GPE and GPE Analogues as Promising Neuroprotective Agents

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Abstract: The tripeptide glycine-proline-glutamate (GPE) is the naturally cleaved N-terminal tripeptide of insulin-like growth factor-1 (IGF-1) in brain tissues by an acid protease. Although GPE does not bind to IGF-1 receptors and its mode of action is not clear, *in vitro* studies have demonstrated its ability to stimulate acetylcholine and dopamine release, as well as to protect neurones from diverse induced brain injures. More importantly, GPE has been shown to have potent neuroprotective effects in numerous animal models of hypoxic-ischemic brain injury and neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's diseases. As a consequence, GPE was suggested to be a potential target for the rational design of neuroprotective agents. Unfortunately, the use of GPE as a therapeutic agent is limited because of its unfavorable biochemical and pharmacokinetic properties.

This review will focus on structural modifications performed on the GPE molecule in order to obtain bioactive analogues with increased pharmacokinetic profile useful for the treatment of central nervous system (CNS) injures and neurodegenerative disorders.

Keywords: Glycine-proline-glutamate, GPE analogues, macrocyclic GPE analogues, structure-activity relationships, neuroprotective agents, neurodegenerative disease, hypoxic-ischemic brain injury.

INTRODUCTION

There are potentially a large number of highly conserved tripeptide motifs in proteins in nature, and much effort has undergone to identify these sequences and their biological functions [1]. In this context, insulin-like growth factor 1 (IGF-1) is a naturally occurring peptide and a potent neurotrophic factor in the central nervous system (CNS) [2]. It plays an important role both in the CNS growth and in the regulation of mature neuronal and glial function [3-7]. IGF-1 performs neuroprotective effects by reducing neuronal loss in several regions after injury [8], during development [9], and preventing *in vitro* neuronal apoptosis [10]. Findings suggest that these biological functions are realized by interacting with IGF-1 receptors (IGF-1R) which are widely distributed in the CNS [11]. Ligand-receptor interactions are modulated by six IGF binding proteins (IGFBPs) to which IGF-1 is almost entirely bound [12]. In brain tissues, IGF-1 is cleaved by an acid protease into des-N(1-3)-IGF-1 (des-IGF-1), a truncated IGF-1 comprised of 67 amino acids, and the N-terminal tripeptide motif glycine-L-proline-L-glutamate (glypromate ®, GPE, Fig. **1**) [1, 13-15]. Des-IGF-1 has a potent neurotrophic action in the CNS *via* interaction with the IGF-1R, while GPE exerts its main biological functions through a different mechanism [16]. Data suggest that GPE bioactivity is not mediated by IGF-1R, since the presence of amino-terminal glycine and carboxy-teminal glutamate GPE can activate N-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamic acid receptor belonging to the family of ligand gated ion channel receptors [13]. In fact, synthetic GPE is able to stimulate the potassium-evoked release of dopamine from rat striatal slices by NMDA receptor interactions, but not by (RS)-2-amino-3-3-hydroxy-5-methylisoxazol-4-yl propionic acid (AMPA) or kainate glutamate receptorial subtypes [13]. In addition, GPE is more potent in stimulating the release of acetylcholine from rat's brain tissue by several orders of magnitude compared to IGF-1 [17]. This action is probably mediated by another mechanism that should involve a novel ion-channel-associated receptor.

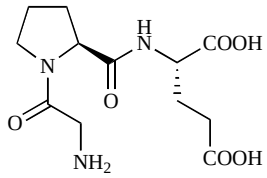

Fig. (1). Structure of H-Gly-Pro-Glu-OH (GPE).

NEUROPROTECTIVE ACTIVITY OF GPE

Current reports have provided the evidence that GPE is not only an *in vitro* neuromodulator agent but also a neuroprotective tripeptide [18, 19]. The dose-dependently neuroprotective effect of GPE was observed for the first time after NMDA-induced neuronal injury *in vitro* CA1-2 hippocampal neurons by Saura *et al*. [20]. Later *in vivo* studies demonstrated that GPE can act as a neuronal rescue agent following hypoxic-ischemic (HI) brain injury after central administration [18]. In particular, intracerebro-ventricular (i.c.v.) administration of GPE 2 h after HI brain injury in adult rat protects neurons in the cerebral cortex, in the CA1-2 subregions of hippocampus and prevents the loss specific subtypes of striatal neurons (choline acetyltrans-ferase, glutamate decarboxylase, neuronal nitric oxide

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1389-5575/12 $58.00+.00

synthase, and somatostatin-immunopositive neurons) [18]. To achieve neuroprotection by central administration of GPE in the infant rat a dose of 30 μg is necessary, a 10-fold higher dose than that used in adult rat 2 h after HI injury into the lateral ventricle [18, 19]. Administration of higher doses in infant rats is probably due to the difference in the age-related bioavailability and functionality of the site of action of GPE [19]. Furthermore, Guan and coworkers [21] demonstrated that the central penetration of GPE is injury-dependent since GPE levels in the cerebrospinal fluid (CSF) were found to be increased only in the HI injured treated rats [21, 22]. The rise of permeability of blood-brain barrier (BBB) to GPE is likely due to the loss of basal lamina caused by the activation of matrix metalloproteinases (MMP)-2 and -9 after HI injury [23, 24].

