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# Phosphonate and Phosphinate Analogues of *N*-Acylated γ-Glutamylglutamate: Potent Inhibitors of Glutamate Carboxypeptidase II

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**Abstract**—Phosphonate and phosphinate analogues of *N*-acylated γ-glutamylglutamate were tested for the ability to inhibit glutamate carboxypeptidase II (GCP II). All of the compounds inhibit GCP II with $IC_{50}$ values in the low nanomolar range. The comparison of the results to previously reported inhibitory studies of the same compounds toward folylpoly-γ-glutamyl synthetase (FPGS) and γ-glutamyl hydrolase (γ-GH) provides insight into structural and mechanistic features of each enzyme. Potential utility of these compounds as diagnostic agents and probes to understand folate or antifolate poly-γ-glutamates metabolism is also described. © 2002 Elsevier Science Ltd. All rights reserved.

Folates, a family of vitamins, play an important role as carriers of one-carbon units in the biosynthesis of various cellular components, including methionine, thymidylate and purine nucleotides.[1] Folates are present in cells predominantly as poly-γ-glutamate conjugates. The physiological significance of poly-γ-glutamylation in cells is underscored by previous biological studies on poly-γ-glutamate conjugates.[2] Compared to monoglutamates, the folate poly-γ-glutamates are very inefficiently transported across the cell membrane and thus better retained in cells. In addition, the poly-γ-glutamates generally are better intracellular co-substrates for most folate-dependent enzymes than are the monoglutamates. Methotrexate and other classical antifolates also undergo intracellular poly-γ-glutamylation, which is implicated in their enhanced cytotoxicity. On the other hand, inefficient poly-γ-glutamylation is believed to be one of the mechanisms of resistance to these antifolates in cancer chemotherapy.[3]

The poly-γ-glutamylation of folates and antifolates is catalyzed by an ATP-dependent enzyme, folylpoly-γ-glutamyl synthetase (FPGS). Like other ATP-dependent ligases, FPGS-catalyzed poly-γ-glutamylation proceeds through the formation of an acyl phosphate at the γ-carboxylate of a terminal glutamate. The acyl phosphate is subsequently attacked by an incoming glutamate.[4] FPGS is unique in that it adds glutamate residues, one at a time, to produce poly-γ-glutamates. Cellular folate and antifolate poly-γ-glutamates can be hydrolyzed by the cysteine peptidase γ-glutamyl hydrolase (γ-GH).[5] γ-GH exhibits either exopeptidase (removal of a terminal glutamate residue) or endopeptidase (hydrolysis of an internal peptide bond within the poly-γ-gultamate chain) activity depending on the species and tissue of origin. Thus, FPGS and γ-GH are believed to be involved in the regulation of intracellular concentration of folate coenzymes (Fig. 1).

More recently, glutamate carboxypeptidase II (GCP II), also known as *N*-acetylated α-linked acidic dipeptidase (NAALADase) or prostate specific membrane antigen (PSMA), was found to exhibit poly-γ-glutamyl exopeptidase activity toward both folate and methotrexate poly-γ-glutamates.[6] GCP II is a metalloprotease with an apparent molecular weight of 94 to 100 kDa. Through sequence alignment with other metalloproteases, Rawlings and Barrett assigned the catalytic domain of GCP II to peptidase family M28.[7] Peptidases in this family contain two metal ions forming a co-catalytic active site with metal ligands.

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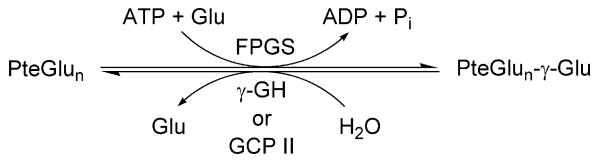


