Letters

Design of Remarkably Simple, Yet Potent Urea-Based Inhibitors of Glutamate Carboxypeptidase II (NAALADase)

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Introduction. The amino acid glutamate is present in high concentrations in the mammalian brain, and it acts as the major excitatory neurotransmitter in the CNS. Through its actions on both ionotropic and metabotropic receptors, glutamate plays an important role in a variety of physiological functions including learning, memory, and developmental plasticity. Excessive activation of glutamate receptors or disturbances in the cellular mechanisms that protect against the adverse consequences of physiological glutamate receptor activation have been implicated in the pathogenesis of a host of neurological disorders. Although several drugs designed to attenuate the pathological consequences of excessive glutamate activation have been shown to reduce injury in experimental models of cerebral ischemia, so far none of these compounds has proven to be effective in the clinical treatment of stroke.[1]

N-Acetyl-L-aspartyl-L-glutamate (NAAG) is a peptide neurotransmitter that is widely distributed in the mammalian nervous system.[2] NAAG is both an agonist at metabotropic glutamate receptors (mGluR3)[3] and a mixed agonist/antagonist at the *N*-methyl-D-aspartate (NMDA) receptor.[4] NAAG is hydrolyzed by the neuropeptidase glutamate carboxypeptidase II (GCPII; also known as N-acetylated α-linked acidic dipeptidase, NAALADase, or NAAG peptidase) to liberate *N*-acetylaspartate and glutamate both in vitro and in vivo.[5] The role of this metalloprotease GCPII is thus thought to be twofold: (1) to terminate the neurotransmitter activity of NAAG and (2) to liberate glutamate which is then able to act at the various glutamate receptor subtypes. Alterations in the levels of GCPII and NAAG have been observed in disorders that are linked to abnormalities in glutamatergic neurotransmission.[6]

As a consequence of these findings, it has been hypothesized that the inhibition of GCPII might provide an effective strategy for achieving neuroprotection in cases of cerebral ischemia by increasing the levels of NAAG while decreasing the levels of glutamate. In fact, recent work by Slusher et al. led to the demonstration that the GCPII inhibitor 2-PMPA provides significant protection against injury in rats after transient middle cerebral artery occlusion (MCAO).[7] Furthermore, in the rat MCAO model, 2-PMPA decreased glutamate levels while increasing NAAG levels, as would be predicted for a compound working as a GCPII inhibitor. As a therapeutic target, GCPII inhibition has been suggested to have potential benefits over receptor-based strategies, as it represents an upstream mechanism of glutamate regulation that could reduce transmission at a number of glutamatergic receptors rather than inhibiting a single receptor subtype.[8] Equally important, NAAG is colocalized in neurons with small amine transmitters including GABA and dopamine, and it has been shown to act on presynaptic receptors to regulate transmitter release.[9]

In our previous work,[10] starting from NAAG, we designed a dually acting ligand, 4,4′-phosphinicobis-(butane-1,3-dicarboxylic acid) (**1**), which acts both as an mGluR3-selective agonist (∼30 μM) and as a potent inhibitor of GCPII (21.7 ± 2.1 nM). From this novel lead compound, we now chose to investigate the activity of structures comprising two amino acids joined through their NH_2 groups by a urea linkage (Figure 1). We envisaged that the urea group would serve as a suitable replacement for the central $CH_2P(O)(OH)CH_2$ present in the lead structure. The impetus to pursue this chemistry was driven largely by the ease of synthesizing such structures, thereby facilitating further SAR analysis.