The neuroprotective activity of GPE was also studied following its peripheral administration. In this context, the treatment with GPE (15 mg/kg i.p.), 30 min after HI injury, improved the neuronal survival in the CA1-2 subregions of hippocampus in a dose-dependent way. A similar dose in the infant rat (12 mg/Kg i.p.) produced the same neuroprotective effect [25]. The degree of neuroprotection of GPE, following a single bolus injection, is variable because of its short-life in plasma, which is estimated to be less than 2 or 4 minutes after single bolus i.v. or i.p. administration to normal wistar rats, respectively [25, 26]. However, coadministration of GPE with a peptidase inhibitor cocktail extends the GPE half-life until to 3 h after i.c.v. administration, suggesting its enzymatic stability in brain tissues [25]. According to these findings, a continuous intravenous infusion seems to be the most appropriate mode of drug delivery for achieving and maintaining appropriate GPE blood levels to get neuroprotection after HI injury. Furthermore, data reported that a delayed 4 h infusion (12 mg/kg) of GPE at either 3 or 7 h after HI injury showed a similar degree of neuroprotection compared with earlier administration at 1 h after injury [21], suggesting that GPE owns a wide window of treatment to 7-11 h after HI injury. Treatment was not effective when the 4 h infusion was initiated at 24 h after HI injury. Thus, GPE could be proposed as a useful pharmacological tool for the treatment of acute, rather than chronic, neurological conditions avoiding the side effects due to the drug accumulation [21].

Although the neuroprotective effects of GPE after HI injury have been extensively investigated, its mechanism of action has not been clearly defined yet. Guan *et al.* [21] suggested that GPE neuroprotective effect could be associated with the inhibition of both caspase-3-dependent and -independent neuronal apoptosis. In this context, their studies demonstrated that GPE treatment 1-5 h after HI injury significantly reduced the tissue damage, as well as TUNEL and caspase 3-positive cells, suggesting the inhibition of both neuronal necrosis and apoptosis. In addition, treatment with GPE promotes astrocyte survival, strongly suppresses microglia proliferation following HI injury, and improves long-term somatofunction [21, 22, 27, 28]. GPE involvement in all these events probably emphasizes its mechanism of neuroprotection after HI injury.

Further studies were performed in order to explore the role of GPE in chronic neurodegenerative diseases. GPE can act as a neuronal rescue agent in Parkinson's animal models preventing the loss of TH positive neurons and TH immunoreactive processes after 6-hydroxydopamine (6-OHDA) induced degeneration of nigral neurons in adult rats. In particular, i.c.v. administration of GPE (3 μg) 2 h after 6-OHDA lesion reduced the number of TH immunopositive neurons in the ipsilateral CNS suggesting that the degree of protection is related to the severity of the lesion [29]. Moreover, other studies confirmed that the peripheral administration of GPE after onset of nigrostriatal dopamine depletion improves the long-term recovery of parkinsonian motor deficits, independent of neuronal outcome [30]. Although the neuroprotective mechanism of GPE after 6-OHDA lesion is not yet clear, this molecule could be a suitable candidate for the treatment of Parkinson's disease.

Few data are available on the role of GPE in Alzheimer's disease (AD). It is known that concentrations of somatostatin (SRIF), an important neurotransmitter implicated in learning and memory, are decreased in the temporal cortex of patients with AD [31]. In fact, i.c.v. beta amyloid fragment 25-35 (Aβ25-35) administration for 14 days (300 pmol/day) to ovariectomized rats produced a marked reduction in SRIF content, SRIF receptor density and inhibitory effect of SRIF on adenylyl cyclase activity. An *in vivo* study carried out by Aguado-Llera *et al*. indicated that i.p. administration of three doses (300 μg) of GPE on days 0, 6 and 12 resulted in a partial recovery of the parameters affected by Aβ25-35 administration [32]. The increase of SRIF receptor density after GPE treatment in Aβ-treated rats could be explained by the ability of this tripeptide to stimulate dopaminergic neurotrasmission [13], as dopamine stimulation leads to a rise in the number of SRIF receptors [33]. In these pathological conditions, the neuroprotective effect of GPE may be also related with modulation of intracellular calcium signaling and blockage of apoptotic process [34]. The activation of the mentioned pathways indicates that GPE may provide a new approach for the treatment of some symptoms of AD even though the exact GPE mechanism in AD should undergo further investigation.

Finally, some researchers also found that GPE is a neuronal rescue factor for striatal neurons in excitotoxin animal model of Huntington's disease [35]. In particular, GPE shows neuroprotection in models lesioned with quinolinic acid rescuing all the three major striatal neuronal phenotypes (projections neurons, cholinergic interneurons, and NADPH interneurons) which degenerate in Huntington's disease [35].

To date, the molecule of GPE that was intensively studied by Neuren Pharmaceuticals is undergoing phase III trials for the treatment of neurological injury resulting from cardiac surgery. Current findings report that a significant proportion of patients who undergo coronary artery bypass grafting (CABG) develops some degree of decline in cognitive functions during the early postoperative period [36, 37]. Continuous intravenous infusion of GPE for a period of 4 h following cardiac surgery significantly reduces brain damage. This is an important advantage in preventing the damage resulting from cardiac surgery [38]. There are

currently no drugs approved to reduce cognitive impairment following cardiac surgery, thus GPE could be a suitable candidate for the treatment of these pathological conditions.

STRUCTURE-ACTIVITY RELATIONSHIPS

Tripeptide motifs represent potentially important starting points for design of small molecule biological modulators [1]. In this context, we have extensively studied biologically relevant endogenous tripeptides as glutathione (GSH), thyrotropin-releasing hormone (TRH) or chemotactic tripeptides in order to obtain more stable and potent analogues compared to the parent molecule [39-44]. The molecule of GPE, as well as GSH or TRH, contains three aminoacids and represents an optimal lead compound for chemical modifications in order to obtain analogues with improved stability and activity.