**Figure 1.** Metabolic interconversion of folate poly-γ-glutamates mediated by FPGS, γ-GH, and GCP II.

In prostatic cells, two forms of GCP II have been characterized. The cytosolic form lacks a transmembrane region and thus exists in the cell cytoplasm. The membrane form is a type II membrane protein and its catalytic domain is located in the extracellular region of the enzyme. Human PSMA was originally characterized as the antigen of the 7E11C-5 monoclonal antibody. Since PSMA is expressed in very high concentration in prostate cancer cells, the antibody has been used as a ligand of the imaging agent for the diagnosis of prostate cancer.[8] GCP II also catalyzes the hydrolysis of the neuropeptide *N*-acetylaspartyl glutamate (NAAG) in the nervous system to release glutamate, one of the major excitatory neurotransmitters in the brain.[9] In the nervous system NAAG is believed to be one of the major sources of glutamate and GCP II is believed to play a key role in modulating the release of glutamate. Thus, GCP II has been a target for the treatment of neurological disorders associated with excess glutamate toxicity such as stroke, spinal cord injury, chronic pain, and peripheral neuropathies.[10] For example, 2-(phosphonomethyl)pentanedioic acid,[11] a potent and selective inhibitor of GCP II, was found to protect against ischemic injury in a neuronal culture model of stroke and in rats after transient middle cerebral artery occlusion.[12] Despite the well-defined role of GCP II in the brain, the role of GCP II as a folate poly-γ-glutamate hydrolase is not well understood.

Inhibitors of FPGS, γ-GH and/or GCP II could thus be useful tools for understanding the catalytic mechanism and physiological role of each of the three enzymes. We have previously reported the synthesis and biological evaluation of phosphonate and phosphinate analogues of *N*-acylated γ-glutamylglutamate **1**–**4** (Fig. 2).[13,14] We found that compounds **3** and **4** were potent inhibitors of FPGS-catalyzed poly-γ-glutamylation while **1** and **2** exhibited significantly lower potencies. In contrast, neither **1** nor **3** inhibited γ-GH-catalyzed hydrolysis of poly-γ-glutamate, suggesting that γ-GH catalysis does not involve a direct attack by $H_2O$ but may involve an acyl enzyme intermediate.

Although GCP II catalyzes a hydrolysis reaction similar to that of γ-GH, its catalytic mechanism is believed to be distinct and involve an active site zinc ion. Phosphonate monoesters and phosphinates are postulated to mimic unstable tetrahedral intermediates formed during metalloprotease-catalyzed peptide hydrolysis and have proven to be very effective inhibitors of this class of peptidases.[15] In this paper, we report the exploration of inhibitory activity of the four phosphorous-containing transition-state analogues **1**–**4** toward GCP II. The results were compared to those of FPGS and γ-GH to better understand the structure–activity relationships for each enzyme.

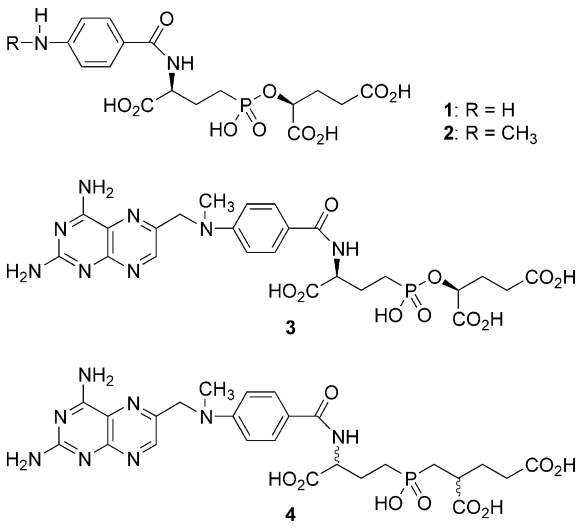


**Figure 2.** Structures of phosphonate and phosphinate analogues of *N*-acylated γ-glutamylglutamate.

The ability of **1**–**4** to inhibit GCP II was evaluated using *N*-acetyl-L-aspartyl-[$^3$H]-L-glutamate as a substrate.[16] Table 1 shows the $IC_{50}$ values for the GCP II assay along with previously reported data on FPGS and γ-GH assays. The compounds **1**–**4** were found to be potent inhibitors of GCP II with $IC_{50}$ values of 3, 4, 4, and 5 nM, respectively.

