76 (CH_2 δ-Pro), 52.27 (OMe), 66.09 (Ph-CH_2O), 66.44 (C α-Pro), 127.76–128.31 (5 × CH Ar), 136.55 (C Ar), 156.19–173.94 (3 × CO). Anal. ($C_{17}H_{22}N_2O_5$) C, H, N.

2.2. Z-Gly-Pro^{Me}-OH (*5*)

LiOH (407 mg, 17.31 mmol) was put into a stirring solution of **4** (3.86 g, 11.54 mmol) in MeOH (58 mL) and H_2O (19.4 mL) and left for 4 h at room temperature. After evaporation, the residue was taken up with water, acidified with HCl 1 N, and then extracted with Et_2O (×3) and DCM/EtOH (2:1) (×3). The organic layers were combined and evaporated under reduced pressure giving compound **5**. Yield: 43%; R_f = 0.42 (AcOEt); $[\alpha]_D$ = −76.7° (c = 1, MeOH); HPLC purity: 95%; ^{1}H NMR ($CDCl_3$) δ: 1.51 (3H, s, α-CH_3), 1.78–2.17 (4H, m, β- and γ-Pro), 3.45–3.50 (2H, m, δ-Pro), 3.78–3.98 (2H, m, Gly), 5.05 (2H, s, Ph-CH_2), 6.10–6.12 (1H, J = 6.6 Hz, d, NH), 7.26–7.29 (5H, m, Ar), 9.08 (1H, br s, COOH); ^{13}C NMR ($CDCl_3$) δ: 21.25 (α-CH_3), 23.86 (CH_2 γ-Pro), 38.19 (CH_2 β-Pro), 43.54 (CH_2 α-Gly), 47.21 (CH_2 δ-Pro), 66.50 (Ph-CH_2O), 66.81 (C α-Pro), 127.94–128.43 (5 × CH Ar), 136.45 (C Ar), 156.62–176.58 (3 × CO). Anal. ($C_{16}H_{20}N_2O_5$) C, H, N.

2.3. General method for the synthesis of protected GP^{Me}X tripeptides (*9*–*11*)

TEA (0.7 mL, 5.02 mmol) was slowly added under nitrogen atmosphere to a stirred solution of **5** (503 mg, 1.57 mmol) and the suitably protected amino acid (**6**, **7**, or **8** respectively) (2.04 mmol) in dry DCM (84 mL) and left for 10 min at room temperature. After the addition of bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BopCl) (504 mg, 1.98 mmol) the reaction mixture was kept for 17 h at room temperature [21]. The resulting solution was washed with HCl 1 N, $NaHCO_3$, dried over anhydrous $MgSO_4$, and the solvent was removed. The residue was chromatographed on silica gel with $CHCl_3$/MeOH (95:5) as eluant providing the corresponding fully protected tripeptides **9**–**11**.

2.4. Z-Gly-Pro^{Me}-Arg(NO$_2$)-OBzl (*9*)

Yield: 71%; R_f = 0.37 ($CHCl_3$/MeOH, 95:5); $[\alpha]_D$ = −58.4° (c = 1, MeOH); HPLC purity: 96%; ^{1}H NMR ($CDCl_3$) δ: 1.62 (3H, s, α-CH_3), 1.65–1.83 (4H, m, β- and γ-Arg), 2.00–2.36 (4H, m, β-and γ-Pro), 3.13–3.21 (2H, m, δ-Arg), 3.59–3.62 (2H, m, δ-Pro), 3.86–4.06 (2H,