In fact, the molecule of GPE undoubtedly appears to be an attractive and promising pharmacological tool for the control of neurological disorders, but due to a poor stability and a low bioavailability, its delivery to the CNS is limited. HPLC studies indicate that the proteolysis of GPE initially involves the removal of the C-terminal glutamate, followed by the rapid proteolysis of the remaining dipeptide, Gly-Pro, into its constituent amino acids. Exopeptidases, such as carboxypeptidases, peptidyldipeptidases, dipeptidases, and aminopeptidases are responsible for degradation of the tripeptide in rat plasma [25]. The facility of enzymatic hydrolysis of GPE and the difficulty in crossing cell membranes limit its therapeutic potential. According to this, continuous administration of high doses could be necessary to reach a therapeutic value [21, 26].

In the current review, we will focus on different experimental approaches to transform GPE into a potential drug with improved bioavailability retaining neuroprotective activity. Our reading of literature has indicated that the structural simplicity of GPE lends itself to several chemical modifications, as well as general approaches for structural modification of peptides (e. g. aminoacid replacement, introduction of amide bond surrogates, cyclization) (Fig. **2**). We will pay particular attention to the replacement and/or modification of every aminoacid of the GPE sequence. At the present, few data are available about a clear structure-activity relationship and biological consequences. Nevertheless, structure-activity relationships should be proposed based on biological results reported in literature.

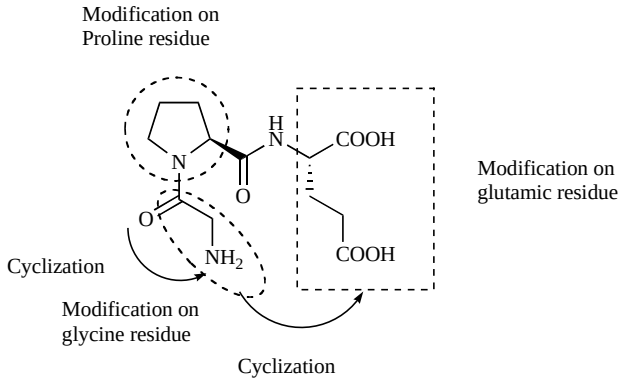


Fig. (2). Structural modification performed on GPE molecule.

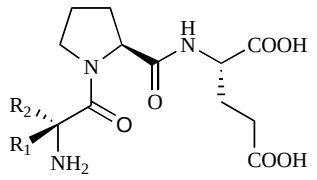
MODIFICATION AT THE GLYCINE MOIETY

In order to elucidate the effect of a single aminoacid changes on neuroprotective activity, a variety of GPE analogues in which the Gly residue is modified or replaced by other aminoacid has been synthesized (Fig. **3**). The approach of introducing one or two methyl group, having D- or L- stereochemistry in place of the α-hydrogens of glycine, was first considered. The introduction of one or two methyl groups confers to compounds **1-3** increased lipophilicity and stability to specific Gly-Pro linkage proteases [45]. Replacement of Gly moiety with other aminoacids, as Phe, Asp, Lys, Nle, and Ile, afforded another series of GPE derivatives **4-8** [46, 47]. The introduction of saturated rings, such as cyclopentyl and cyclohexyl, was considered in compounds **9-10** to improve GPE stability. Alkylation of the amino-terminal glycine residue with a methyl group or saturated rings (pyrrolidine and piperidine) afforded compounds **11-14** characterised by enhanced transmembrane permeability [45].

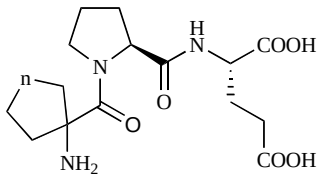
In order to understand if the neuroactivity of GPE analogues was correlated to glutamate receptor binding affinity compounds **1-8** have been tested. Binding studies to glutamate receptors demonstrated that compounds **1-2** and **4** are able to displace L-[^{3}H]glutamate from rat synaptic membranes with one order of magnitude higher than GPE. No displacement of L-[^{3}H]glutamate was observed for derivate **8**, while compounds **5-7** show the same ability of endogenous GPE [46]. Affinity binding studies for compounds **9-14** were not performed. All GPE analogues **1-14** were further investigated to evaluate their neuroprotective effects using striatal cells injured by okadaic acid. Compound **2**, containing L-Ala residue in place of glycine, showed the highest recovery value of 26.4% at concentration of 10 μM respect to GPE that showed neuroprotective activity (recovery value of 20.1%) at higher concentrations (1 mM). N,N-dimethyl-Glu-Pro-Glu-OH **12** showed neuroprotective activity at 1 mM with a recovery value of 30-35% comparable to the parent peptide GPE (recovery value from 25% to 40%) at the same concentration. Lower neuroprotective values (<20%) were obtained with 1 mM of the analogues **13** and **1**. None of the other analogues exhibited neuroprotective activity [45]. These data seem to indicate a slight correlation of ability to displace L-[^{3}H]glutamate from rat synaptic membranes and neuroactivity only for compound **2** suggesting that only the introduction of small groups (e.g. methyl) on glycine portion does not significantly alter selectivity to glutamate receptors and activity. The insertion of acidic, basic, or high aliphatic aminoacid seems to be crucial for the prevention of neuronal cell protection.

