Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1521–1524

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

EVALUATION OF PHOSPHORUS-CONTAINING INHIBITORS OF γ-GLUTAMYL HYDROLASE

Chester E. Rodriguez, H. Michael Holmes, Karyn L. Mlodnosky, Vinh Q. Lam, and Clifford E. Berkman*

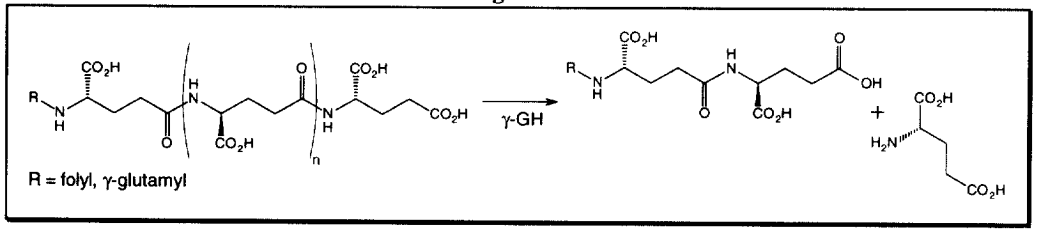
Department of Chemistry & Biochemistry, San Francisco State University
1600 Holloway Avenue, San Francisco, CA 94132, U.S.A.

Received 3 April 1998; accepted 11 May 1998

Abstract: Several putative, phosphorus-containing inhibitors of γ-glutamyl hydrolase were synthesized and evaluated for inhibitory activity. The phosphonamidoic acids were shown to be weak competitive inhibitors while both a phosphoramidate diester and a phosphonamidate ester were shown to be potent time-dependent inactivators, presumably through irreversible phosphorylation of an active site nucleophile.

Resistance to methotrexate (MTX) by human sarcoma cell lines has recently been attributed to increased cellular levels of the enzyme γ-Glutamyl Hydrolase (γ-GH)[1] In addition, many solid tumors, including breast cancer and hepatoma, have been shown to secrete high levels of this enzyme.[2] The scope of γ-GH's activity includes the hydrolytic cleavage of poly- and folylpoly-γ-glutamates (Figure 1).[3] As a result, folyglutamates including MTX, which are normally retained in cells as a result of γ-glutamyl synthetase-mediated polyglutamylation, may be susceptible to elimination from cells as terminal glutamyl residues are hydrolytically cleaved by the action of γ-GH. Therefore, specific inhibitors of γ-GH could be therapeutically invaluable for MTX-resistant tumors by countering their mode of resistance.

Figure 1



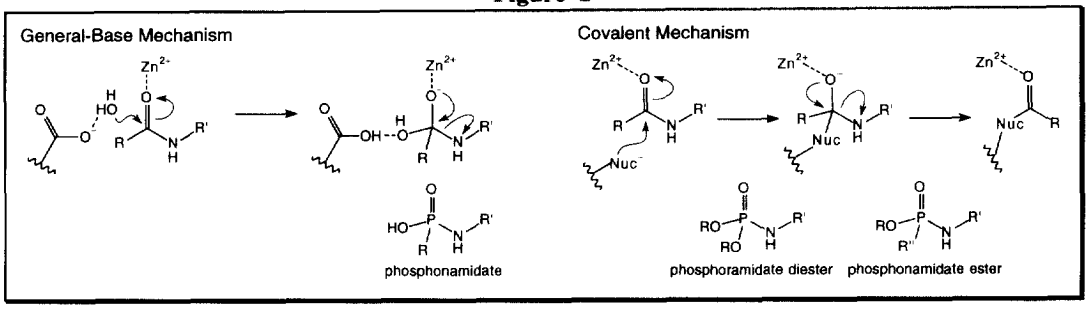
The specific mechanism for this enzyme is presently unknown, yet in many tissues γ-GH has been shown to be sulfhydryl and Zn^{2+} dependent.[4] Thus, it has been postulated that this enzyme is a metalloprotease and more specifically, a cysteine metalloprotease. The crystal structure for the bacterial form of γ-GH has recently been solved and although it has been suggested that it too is a Zn^{2+} metalloprotease, the mechanism remains to be determined.[5] Therefore, based on the current understanding, it is reasonable to propose that the specific hydrolytic activity occurs via either one of two types of mechanisms: general-base or covalent. A general-base mechanism is anticipated to be similar to that of other metalloproteases such as thermolysin or carboxypeptidase A (Figure 2) whereby a water molecule is polarized by a basic residue and attacks the carbonyl of the amide bond to generate a transitory tetrahedral intermediate. Phosphonamidates have been shown to be extremely potent competitive inhibitors of general-base metalloproteases by acting as transition-state or tetrahedral-intermediate mimics.