The high potency of these inhibitors can be attributed to the interaction of the dipeptide portion of the molecule with the catalytic zinc ion(s) as well as both primed and unprimed regions of the active cleft. Indeed, the double-reciprocal plot of velocity (v) versus substrate concentration ([S]) for inhibitor **3** shows a pattern characteristic of competitive inhibition (Fig. 3) and suggests that the compound **3** occupies the substrate-binding site of GCP II. The inhibition constant, $K_i$, of **3** to GCP II was 2 nM.[17]

The fact that all of these compounds exhibited similar potency despite structural difference in their *N*-acyl group suggests that a pteroyl portion of the inhibitors is not involved in the interaction of the molecules with GCP II. This is consistent with the competition assay studies with *N*-acetyl-L-aspartyl-[$^3$H]-L-glutamate by Coyle's group which showed no significant difference in

**Table 1.** Inhibition of GCP II, FPGS, and γ-GH by compounds **1**–**4**

| Compd | GCP II $IC_{50}$, nM | FPGS $IC_{50}$, nM | γ-GH $IC_{50}$, nM |
|---|---|---|---|
| **1** | 3 (±1) | 8600[a] | >200,000[a] |
| **2** | 4 (±2) | 5700[a] | nd[c] |
| **3** | 4 (±1) | 120[a] | >200,000[a] |
| **4** | 5 (±3) | 20[b] | nd[c] |

[a]See ref 13.
[b]See ref 14.
[c]Not determined.

affinity between pteroyl, *p*-aminobenzoyl, and non-substituted poly-γ-glutamates.[18] They also reported that the degree of poly-γ-glutamylation does not affect affinity as long as the molecule possesses two or more glutamyl residues. From these findings, one could postulate that during the course of hydrolysis, only the terminal and penultimate glutamate residues interact with the enzyme and that the rest of the molecule sticks out of the specificity pocket of the enzyme. A similar mode of binding can be applied to our phosphorous-containing transition-state analogue inhibitors. Rawlings and Barrett identified several positively charged residues that are potentially located in the specificity pocket of GCP II.[7] Some of these residues are likely to form electrostatic interactions with the three carboxylate groups of these inhibitors (Fig. 4).

The mode of binding to GCP II proposed for the phosphorous-containing transition state analogue inhibitors suggests that a wide variety of substituents can be incorporated into the N-terminus of phosphonate or phosphinate analogue of γ-glutamylglutamate without causing a significant loss in inhibitory potency. For example, the incorporation of an appropriate analytical marker would provide a GCP II inhibitor-based imaging agent for prostate cancer,[19] which may provide an alternative to conventional monoclonal antibody-based agents.[8]

The comparison of these results to those of FPGS and γ-GH gives us a better insight into the structural and mechanistic features of each enzyme. Unlike GCP II, a pteroyl group is essential for the strong binding of the inhibitor to FPGS as compounds **3** and **4** potently inhibit FPGS. The results can be attributed to the structural differences between these two enzymes. Smith's group recently reported a high-resolution crystal structure of a ternary complex of FPGS formed with β,γ-methylene-ATP and 5,10-methylenetetrahydrofolate.[20] They identified the folate-binding site where the pteridine ring is sandwiched between Phe and Tyr residues. Such a folate-binding site is unlikely to exist in GCP II because all of the four phosphorous-containing inhibitors **1**–**4** show similar inhibitory potency in GCP II assays regardless of the presence or absence of a pteridine ring. The difference in inhibitory profile between FPGS and GCP II allowed us to design dual (both FPGS and GCP II) inhibitors (e.g., **3** and **4**) or selective GCP II inhibitors (e.g., **1** and **2**) by the addition or removal of a pteroyl group from the dipeptide moiety. These inhibitors are potentially useful as probes to understand physiological role of each enzyme.