MODIFICATION AT PROLINE MOIETY

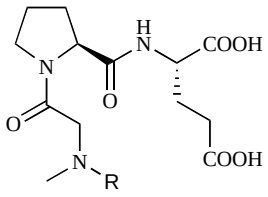
Being the only cyclic proteinogenic amino acid, proline plays a particular role in determining the structural and conformational properties of peptides and proteins [48]. Unlike other peptide groups, which predominantly adopt the *trans* form, the amide bond between Xaa-Pro readily exists in the *cis* as well as the *trans* form, even in absence of structural constraints [49]. Significantly, *cis-trans* isomerisation of this peptide bond is essential for regulating



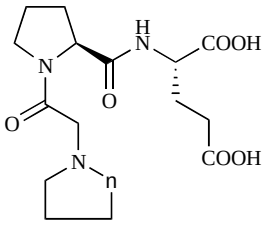
1 R_1 = H, R_2 = CH_3 (D-Ala)
2 R_1 = CH_3, R_2 = H (L-Ala)
3 R_1 = R_2 = CH_3
4 R_1 = CH_2Ph (Phe), R_2 = H
5 R_1 = CH_2COOH, R_2 = H (Asp)
6 R_1 = $(CH_2)_4NH_2$, R_2 = H (Lys)
7 R_1 = $(CH_2)_3CH_3$, R_2 = H (Nle)
8 R_1 = $CH(CH_3)CH_2CH_3$, R_2 = H (Ile)



9 n = 1
10 n = 2



11 R = H
12 R = CH_3



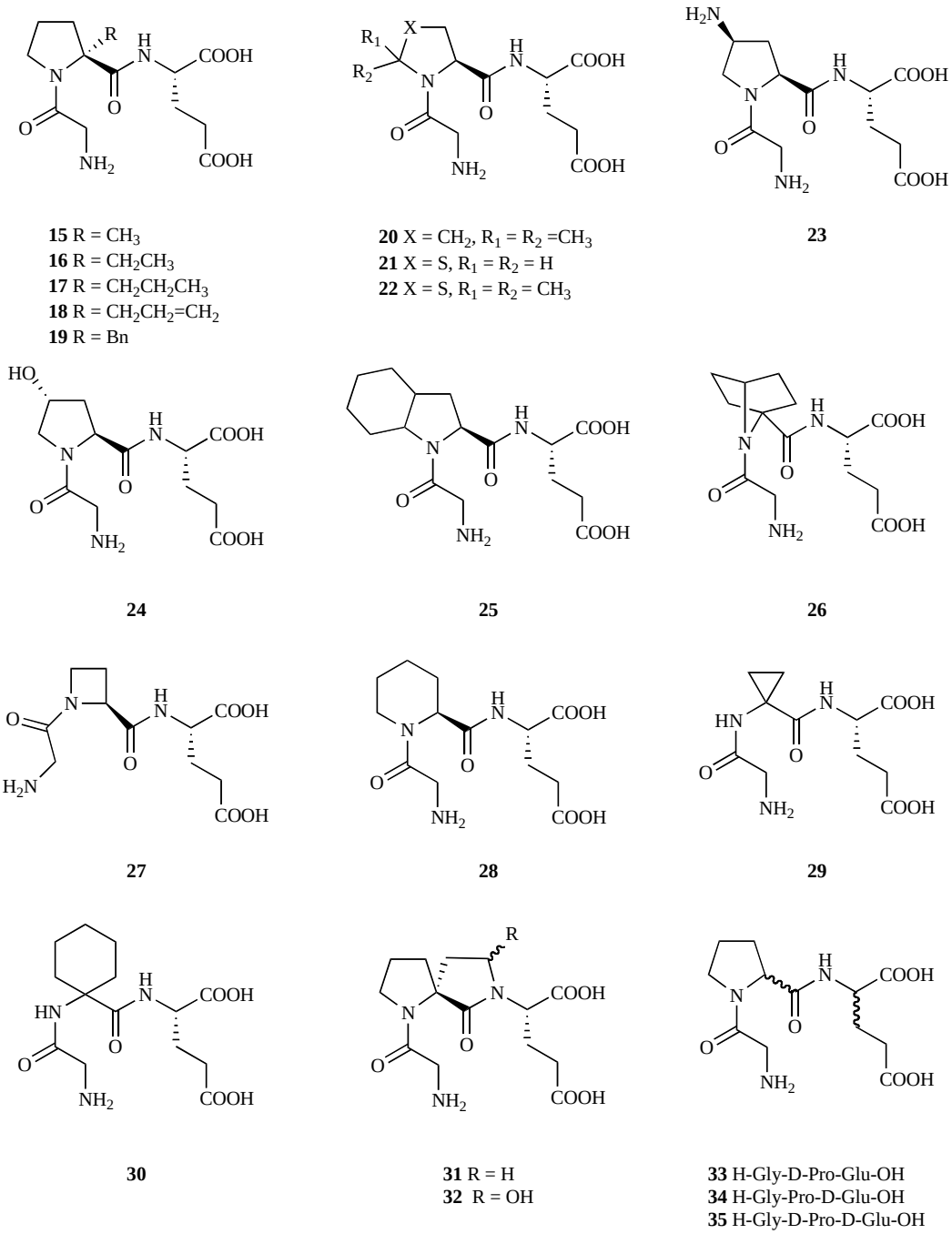
13 R= H, n = 1
14 R = H, n = 2

Fig. (3). Chemical structures of GPE analogues modified at glycine moiety.