Results and Discussion. The compounds that have been prepared are shown in Table 1. For comparison purposes, we also provide published data[6a] for some related dipeptide structures. First, we explored the activity of Glu-C(O)-Glu, where the Glu's are of the *S*-configuration. In general, these symmetrical ureas were prepared (Scheme 1) by reacting the appropriate amino acid benzyl ester with triphosgene/Et_3N at −78

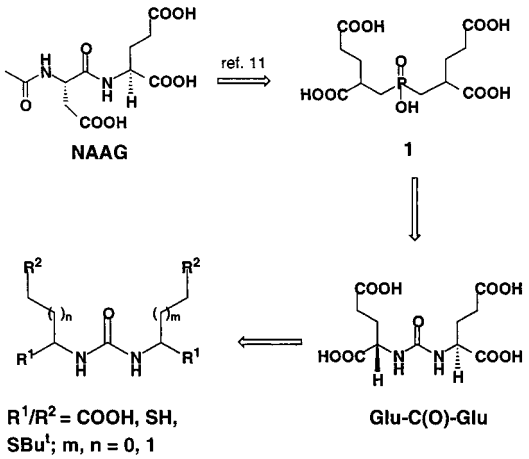

Figure 1. Rational design of urea-based GCPII inhibitors.

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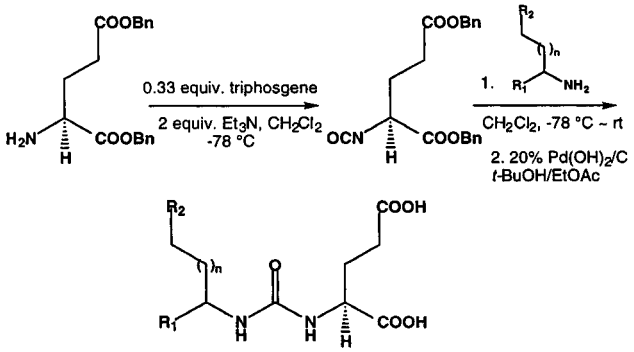
Table 1. Inhibitory Activity of Dipeptides and Ureas Against Expressed Rat GCPII[a]

compound	IC_{50}
PMPA	5.1 ± 0.6 nM
$[HO_2C(CH_2)_2CH(CO_2H)CH_2]_2P(O)(OH)$	21.7 ± 2.1 nM (ref 10)
(*S*)-Glu-(*S*)-Glu	0.75 μM (ref 6a)
(*S*)-Glu-C(O)-(*S*)-Glu	47 ± 4.5 nM
(*R*)-Glu-C(O)-(*R*)-Glu	67% inhib at 100 μM
(*R*)-Glu-C(O)-(*S*)-Glu	25% inhib at 1 μM
(*S*)-Glu-C(O)C(O)-(*S*)-Glu	9% inhib at 1 μM
(*S*)-Asp-(*S*)-Asp	42% inhib at 100 μM (ref 6a)
(*S*)-Asp-C(O)-(*S*)-Asp	3.8 μM
(*S*)-Asp-(*S*)-Glu	2.4 μM (ref 6a)
(*S*)-Asp-C(O)-(*S*)-Glu	46.1 ± 1.4 nM
t-BuNHC(O)-(*S*)-Glu	10% inhib at 1 μM
Gly-C(O)-(*S*)-Glu	46% inhib at 1 μM
(*R*)-Cys-C(O)-(*R*)-Cys	inactive at 1 μM
(*t*-Bu)Cys-C(O)-(*S*)-Glu	29 ± 6 nM
(*R*)-Cys-C(O)-(*S*)-Glu	6.9 ± 0.4 nM

[a] IC_{50} data were obtained using a substrate concentration of 5 μM.