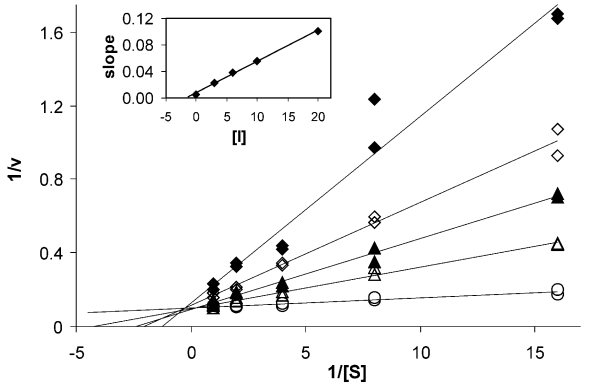


**Figure 3.** Double-reciprocal plot of the hydrolysis of NAAG by GCP II in the presence of compound **3**. The units of substrate concentration are micromolar, while the units of velocity are picomolar per second. Concentrations of **3** were 0 (○), 3 (△), 6 (▲), 10 (◇), and 20 nM (◆). Inset: Replot of the double-reciprocal plot, $K_{mapp}/V_{max}$ versus [I].

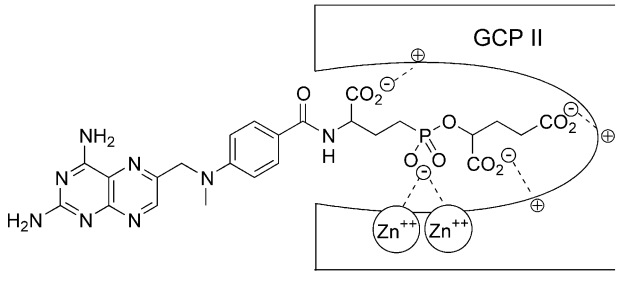


**Figure 4.** Predicted binding mode of inhibitor **3** to the active site of GCP II. Three positively charged residues that are potentially interacting with carboxylates of the inhibitor are shown as '⊕'.

As previously reported, neither **1** nor **3** potently inhibits γ-GH.[13] The lack of inhibitory activity against this cysteine protease[21] is in good agreement with the fact that phosphonates are generally more effective inhibitors of metalloproteases. These two phosphonates may be useful probes for not only understanding the physiological role of GCP II but also for determining whether γ-GH or GCP II is responsible for poly-γ-glutamate hydrolysis in tissues of interest. Compound **1** is particularly attractive for such purposes because it lacks the ability to inhibit FPGS, which catalyzes the reverse reaction.

The critical issue in studying biological significance of folate and antifolate poly-γ-glutamates is to understand the mechanism and physiological role of each of the three enzymes, GCP II, FPGS, and γ-GH. Inhibitors of FPGS, γ-GH and/or GCP II are potentially very useful in addressing this particular issue. The major focus of the work described here was the evaluation of compounds **1**–**4** as GCP II inhibitors and comparison of these results to those of FPGS and γ-GH. The potent inhibition of GCP II by compounds **1**–**4** coupled with previously reported data on FPGS and γ-GH provide insight into the distinguishing mechanistic and structural features of the three enzymes. These compounds or their analogues will be useful tools in further dissecting the metabolism of folate or antifolate poly-γ-glutamates and its physiological importance.

## Acknowledgements

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16. The GCP II assay was carried out as outlined previously[9] with modifications. Radiolabeled NAA[$^3$H]G (30 nM, 15 Ci/mmol) and inhibitors (10 pM to 100 μM) were incubated with purified recombinant GCP II[22] (20 pM) in Tris buffer (pH 7.6, 50 mM) containing $CoCl_2$ (1 mM) in a total volume of 50 μL at 37 °C for 15 min. The reaction was terminated with phosphate buffer (0.1 M, pH 7.4, 50 μL) and the material was applied to a strong anion exchange mini column (AG 1-X8 anion exchange resin, 200–400 mesh; formate form). [$^3$H]Glutamate was eluted with formate (1.0 M) while unreacted NAA[$^3$H]G remained bound to the column. The eluate was transferred to a solid scintillator coated plate and dried. The radioactivity was measured with a top scintillation counter. All the GCP II assays were performed in triplicate.
17. $K_i$ value of compound **3** was determined from the initial velocity of the hydrolysis at NAAG concentrations ranging from 60 nM to 1 μM. An apparent value of $K_m$ ($K_{mapp}$) was obtained from the reciprocal plot (1/v vs 1/[S]) for each set of data. The $K_i$ value was obtained directly from a replot of the slopes of the reciprocal plots ($K_{mapp}/V_{max}$) versus inhibitor concentration.[23]
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