many important biological processes, including protein folding, recognition, and signal transduction [50-52]. Structural analogues of proline can be used to examine the folding pathways and bioactive conformation of prolyl-containing therapeutic targets [53, 54]. In this context, several mimetics of proline were directly inserted into GPE primary sequence in place of the native proline residue (Fig. **4**). Structurally, the most commonly used proline-mimetics are derivatives of proline itself with an alkyl group in C^{α}-position. In order to investigate the influence of proline on stability and potency, the proline residue in the native GPE was replaced by α-methyl proline (**15**), α-ethyl proline (**16**), α-propyl proline (**17**), α-allyl proline (**18**) and α-benzyl proline (**19**) [55]. The insertion of 2-alkyl proline in GPE analogues **15-19** promoted the *trans* conformation and ensured higher stability to protease. Surprisingly, the replacement of proline with α-methyl proline afforded the best GPE analogue glycyl-L-2-methylprolyl-L-glutamic acid (NNZ-2566, **15**) with increased half-life in blood and brain, while maintaining neuroprotective efficacy [56]. In fact, NNZ-2566 displayed a half-life of 49 min and 74 min in rat blood and brain, respectively, while the half-life of the native GPE is less than 2 min [26, 38]. NNZ-2566 has a pharmacokinetic profile suitable for both intravenous infusion and microemulsion for oral delivery [38]. Furthermore, neuroprotective studies in numerous *in vitro* and *in vivo* models of brain injury confirmed the efficacy of NNZ-2566 in decreasing both inflammation and apoptosis, reducing the consequences of brain injury when administered several hours after the beginning of the insult. In this context, Wei and co-workers [57] observed that NNZ-2566 effectively suppresses the expression of inflammatory cytokine and inhibits both acute and delayed neuroinflammation following ballistic-like brain injury (PBBI) in rats. In addition, NNZ-2566 was demonstrated to be effective in reducing the incidence and severity of non-convulsive brain seizures and in restoring motor coordination in animals with traumatic brain injury [58]. Finally, strong neuroprotection after stroke was observed in rats when NNZ-2566 was administered in combination with caffeinol [59] suggesting that this pharmacologic approach could be potentially effective in these pathological conditions. To date, NNZ-2566 is undergoing phase II trials and Neuren Pharmaceuticals has initiated development of its oral form for the treatment of cognitive deficits following traumatic brain injury (TBI).

In order to investigate the relationship between *cis/trans* isomerism and bioactivity, the native residue of proline of GPE was also substituted by 5-alkyl-proline and pseudoprolines (ΨPro), as proline mimetics of tailored structural and functional properties [60, 61]. In this context, incorporation of 5,5-dimethyl-L-proline (dmP) into GPE sequences constraints the Xaa-Pro amide bond into a *cisoid*-arrangement and can be used to permanently lock a bioactive *cis*-prolyl conformation and potentially enhance activity. In fact, analogue **20** containing the residue dmP adopts *cis* conformation (*cis:trans*, 72:28) at the Gly-Pro bond as confirmed by NMR analysis (Fig. **4**) [55].

Another structural motif introduced into the linear sequence of GPE is represented by synthetic mimetics of thiazolidine, characterized by a 5-membered saturated ring



15 R = CH_3
16 R = CH_2CH_3
17 R = $CH_2CH_2CH_3$
18 R = $CH_2CH_2{=}CH_2$
19 R = Bn

20 X = CH_2, $R_1 = R_2 = CH_3$
21 X = S, $R_1 = R_2$ = H
22 X = S, $R_1 = R_2 = CH_3$

23

24

25

26

27

28

29

30

31 R = H
32 R = OH

33 H-Gly-D-Pro-Glu-OH
34 H-Gly-Pro-D-Glu-OH
35 H-Gly-D-Pro-D-Glu-OH

Fig. (4). Chemical structures of GPE analogues modified at proline moiety.

with a sulphur atom in place of γCH_2 position of proline. Analogue **21**, obtained by replacing the native proline residue with unsubstituted thiazolidine ring, preserves the isomerism *cis:trans* in the same ratio observed for the GPE (*cis:trans*, 20:80) [62], while the introduction of disubstituted thiazolidine strongly favours a *cis*-configured amide bond as observed in the analogue **22** (*cis-trans*, 85:15) (Fig. **4**) [55]. In addition to influencing the conformation of the amidic Xaa-Pro bond, disubstitution also reduces the chemical stability of the thiazolidine core, which will readily revert to parent acyclic cysteine under mild experimental conditions [60]. To elucidate the pharmacological profile of these closely related GPE analogues, binding assays and neuroprotection studies were performed. Binding affinity studies for glutamate receptors showed that analogues **15** and **20** could displace L-[^{3}H]glutamate from rat brain synaptic membranes with K_i values of 7.96±1.83 μM and 3.79±0.53 μM, respectively, while compounds **21-22** showed no ability even at concentrations of 100 μM. As noted by these data, the *cis-trans* isomerism at the Gly-Pro bond is not correlate with glutamate receptor binding affinity because both compounds **15** and **20**, respectively with *trans* and *cis* conformation, displayed good affinity for the glutamate receptors. The presence of a sulfur atom in the proline ring is

probably responsible for the lack of affinity for glutamate receptor in both thiazolidine derivatives **21-22** [63]. Compared with derivatives **21-22**, neuroprotective studies performed on analogues **15** and **20** showed that both of them can significantly prevent, at concentration of 10 and 100 μM, the death of hippocampal neurones caused by NMDA (100 μM) excitotoxicity. However, compounds **21-22**, with no affinity for glutamate receptors, increase the neuronal survival after oxygen-glucose deprivation compared with analogues **15** and **20** [63, 64]. In particular, GPE analogue **22** produced 100% protection against the loss of neuronal cell viability caused by okadaic acid in a concentration-dependent manner from 10 nM to 10 μM [65]. Based on these results, the introduction of pseudoproline in compound **22** significantly alters the binding affinity to NMDA receptor; on the other hand, it maintains the neuroprotective activity, suggesting that this structural analogue of GPE should be further investigated for its mode of action.