Alternatively, a covalent mechanism could be envisioned whereby a nucleophile in the active site is temporarily acylated during the hydrolysis of the amide, similar to serine esterases and peptidases (Figure 2).

PII: S0960-894X(98)00253-4

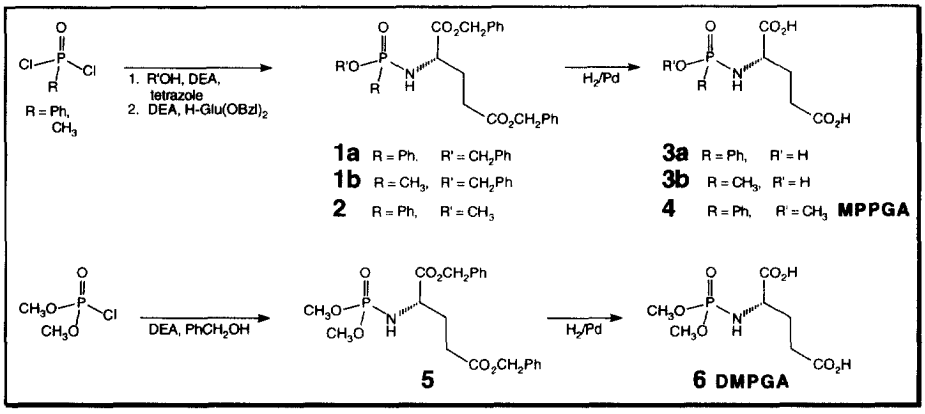
Phosphoramidate diesters and phosphonamidate esters, analogous to the phosphate and phosphoramidate esters inhibitors of serine esterases, would be likely inhibitors of a such a mechanism by phosphorylating an active site nucleophile.

Figure 2



In order to develop specific inhibitors of γ-GH as well as to explore its mechanism of action, we synthesized representative structures from each putative class of mechanistic inhibitors of γ-GH as outlined in Scheme 1.[6] We recently developed a two-step, one-pot method utilizing catalytic tetrazole to procure the phosphonamidate ester intermediates **1** and **2** in high yield.[7] The intermediates (**1**, **2**, and **5**) were deprotected via catalytic hydrogenation to obtain the desired, putative inhibitors **3**, **4**, and **6**, respectively.

Scheme 1

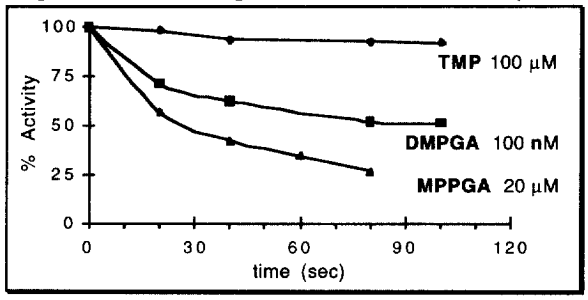


Employing a bacterial form of γ-GH as an enzymatic model for the tumor cell form, the phosphonamidoic acids **3** were examined for inhibitory activity utilizing a spectrophotometric method to directly observe the consumption of MTX.[8] From Lineweaver–Burk and Dixon analyses, it was determined that these compounds were competitive inhibitors of γ-GH, albeit very weak (K_i = 0.95 mM and 0.93 mM for **3a** and **3b**, respectively).[9,10]

Because it was anticipated that the phosphonamidate ester **4** [N-(methyl phenylphosphonyl)glutamic acid, **MPPGA**] and the phosphoramidate diester **6** [N-(dimethyl phosphonyl)glutamic acid, **DMPGA**] would function as irreversible inhibitors, they were examined for time-dependent inhibition. Briefly, γ-GH was incubated with each and at selected time intervals, aliquots were assayed for remaining activity.[11] The results shown in Figure 3 clearly indicated time-dependent inhibitory behavior by both **MPPGA** and **DMPGA**. To further determine that