Scheme 1. Synthesis of Unsymmetrical Ureas: AA-C(O)-AA′



°C, followed by warming to room temperature. After purification by column chromatography or recrystallization, the intermediate tetraester was transformed to its free acid by hydrogenolysis. All new compounds were assayed for their ability to inhibit rat GCPII stably expressed in Chinese hamster ovary (CHO) cells using conditions identical to those reported previously. The readily synthesized compound (*S*)-Glu-C(O)-(*S*)-Glu was quite active, with an IC_{50} value of 47 nM against expressed rat GCPII. Thus, this ligand is only 2-fold less potent than our lead phosphinate. Glu-C(O)-Glu made from (*R*)-Glu gave only 67% inhibition when tested at 100 μM, while (*R*)-Glu-CO-(*S*)-Glu gave 25% inhibition at 1 μM, thus demonstrating the specificity of the enzyme for *S*-configured amino acids. The corresponding dipeptide (*S*)-Glu-(*S*)-Glu has been reported to have some inhibitory activity toward GCPII, but it is 16-fold less potent with an IC_{50} of 0.75 μM.[6a,11] Interestingly, when we examined (*S*)-Asp-C(O)-(*S*)-Asp, this compound was found to be relatively inactive, with an IC_{50} of 3.8 μM. The corrresponding dipeptide (*S*)-Asp-(*S*)-Asp has been reported to inhibit only 42% of enzyme activity at 100 μM.[6a] Next, we examined the activity of (*S*)-Asp-C(O)-(*S*)-Glu and found this compound to be comparable in activity (IC_{50} = 46 nM) to (*S*)-Glu-C(O)-(*S*)-Glu. Thus, the presence of a single fragment having a three-carbon spacer between two of the carboxyl groups appears to be essential for high inhibitory potency. The possibility to replace the urea linker by a larger spacer group, namely an oxalamide, was explored. This particular analogue, (*S*)-Glu-C(O)C(O)-(*S*)-Glu, proved to be inactive. Last, we examined the ability to replace one of the Glu fragments by other amino acids, or even a simple amine. The preparation of these unsymmetrical ureas was brought about by first treating the tosylate salt of dibenzyl glutamate with triphosgene/Et_3N at −78 °C followed by addition of the second amine component and warming to room temperature. Deprotection was then effected through catalytic hydrogenation as well as the use of TFA/$Hg(OAc)_2$/anisole followed by H_2S in the case of cleavage of the *t*-Bu group from Cys (Scheme 1).

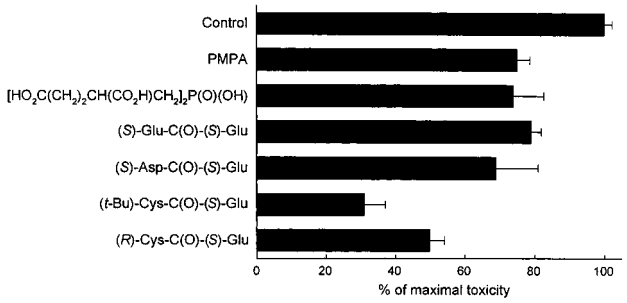


Figure 2. Protective effects of novel ureas against neuronal cell death induced by NMDA in primary cultures of mouse cortical neurons. Cultures of cortical neurons were treated for 10 min with 20 μM NMDA, followed by treatment with culture medium collected from cultures of cortical astrocytes pretreated for 24 h with the indicated compounds at 1 μM concentration. Neuronal viability was assessed 24 h later by measurements of LDH accumulation. Results are expressed as a percent of cell death induced by NMDA alone. Bars represent mean values ± SEM from 16−32 determinations.

As shown in Table 1, the urea derived from *tert*-butylamine + (*S*)-Glu proved inactive, as did Gly-C(O)-(*S*)-Glu. In light of the ability of certain sulfur-containing ligands to act as potent peptidase inhibitors (e.g., captopril for angiotensin converting enzyme) through the ability of the sulfur atom to coordinate with a zinc atom present in the active site, we felt it would be valuable to explore the activity of (*R*)-Cys-C(O)-(*S*)-Glu. Remarkably, this tricarboxylic acid proved to be 6-fold more potent than (*S*)-Glu-C(O)-(*S*)-Glu. Even the *tert*-butylthio-containing precursor molecule (*t*-Bu)Cys-CO-(*S*)-Glu proved to be more active with a K_i of 29 nM. Note, however, that the related urea (*R*)-Cys-C(O)-(*R*)-Cys was inactive when tested at 1 μM. Thus the present SAR reveals that a urea containing at least one glutamate residue plus a second residue bearing a carboxyl group in addition to another group (SR or CO_2H) represents the minimum requirement to achieve effective GCPII inhibition.