Other researchers investigated the direct insertion of several proline-homologues on GPE molecule and their effect upon the structural and biological profiles of the resulting **23-30** derivatives (Fig. **4**) [46]. Following this key substitution, *cis*-aminoproline and *trans*-hydroxyproline were incorporated at position 2 of GPE (compounds **23** and **24**, respectively) with slight influence on the binding affinity for glutamate receptors, while the insertion of octahydroindole-2-carboxylic acid (Oic) does not determine substantial changes (analogue **25**). GPE analogues **27**, **29** and **30,** containing azetidine-2-carboxylic acid (Aze), 1-amino-1-cyclopropane carboxylic acid (Acp), 1-amino-1-cyclohexane carboxylic acid (Ach) residues in position 2 of GPE, respectively, showed no significant improvement binding affinity to the glutamate receptor. The most active compounds **26** and **28**, containing the conformationally restricted proline residue b_7Pro and pipecolic residue, showed a 60-fold and 13-fold increase in binding affinity to the glutamate receptor, respectively. Neuroprotection studies, performed on rat hippocampal neurons insulted by NMDA, highlighted that the compounds with greater affinity for the receptor (analogues **26** and **28**) are not the most active, while compounds **27**, **29-30** maintain the neuroprotective activity at 100 μM, with recovery values ranging from 27% to 34%. Also in this series of compounds there is no correlation between glutamate receptor affinity and neuroprotective activity [46].

A third series of GPE analogues was obtained by introducing the spirolactam system (Fig. **4**). The conformationally restricted analogues **31-32**, obtained through a bridge between the α-carbon of proline ring and the nitrogen of glutamate moiety, adopted preferentially the *trans* conformation [55, 64]. Binding affinity and neuroprotective studies performed on compounds **31-32** underlined that the conformational restriction is negative for bioactivity [64].

Systematic replacement of the amino acids in GPE sequence with a D-amino acid was investigated to explore the individual contributions of amino acids to biological activity and glutamate receptor binding affinity. The replacement of L-proline and L-glutamic acid with D-proline and D-glutamic acid, respectively, was performed on compounds **33-35** (Fig. **4**). Although these compounds have no affinity for glutamate receptors, they show better neuroprotection percentage than GPE in both NMDA exicitotoxicity and oxygen-glucose deprivation assays at both concentration of 10 and 100 μM. These data probably suggest that the correct conformation is detrimental for affinity to NMDA receptor but not for bioactivity [63, 64].

In summary, the proline is a crucial amino acid for the biological activity of GPE. The structural properties of proline and its derivatives result in characteristic and unique constraints on the conformational space of GPE sequences containing restricted proline-analogues, proline-homologues or D-analogues. All studies performed on these analogues confirmed that there is no correlation between affinity for glutamate receptors and neuroactivity.

MODIFICATION AT GLUTAMIC ACID MOIETY

The mechanism of action of GPE is not yet clear, but it is certain that the presence of amino-terminal glycine and carboxy-teminal glutamate of GPE may activate NMDA receptor [4]. In order to examine the conformational requirements of L-Glu for stimulating glutamate receptor subtypes and to obtain more stable GPE analogues, two series of compounds, modified at α- and γ-carbon of glutamic acid, respectively have been recently developed (Fig. **5**) [62, 66].

The first series of analogues **36-38** was obtained through successive replacement of α-carboxylic group with hydrogen, a methyl group or two methyl groups, respectively [62]. These compounds, devoid of their amino acid character, demonstrate increased proteolytic resistance and lipophilicity that facilitate their transport through membranes. Similar results were obtained with the introduction of amide group in place of the α-carboxylic one (compounds **39-41**). GPE analogue **42** was obtained by the insertion of α-methyl glutamic acid, which confers higher metabolic stability. All the chemical modifications performed on analogues **36-42** do not significantly alter the *cis-trans* isomerism observed in GPE and may confer resistance to the action of peptidase. Only the analogue **41** at 10 μM retains neuroprotective activity with recovery value of 58% in survival assay using striatal cells injured by okadaic acid respect to GPE (at concentration of 1 mM, recovery value of 30.5%). Probably, the presence of tertiary amide in α-carboxylic acid allows to preserve the neuroprotection. However, none of the other compounds showed neuroprotective activity except analogue **37**, with low recovery values (20%) against striatal cell survival post apoptosis-induced injury at concentration of 1 mM [62].

The second series of GPE analogues was obtained by changing the side chain of glutamic acid in γ-position (Fig. **5**) [66]. Removal of the γ-carboxylic group of GPE confers to analogue **43** improved metabolic stability and decreased polarity, thus facilitating penetration through membranes. Amide groups and alcohol function were also introduced in GPE analogues **44-46** and **47**, respectively, in order to reduce the overall charge in the parent molecule. Analogue **48** is characterized by a complete removal of glutamic acid side chain, while analogue **49** shows both carboxylic