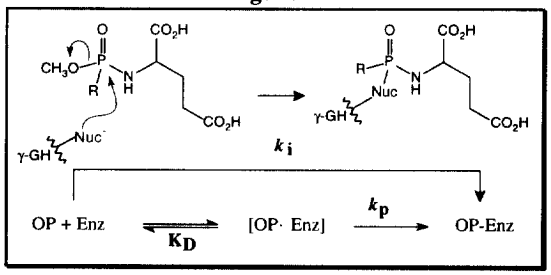
this inhibition was specific, trimethyl phosphate (**TMP**; $[CH_3O]_3PO$) was also examined for nonspecific time-dependent inhibition of γ-GH. Because the results showed that **TMP** did not elicit time-dependent inactivation of the enzyme even for concentrations 1000-fold greater than effective concentrations of **DMPGA** (Figure 3) it was concluded that the glutamyl portion of **DMPGA** and **MPPGA** must impart specificity for these inhibitors toward the active site.

Figure 3. Time-Dependent Inactivation of γ-GH



These results suggest that the most probable mechanism of inhibition or inactivation of γ-GH by **MPPGA** and **DMPGA** is irreversible phosphorylation of an active site nucleophile as shown in Figure 4. Presumably the mechanism of such time-dependent inhibition of γ-GH is similar to the well studied irreversible inactivation of serine esterases by organophosphate inhibitors. In the case of γ-GH, an active site nucleophile (Nuc^-) is presumed to attack the phosphorus center of **MPPGA** or **DMPGA** simultaneously displacing a leaving group. Based on this presumption, the kinetic parameters k_i, K_D, and k_p for both **DMPGA** and **MPPGA** were be determined (Table 1).[12]

Figure 4



Based on the inactivation data in Table 1, the inhibitory potency (k_i) of **DMPGA** is approximately 40-fold greater than that of **MPPGA**. From closer inspection of the kinetic parameters, the basis for this difference is primarily due to differences in the dissociation constants K_D. Presumably, the greater K_D for **MPPGA** is due to greater steric effects imparted by the phenyl ligand of **MPPGA** in the active site compared to that of a single methoxy group of **DMPGA**. Although the differences in the phosphorylation rate constants (k_p) are small, the greater value for **MPPGA** can be attributed to the general greater reactivity of phosphonates over that of

Table 1. Inactivation of γ-GH*

inhibitor	k_i $M^{-1}min^{-1}$ ($x10^6$)	K_D M^{-1} ($x10^{-6}$)	k_p min^{-1}
DMPGA	10.9 (0.12)	0.223 (0.02)	2.42 (0.10)
MPPGA	0.264 (0.11)	27.4 (0.40)	7.24 (0.42)

*standard error in parentheses

phosphates to nucleophilic events such as hydrolysis.[13] It should also be noted that **MPPGA** exists as a mixture of two diastereomers (approximately 50/50 by ^{31}P NMR) due to the chiral phosphorus center because the stereogenic center at the α-carbon of the glutamyl moiety is fixed with an *S*-configuration. Therefore, the kinetic parameters for **MPPGA** may be the result of interactions of both stereoisomers with the enzyme and that their individual contributions can only be determined by separation and evaluation of each diastereomer.

In conclusion, the results from the inhibition data herein further support the proposition of a covalent rather than a general-base mechanism for bacterial γ-GH activity. Although it appears that the inhibitory action of both the phosphoramidate diester **DMPGA** and the phosphonamidate monoester **MPPGA** occur via irreversible phosphorylation of γ-GH, the putative active site residue that is phosphorylated has yet to be determined and such studies are currently being investigated in our laboratory. These results now warrant elaborations of the chemical structure of the irreversible inactivators to develop more potent inhibitors, ultimately resulting in chemotherapeutic strategies to thwart cellular resistance to antifolates.

