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The neuroprotective activity of GPE tripeptide analogues does not correlate with glutamate receptor binding affinity

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Abstract—The influence of several modifications on the GPE tripeptide structure upon the binding to GluRs and on their neuroprotective effects has been studied. The results indicated that the prevention of neuronal death showed by GPE and some analogues is not directly related to their affinity at glutamate receptors.

The tripeptide Gly-Pro-Glu (GPE, **1**) is endogenously formed from the acid protease-mediated metabolism of IGF-1 (insulin-like growth factor type 1).[1–3] Unlike IGF-1 and des(1–3)IGF-1, GPE neither binds to IGF-1 receptors nor has any neurotrophic effect, but there is evidence that it displays remarkable neuromodulatory activities in the CNS. Thus, GPE stimulates acetylcholine and dopamine release in the rat cortex and striatum,[4,5] and protects neurons of different brain regions from diverse induced injuries (hypoxia-ischemia, glutamate, quinolinic acid, etc.).[5–8] Furthermore, GPE shows neuroprotective effects in animal models of neurodegenerative processes, such as Huntington's, Parkinson's, and Alzheimer's diseases.[5,9,10] Although the mode of action of this tripeptide in neuroprotection remains unclear, initial structural considerations suggested that GPE could interact with one or more glutamate receptors (GluRs) and, in fact, it was demonstrated that it binds to the *N*-methyl-D-aspartate (NMDA) receptors, but not to the (2*S*)-2-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or kainate (KA) receptors.[1] In this sense, both agonistic and antagonistic effects of GPE on NMDA receptors have been reported.[1,11] It has also been shown that GPE stimulates the potassium-evoked release of acetylcholine and dopamine through interaction with NMDA receptors, along with other unknown non-NMDA receptor-mediated pathways.[1,6,12] Studies on metabolic stability have demonstrated that GPE has very short half-life in plasma, which hampers its pre-clinical and clinical trials.[13,14]

The structural simplicity of GPE converts this tripeptide into a promising starting point for the search of non-peptide mimics with improved bioavailability. In the process to achieve this purpose, through the rational design of peptidomimetics, the study of the bioactive conformation is a key step. In a previous communication,[15] we have addressed this issue by exploring the influence of both the amino acid side-chain orientation and the *cis*/*trans* isomerism at the Gly-Pro peptide bond on the binding of GPE to glutamate receptors. The significance of the amino acid side-chain orientation was investigated by the sequential replacement of both the L-Pro and L-Glu residues by their enantiomeric forms D-Pro and D-Glu in **2**–**4**, while the importance of the *cis*/*trans* isomerism was studied by the replacement of the Pro residue by pseudoprolines[16,17] [(*R*)-thiazolidine-4-carboxylic

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acid (Cys[$\Psi^{H,H}$pro]) and (*R*)-2,2-dimethylthiazolidine-4-carboxylic acid (Cys[$\Psi^{Me,Me}$pro])[18] in analogues **5** and **6**, and by alkylprolines [(*S*)-2-methylproline (P^{Me})[19] and (*S*)-5,5-dimethylproline (dmP)[20]] in analogues **7** and **8**, respectively. The binding affinity of analogues **2**–**8** for GluRs was evaluated by the measure of the displacement of L-[^{3}H]Glu from rat brain synaptic membranes. In this evaluation, only the GPE analogues **7** and **8**, which incorporate alkylprolines inducing preferentially the *trans* and *cis* rotamer, respectively, at the Gly-Pro bond, showed noticeable affinities, which were higher than that displayed by GPE, and comparable to that of D-AP5, one of the most potent and selective competitive NMDA antagonists.[21] Taking into account these results, GPE analogues **7** and **8** were also evaluated as neuroprotective agents in cultured rat hippocampal neurons. These compounds reduced the neuronal death induced by NMDA administration with a similar potency to that of GPE, and, in particular, the P^{Me} derivative **7** increased significantly the neuronal survival after oxygen–glucose deprivation (OGD) injure.

In a recent communication on other series of GPE analogues, we have shown that their neuroprotective activity does not seem to be directly related to the binding affinity at GluRs.[22] On account of these results, now we have determined and reported herein the neuroprotective activity of GPE analogues **2**–**6**. In addition, to further assess the significance of the configuration of the dmP residue of **8**, and that of the volume of the Me substituent in the P^{Me} residue of **7**, their analogues **9** and **10**, incorporating D-dmP and 2-propyl-L-proline (P^{Pr}), respectively, have been prepared and evaluated. Moreover, taking into account the high propensity of the Pro residue to induce folded conformations,[23] and specially reverse turns,[24] we have also prepared and evaluated the analogue **11**, where the Pro-Glu peptide bond has been restricted into a [4.4]-spirolactam β-turn mimetic[25] (Fig. 1).