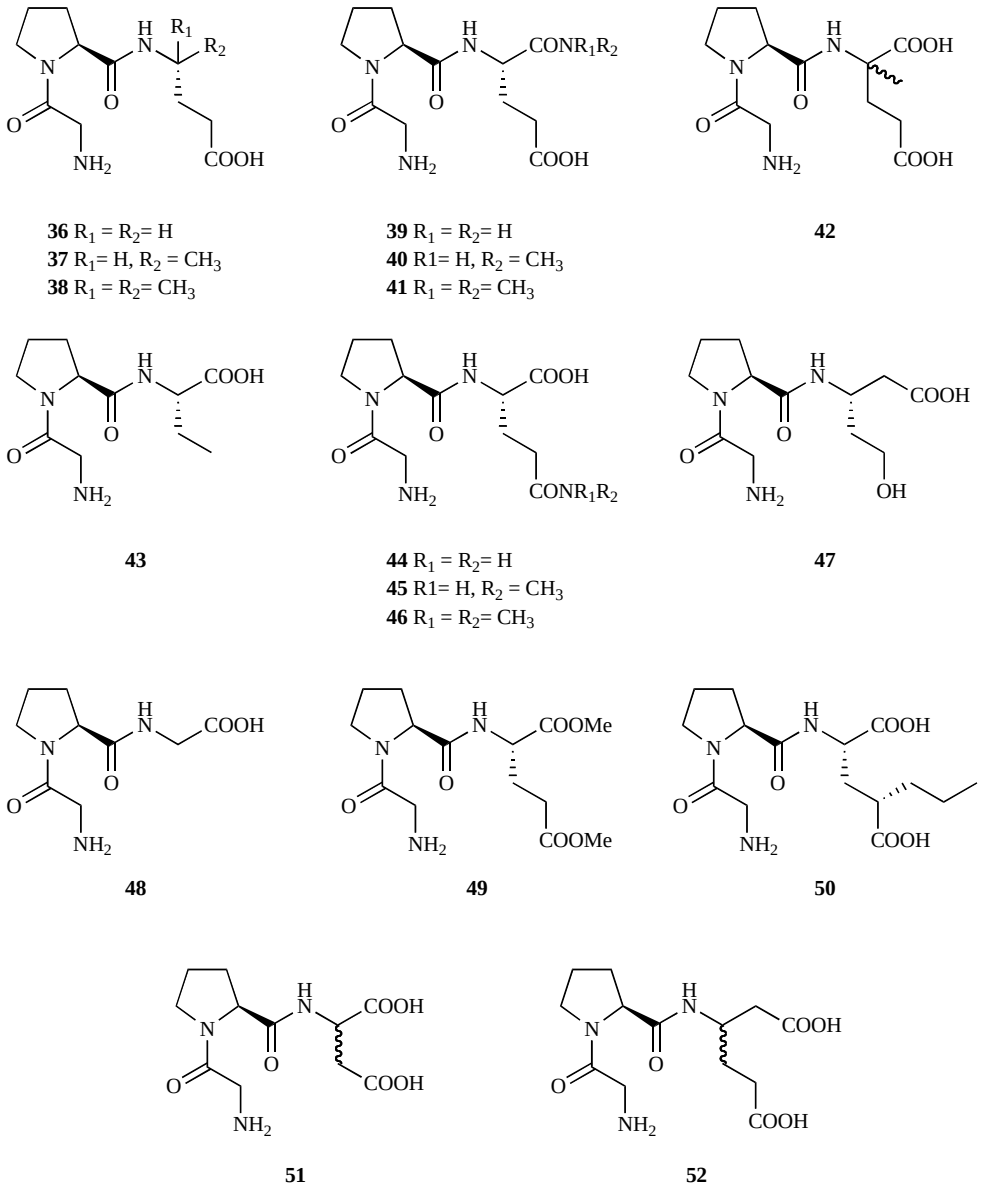


Fig. (5). Chemical structures of GPE analogues modified at glutamate moiety.

functions esterified by methyl ester, resulting in a more lipophilic peptide that may slowly release GPE *in vivo* following esterase action. Another undertaken strategy involved the inclusion of a propyl residue in γ-position to improve lipophilicity (analogue **50**). All compounds of this second series (analogues **43-50**) were subjected to neuroprotection studies using striatal cells injured by okadaic acid [66]. As a notable result, only GPE analogue **48** showed recovery value (20%) at 1 mM concentration comparable to GPE, whereas compounds **44**, **47** and **49** displayed lower recovery values (from 10% to 15%). All the other compounds were devoid of neuroprotective activity. Although none of the compounds **43-50** was subjected to binding affinity studies for glutamate receptors, the results demonstrate that compound **48**, avoid of γ side chain, is the most active suggesting that the presence of side chain is not essential for the overall neurobioactivity [59].

In a third series of analogues, the native residue of glutamic acid was replaced by L- or D-aspartic acid and L or D-homoglutamic acid obtaining compounds **51** and **52**, respectively (Fig. **5**) [63]. These substitutions were performed in the attempt to identify the influence of side chain length on bioactivity. In order to further elucidate the pharmacological profile of these closely related GPE analogues the affinity for the NMDA receptor of analogues **51** and **52** was investigated. Both compounds at 100 μM concentration have not affinity for glutamate receptors suggesting that the correct orientation and length of side chain is crucial for the interaction with the receptor.

MACROCYCLIC ANALOGUES OF GPE

The determination of the optimal biologically active conformation of a peptide is critical in the design of new highly potent pharmaceuticals and is usually hindered by the flexibility of the backbone and the side chains of peptides.