For purposes of comparison, we note that PMPA has been reported to be a potent inhibitor of GCPII with a K_i of less than 1 nM[8c] against brain membrane peptidase activity and K_i values up to 4.6 nM when tested against cloned GCPIIs.[8e] PMPA was assayed in parallel with the compounds reported herein and found to have an IC_{50} of 5.1 ± 0.6 nM, reflecting an inhibitory activity very close to that obtained for (*R*)-Cys-C(O)-(*S*)-Glu.

As shown in Figure 2, we have investigated the action of some of these novel ureas for their ability to block NMDA toxicity in vitro. This was done using a published protocol in which cultures of cortical glial cells are first treated with the test compound in order to induce the release of protective factors into the medium.[12] In parallel, cultures of cortical neurons (without glia) are

exposed for 10 min to the excitotoxic action of NMDA (20 μM). The NMDA is removed by washing, and the neurons are treated with the medium collected from the treated glial cells. Toxicity is then assessed 24 h after NMDA treatment by the measurement of lactate dehydrogenase (LDH) activity, which serves as a marker for dying cells. In this test system 1 μM PMPA afforded only about 25% neuroprotection. Consistent with data published on PMPA in a similar NMDA-based neurotoxicity system, 1 μM PMPA afforded about 25% neuroprotection in our experiments.[13]

Among the potent GCPII inhibitors tested herein, three compounds, $[HO_2C(CH_2)_2CH(CO_2H)CH_2]_2P(O)$-(OH), (*S*)-Glu-C(O)-(*S*)Glu, and (*S*)-Asp-C(O)-(*S*)Glu, produced at 1 μM a similarly modest neuroprotection ranging from 21% to 31%. In contrast, the two Cys-containing compounds, (*t*-Bu)Cys-C(O)-(*S*)-Glu and (*R*)-Cys-C(O)-(*S*)-Glu, produced 69% and 50% neuroprotection, respectively. It should be noted that PMPA is substantially more effective in producing neuroprotection in anoxia-based models of neurotoxicity in which the compounds described here have not been tested.[7] Given that PMPA and the compounds described herein each act as peptidase inhibitors, we speculate that their modest to more substantial neuroprotective actions are related to blockade of NAAG hydrolysis. It may be however, that these compounds, including PMPA, have an action in this NMDA-induced toxicity model that is independent of or additive to the inhibition of NAAG hydrolysis. Both the neurons and glia express the peptide and may release it during the medium changes that are associated with this cell culture system. Additionally, the peptidase inhibitors are presented first to the glia and then transferred to the neurons together with whatever factors might be released from the glial cells. Other potential mechanisms include activation of mGluR3 receptors. We have found, for example, that β-NAAG functions as both a peptidase inhibitor and a highly selective mGluR3 antagonist at micromolar concentrations.[14] The precise mechanism of this neuroprotective effect in the widely used NMDA toxicity model therefore remains to be rigorously defined. These data argue in favor of further study of these compounds in other models of neurotoxicity, both in vitro and in vivo, as well as assessment of their potential interactions with metabotropic glutamate receptors.

In conclusion, the present investigation reveals the ability of some remarkably simple compounds to act as potent inhibitors of GCPII, thus offering a new avenue in the rational design of GCPII inhibitors that may lead to effective neuroprotective agents. It is likely that the appendage of other functionality, particularly hydrophobic groups, may lead to further improvements in potency through interaction with accessory hydrophobic pockets. Moreover, because of the possibility to employ GCPII inhibitors in stroke therapy, it will be essential to explore prodrugs or analogues containing carboxylic acid isosteres so as to facilitate blood−brain barrier penetration.

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Supporting Information Available: Spectral and analytical data for all new compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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