The D-dmP-containing tripeptide **9** was synthesized from racemic dmP-OH (**12**),[26] as shown in Scheme 1. After *N*-Boc protection, the coupling of *N*-Boc-dmP-OH (**13**) with *H*-L-Glu(OBn)-OBn, using BOP as activating reagent, led to the dipeptide diastereoisomeric mixture **14**. Then, this mixture was N-deprotected and coupled with Z-Gly-OH, using HATU and HOAt as activating reagents, to obtain the corresponding (1:1) mixture of protected tripeptides **15** and **16**, which were

H-Gly-Pro-Glu-OH (GPE)	**1**	H-Gly-D-Pro-Glu-OH	**2**
H-Gly-Pro-D-Glu-OH	**3**	H-Gly-D-Pro-D-Glu-OH	**4**
H-Gly-Cys[$\psi^{H,H}$Pro]-Glu-OH	**5**	H-Gly- Cys[$\psi^{Me,Me}$Pro]-Glu-OH	**6**
H-Gly-P^{Me}-Glu-OH	**7**	H-Gly-dmP-Glu-OH	**8**
H-Gly-D-dmP-Glu-OH	**9**	H-Gly-P^{Pr}-Glu-OH	**10**

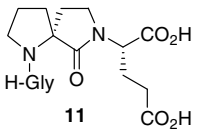

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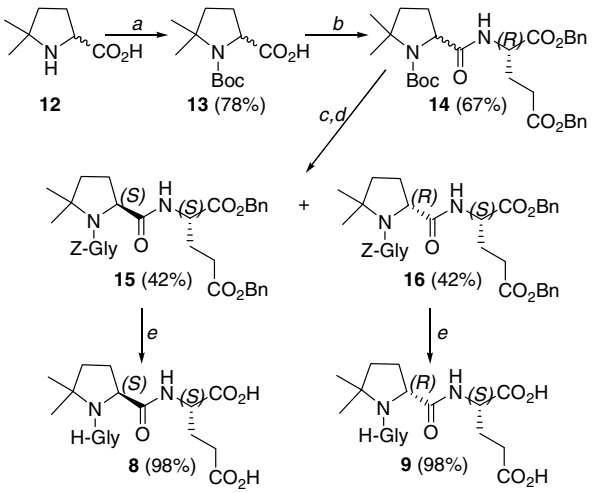
Figure 1. GPE analogues studied.

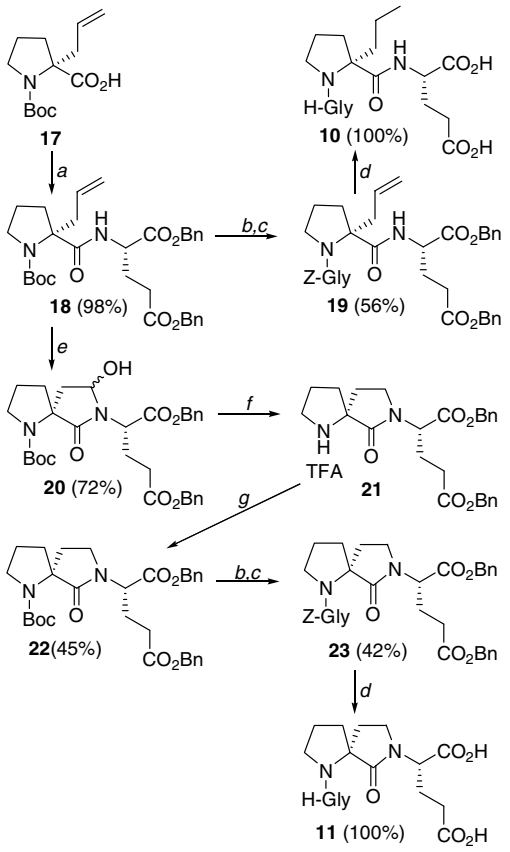


Scheme 1. Reagents: (a) Boc_2O, Et_3N, DMAP, H_2O/dioxane; (b) *H*-L-Glu(OBn)-OBn, BOP, DIEA, CH_2Cl_2; (c) 3.2 M HCl/AcOEt; (d) Z-Gly-OH, HOAt, HATU, DIEA, CH_2Cl_2; (e) H_2, Pd(C), MeOH.