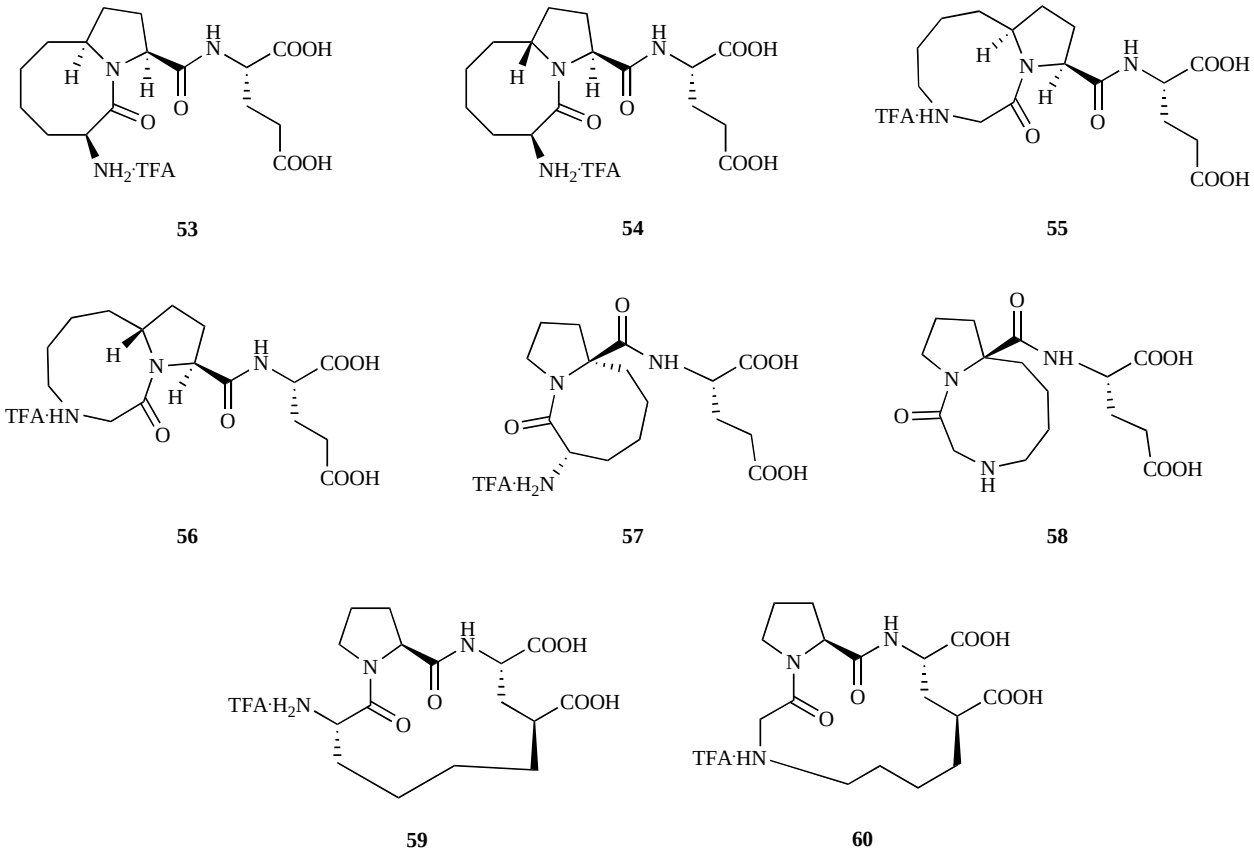


Fig. (6). Chemical structures of macrocyclic GPE analogues.

Anywhere the number of conformational possibilities in peptides can be reduced by introducing conformational constraints to stabilize the backbone conformation and to control side chain orientation [67]. To exploit conformationally rigid GPE analogues and obtain improved stability and activity, Harris *et al.* [68, 69] synthesized macrocyclic derivatives of GPE, in which the residue of proline is forced to assume a precise conformation (Fig. **6**). Macrocyclic GPE analogues **53-54** were obtained through the cyclization of α-carbon of glycine and δ-carbon of proline whereas analogues **55-56** were synthesized by cyclization between the NH terminal of glycine and δ-carbon of proline. All these compounds **53-56** preferentially adopt the *trans* conformation as well as GPE. Macrocyclic analogues **57** and **58**, obtained *via* cyclization between α-carbon of proline and α-carbon of glycine or NH terminal of glycine, respectively, preferentially assume the *cis* conformation. The last two conformationally restricted analogues **59** and **60** were prepared linking the γ-carbon of glutamic acid with α-carbon or NH- terminal of glycine, respectively [68]. In particular, cyclotetradecene **59** mainly exists as *cis* rotamer because of the increased flexibility of the Pro amide bond, when embedded in a larger 14-membered ring. The 13-membered macrocycle **60**, synthesized by Harris *et al.* [68], was obtained as a single *trans* rotamer probably due to the formation of a γ-turn and/or formation of a smaller cycle size.

In order to study neuronal proliferation, neurite growth, and formation of nerve bundles, a series of studies was carried out using cerebellar explants from adult rats treated with glutamate (1 mM) [70]. At sufficiently high concentrations, glutamate is neurotoxic resulting in neuronal cell death. In fact, the glutamate treatment (1 mM) causes in about an 80% loss of cerebellar neurons having neurites compared to vehicle-treated controls. Experiments carried out by Harris *et. al*. [70] showed that the number of cells having neurites significantly increased in a dose-dependent manner following administration of GPE analogues **53** and **55** suggesting that they were able to protect these cells after glutamate-induced neurotoxicity.

Other studies were performed on these macrocyclic GPE analogues in order to investigate their neuroprotective effects after stroke or CABG. Surprisingly, the results showed that the central administration to injured adult rats of a low dose (100 pM) of analogue **55** significantly reduce the tissue damage in each brain region examined compared to the vehicle treated group. Analogue **55** is neuroprotective against neuronal damage caused by HI injury, thus it could be a valid candidate, due to its pharmacological profile, for the treatment of pathologies characterized by neural degeneration or cell death [70].

CONCLUSIONS

The tripeptide glycine-proline-glutamate, the naturally cleaved N-terminal tripeptide of insulin-like growth factor-1, is an important neuromodulator and neuroprotectant agent in the CNS. Although its mode of action is still not clear, *in vitro* studies demonstrated its ability in stimulating

acetylcholine and dopamine release, protecting neurones from diverse induced brain injures and rescuing cell death in numerous animal models of neurodegenerative disorders such as Parkinson's, Alzheimer's and Huntington's diseases. GPE is currently undergoing Phase II clinical trials for reduction of cognitive decline following cardiac surgical procedure. However, its therapeutic potential is limited by its susceptibility to degradation by peptidases. Further researches are needed to design and develop new GPE analogues appropriate for clinical use in terms of stability, metabolism, toxicology, and side effects. In this context, NNZ-2566 represents a promising molecule for development of new more efficient neuroprotective agents. This thesis is further corroborated by the fact that NNZ-2566 is currently being tested in clinical trials for the treatment of cognitive deficits following traumatic brain injury. The better understanding of the mechanism of action associated with structure-activity relationships enable us to rationalize and streamline the process of developing new neuroprotective compounds.

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Received: April 11, 2011 Revised: July 06, 2011 Accepted: August 12, 2011