Acknowledgments: This investigation was supported by a 'Research Infrastructure in Minority Institutions' award from the National Center for Reseach Resources with funding from the Office of Research on Minority Health, National Institutes of Health (Grant #RR11805-02). The authors would also like to extend their gratitude to the Council on Undergraduate Research for the Boehringer Ingelheim sponsored Academic-Industrial Undergraduate Research Partnership (AIURP) Fellowship to K. L. Mlodnosky and to the Dept. of Education for the GAANN fellowship to H. M. Holmes (Grant No. P200A70233).

References and Notes

1. Li, W. W.; Waltham, M.; Tong, W.; Schweitzer, B. I.; Bertino, J. R. In *Chemistry and Biology of Pteridines and Folates*; Ayling, J. E. Nair; M. G.; Baugh, C. M. Eds.; Plenum Press: New York, 1993; pp 635-638.
2. O'Connor, B. M.; Rotundo, R. F.; Nimec, Z.; McGuire, J. J.; Galivan, J. *Cancer Res.* **1968**, *51*, 3874. Rhee, M; Wang, Y.; Nair, M. G.; Galivan, J. *Cancer Res.* **1993**, *53*, 2227. Rhee, M.; Ryan, T.; Galivan, J. *J. Cell. Pharmacol.* **1995**, *2*, 289.
3. Wang, Y.; Dias, J. A.; Nimec, Z.; Rotundo, R.; O'Connor, B. M.; Freisheim, J.; Galivan, *J. Advan. Enzyme Regul.* **1993**, *33*, 207.
4. Silink, M.; Reddel, R.; Bethel, M.; Rowe, P. B. *J. Biol. Chem.* **1975**, *250*, 5982. Yao, R.; Schneider, E.; Ryan, T.J.; Galivan, J. *Proc. Natl. Acad. Sci.* **1996**, *93*, 10134.
5. Rowsell, S.; Pauptit, R. A.; Tucker, A. D.; Melton, R. G.; Blow, D. M.; Brick, P. *Structure*, **1997**, *5*, 337.
6. All compounds were fully characterized by ^{1}H and ^{31}P NMR and mass spectrometry.
7. Mlodnosky, K. L.; Holmes, H. M.; Lam, V. Q.; Berkman, C. E. *Tetrahedron Lett.* **1997**, *38*, 8803.
8. McCullough, J. L.; Chabner, B. A.; Bertino, J. R. *J. Biol. Chem.* **1971**, *246*, 7207.
9. Standard errors for the competitive inhibition K_i values of **1a** and **1b** were 0.06 and 0.04, respectively.
10. Typical experimental procedure: To a 700 μL solution (50 mM Tris, pH 7, 500 μM Zn^{2+}) of 0.015 units γ-GH (Carboxypeptidase G; Sigma Chemical Co., St. Louis MO) was added 100 μL inhibitor solution(**1a** or **2b** dissolved in water) or 100 μL water as control. The enzyme reaction was initiated by the addition of 100 μL substrate (MTX) and monitored at 320 nm for absorbance decreases due to substrate consumption. For Lineweaver-Burk studies, substrate concentrations were varied from 5 to 50 μM and inhibitor concentrations were varied from 0 to 8 mM. For Dixon analyses, inhibitor concentrations were varied from 0 to 8 mM. All data was collected in at least triplicate, averaged, and presented with standard errors.
11. Typical Experimental conditions: To a 540 μL solution of 0.081 units γ-GH (Carboxypeptidase G; Sigma Chemical Co., St. Louis MO) dissolved in water, was added either 60 μL inhibitor solution (10:90 v:v, methanol : water) or 60 μL of a methanol: water solution (10:90 v:v) to serve as a control. At selected time points, 100 μL aliquots of the enzyme-inhibitor (or enzyme-control) were removed and assayed for remaining activity by its addition to a 900 μL substrate solution (120 μM MTX, 50 mM Tris pH 7.0, 500 μM Zn^{2+}) and monitored at 320 nm for absorbance decreases due to substrate consumption. All data was collected in triplicate, averaged, and presented with standard errors.
12. Main, A. R. *Science* **1964**, *144*, 992.
13. Kirby, A. J., Warren, S.G. In *The Organic Chemistry of Phosphorus*, Eaborn, C.; Chapman, N. B., Eds.; Elsevier Publishing Co.: Amsterdam, **1967**; Chapter 10.