chromatographically resolved. Finally, the hydrogenolysis-mediated N- and C-deprotection of separated **15** and **16** provided the GPE analogues **8** and **9**, respectively. Our previously reported synthesis of **8**[15] involved the resolution of racemic dmP-OH (**12**), by crystallization of the L-enantiomer with D-tartaric acid, according to the reported procedure.[20] The NMR analysis of tripeptides **8** and **9** in (90:10) H_2O/D_2O solution showed *cis*/*trans* rotamer ratios at the Gly-dmP peptide bond of (69:31) and (82:18), respectively. The *cis* form of the major preferred conformation was assigned based on the NOE effects observed between the Gly α-H and dmP α-H protons in the respective ^{1}H NMR 1D NOESY spectra.

The P^{Pr}-containing GPE analogue **10** was synthesized from *N*-Boc-2-allyl-L-Pro-OH (**17**), which was previously prepared by applying the Khalil et al.[27] procedure of *N*-Boc-protection of sterically hindered amino acid to 2-allyl-L-Pro-OH.[28] As shown in Scheme 2, the coupling of **17** with *H*-L-Glu(OBn)-OBn, using DCC and HOAt as activating reagents, led to the corresponding dipeptide **18**. *N*-Boc removal in this dipeptide, followed by coupling with Z-Gly-OH, using HATU and HOAt as activating reagents, gave the tripeptide **19**, whose hydrogenolysis-mediated N- and C-deprotection provided the proposed GPE analogue **10** in a 55% overall yield. The NMR analysis of this tripeptide in D_2O solution showed the presence of only one preferred conformation. The NOE effect observed in the ^{1}H NMR spectrum of **10** between the Gly α-H and the Pro^{Pr} 5-H protons indicated the presence of a *trans* conformation at the Gly-Pro^{Pr} peptide bond. It is interesting to note that, while this work was in progress, Harris et al.[29] published an alternative synthesis of **10** in a 32% overall yield from 2-allyl-L-Pro-OMe, although they did not report any data of its biological evaluation.

The spirolactam GPE analogue **11** was prepared by applying our previously developed synthesis of [4.4]-spirolactam β-turn mimetics from *N*-Boc-2-allyl-L-Pro-OH.[30] As shown in Scheme 2, this methodology



Scheme 2. Reagents: (a) *H*-L-Glu(OBn)-OBn, HOAt, DCC, NMM, CH_2Cl_2; (b) 3.2 M HCl/AcOEt; (c) Z-Gly-OH, HOAt, HATU, DIEA, CH_2Cl_2; (d) H_2, Pd(C), MeOH; (e) OsO_4, $NaIO_4$, MeOH/H_2O; (f) $NaBH_4$, TFA; (g) Boc_2O, TEA, DMAP, CH_2Cl_2.

involved the oxidation of the dipeptide **18**, by treatment with OsO_4 and $NaIO_4$ in 2:1 MeOH/H_2O mixture for 24 h, to give the hydroxylactam **20**, whose one-step reduction and N-deprotection by reaction with $NaBH_4$ in neat TFA led to the trifluoroacetate **21**, along with traces of decomposition products. To facilitate the purification of the [4.4]-spirolactam derivative **21**, the Boc-protecting group was reintroduced in **22**, by the treatment of **21** with Boc_2O in the presence of TEA and a catalytic amount of 4-(dimethylamino)pyridine (DMAP). *N*-Boc removal in pure **22**, followed by coupling with Z-Gly-OH and hydrogenolysis-mediated final deprotection as above-mentioned, provided the conformationally constrained tripeptide analogue **11** in a 14% overall yield. Harris et al.[29] have also published an alternative synthesis of **11** in a 10% overall yield from 2-allyl-L-Pro-OMe, but they neither gave any data of its biological evaluation. Similarly to the case of the P^{Pr}-derived tripeptide **10**, the NMR analysis of the spirolactam derivative **11** in D_2O showed the presence of only one preferred conformation, which was assigned as *trans* on the basis of the NOE effects observed between the Gly α-H and the spirolactam 2-H protons.

Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes by the new GPE analogues **9**–**11** was determined, and the results were compared with those previously reported for compounds **1**–**8**.[15] No displacement was found using concentrations up to 100 μM of compounds **10** and **11**. However, the D-dmP-containing GPE analogue **9** displayed one and two orders of magnitude higher affinity for glutamate receptors than its epimer at the dmP residue **8** and GPE (**1**), respectively (see Table 1). This significant increase in the binding affinity was unexpected, taking into account the detrimental effect on the binding affinity at GluRs observed when the Pro residue of GPE was replaced by D-Pro in **2**.[15]

The neuroprotective effects of compounds **2**–**6** and **9**–**11**, regardless of their binding affinity at GluRs, were evaluated in cultured hippocampal neurons exposed to NMDA (100 μM) or oxygen–glucose deprivation (OGD), following described procedures.[31,32] The results are shown in Figure 2 compared with those of GPE and its analogues **7** and **8** previously studied. Surprisingly, the stereochemistry requirements were contrary to those for the GluRs binding affinity. Thus, the GPE diastereoisomers **2** and **3**, which did not bind at all at the higher assayed concentration (100 μM), displayed the best neuroprotection percentage, higher than GPE, in both NMDA excitotoxicity (Fig. 2A) and OGD (Fig. 2B) assays, while, the D-dmP-containing GPE analogue **9**, which, as above-mentioned, showed the higher GluRs binding potency, did not significantly prevent the neuronal death in both types of assays. Similarly, the influence of the ring puckering and hydrophobicity of the Pro residue on the GluRs binding affinity and on the neuroprotective activity was different. Thus, the pseudoproline-containing compounds **5** and **6**, which did not displace L-[^{3}H]glutamate, displayed neuroprotection percentages slightly lower than GPE in the NMDA excitotoxicity assay, but their effect was higher in the oxygen–glucose deprivation model. As previously suggested,[15] the Gly-Pro peptide bond conformation does not seem to have a significant influence on the neuroprotective activity. However, the conformational restriction at the Pro-Glu peptide bond in compounds **10** and **11** was detrimental both for the binding at GluRs and for the neuroprotection effects.

In conclusion, the results herein reported indicate that the tridimensional structural requirements of GPE for binding to GluRs are different to those required for neuroprotective activity. Therefore, it seems to confirm that the prevention of neuronal death showed by GPE and some analogues is not directly related to their affinity at glutamate receptors.

Table 1. Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes

Compound	K_i (μM)
GPE (*H*-Gly-Pro-Glu-OH) (**1**)	31.24 ± 15.65
H-Gly-P^{Me}-Glu-OH (**7**)	7.96 ± 1.83
H-Gly-dmP-Glu-OH (**8**)	3.79 ± 0.53
H-Gly-D-dmP-Glu-OH (**9**)	0.33 ± 0.07

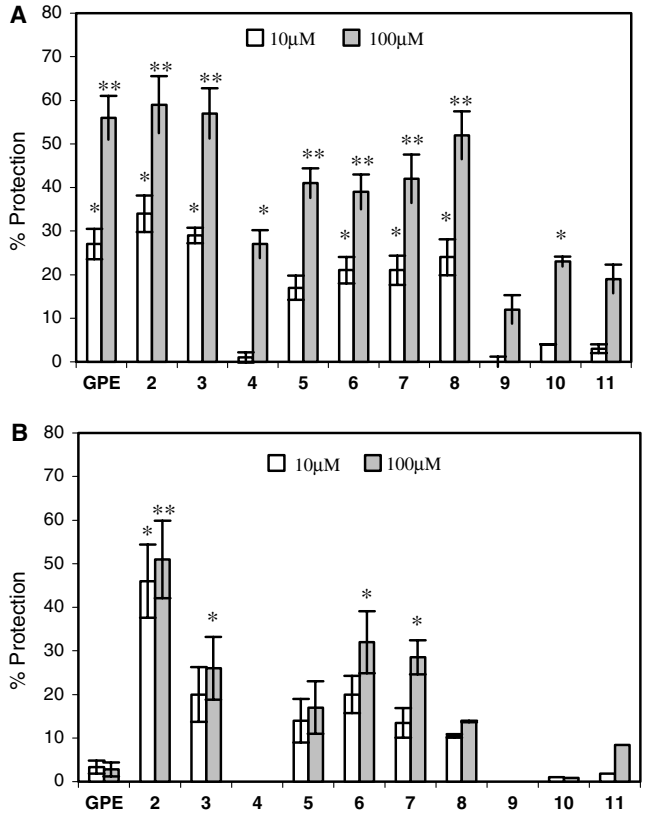


Figure 2. Protection of rat hippocampal neurons from NMDA (100 μM) excitotoxicity (A) and oxygen–glucose deprivation (B). Cell survival was estimated by measuring the activity of mitochondrial dehydrogenase on the tetrazolium derivative MTT. Values are means ± SEM of 8–13 experiments. Asterisks indicate difference from control (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.04.033.

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