THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 27, Issue of November 25, pp. 14689-14694, 1985
Printed in U.S.A.

Development of Selective Inhibitors of Transglutaminase

PHENYLTHIOUREA DERIVATIVES*

(Received for publication, June 3, 1985)

K. N. Lee‡, L. Fesus‡, S. T. Yancey‡, J. E. Girard§, and S. I. Chung

From the Laboratory of Oral Biology and Physiology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205 and the §Department of Chemistry, The American University, Washington, D. C. 20016

For the purpose of developing a transglutaminase inhibitor which could be effective in physiological and pharmacological studies, a series of phenylthiourea derivatives of α,ω-diaminoalkanes were designed, synthesized, and evaluated kinetically as inhibitors of transglutaminases. A homologous series of compounds of the structure phenylthiourea-$(CH_2)_n$-NH_2, where n = 2, 3, 4, 5, and 6, were tested for the inhibition of both guinea pig liver transglutaminase-catalyzed amine incorporation into various glutamine-containing substrates and plasma transglutaminase (factor XIIIa)-catalyzed amine incorporation into fibrin and fibrin cross-linking. It was found that the inhibitory activity of the compounds increases with increasing number of methylene groups in the side chain up to a maximum of n = 5. A further increase in the length of the methylene side chain to n = 6 results in decreased activity. The K_i value (4.9×10^{-5} M) of 1-(5-aminopentyl)-3-phenylthiourea (PPTU) (n = 5) for the inhibition of guinea pig transglutaminase-catalyzed amine incorporation into the B chain of oxidized insulin is in close agreement to its K_m(app) value (7.1×10^{-5} M) obtained using ^{14}C-labeled PPTU. PPTU was also found to be a potent inhibitor of plasma transglutaminase-catalyzed fibrin cross-linking. The finding that the specificity of the alkylamines for inhibition is correlated with the length of their methyl side chains is compatible with those reported for aliphatic amines and monodansylcadaverine analogues (where dansyl is 5-dimethylaminonaphthalene-1-sulfonyl). The phenylthiourea derivatives, however, are far less toxic in mice than monodansylcadaverine as indicated by their LD_{50} values: PPTU, 400 ± 25 mg/kg; and monodansylcadaverine, 160 ± 20 mg/kg.

Transglutaminases are Ca^{2+}-dependent enzymes that catalyze the post-translational modification of proteins through exchange of primary amines for ammonia at the γ-carboxamide group of peptide-bound glutamine residues. These enzymes are widely distributed in body fluids and tissues (for review, see Refs. 1–3) and are thought to play a role in cell activation and differentiation (4–7). A number of potential transglutaminase inhibitors have been developed during the past decade (8–11). Among the transglutaminase inhibitors developed, monodansylcadaverine (MDC[1]) analogues were shown to be among the most effective inhibitors of the transglutaminase-catalyzed amine corporation into various glutamine-containing substrates and also in the cross-link formation between polypeptide chains in *in vitro* assay (8, 11). However, MDC was also shown to inhibit a number of other enzymes, *e.g.* 17-ketosteroid reductase (12), phosphatidylcholine synthetase (13), as well as the calmodulin activation of cyclic nucleotide phosphodiesterase (14) and cell attachment (14). In an effort to develop an inhibitor that is specific for transglutaminases in cellular systems, we have prepared a number of phenylthiourea derivatives of α,ω-diaminoalkanes and examined these as inhibitors of tissue and plasma transglutaminases. Preliminary studies showed that 1-(5-aminopentyl)-3-phenylthiourea (PPTU), at K_i levels, caused no adverse effect on several cellular processes.[2] This report presents studies of the kinetic properties of phenylthiourea (PTU) derivatives of α,ω-diaminoalkanes as inhibitors of the liver transglutaminase-catalyzed methylamine incorporation into various glutamine substrates and of the plasma transglutaminase-catalyzed fibrin cross-linking.

EXPERIMENTAL PROCEDURES[3]

Materials—Guinea pig liver transglutaminase was prepared according to a published procedure (15). The enzyme exhibited 95 ± 5% of the reported specific activity when assayed by hydroxamate formation with the substrate benzyloxycarbonyl-L-glutaminylglycine (Z-Gln-Gly) (15). An extinction coefficient of $E^{1\%}_{280\,nm}$ = 15.8 and a molecular weight of 80,000 were used to determine enzyme concentration (16). Human blood coagulation factor XIII was isolated from 95% clottable fibrinogen prepared from fresh ACD plasma (17, 18). Plasma factor XIIIa (plasma transglutaminase) formed from the zymogen upon activation with thrombin (18) showed 95% of the reported specific activity when assayed by [^{14}C]methylamine uptake by the acetylated B chain of oxidized insulin (18). An extinction coefficient $E^{1\%}_{280\,nm}$ = 13.8 (19) and a molecular weight of 320,000 were used to determine zymogen concentration. [^{14}C]Methylamine (102 mCi/mmol) was purchased from New England Nuclear and was used without further purification. Z-Gln-Gly was a gift from Dr. J. E. Folk, National Institutes of Health, Bethesda, MD. The acetylated B chain

[1] The abbreviations used are: MDC, monodansylcadaverine (where dansyl is 5-dimethylaminonaphthalene-1-sulfonyl); PPTU, 1-(5-aminopentyl)-3-phenylthiourea; PTU, phenylthiourea; EPTU, ethylaminophenylthiourea; Z-Gln-Gly, benzyloxycarbonyl-L-glutaminylglycine.

[2] K. N. Lee, L. Fesus, S. T. Yancey, J. E. Girard, and S. I. Chung, manuscript in preparation.

[3] Portions of this paper (including "Experimental Procedures," Tables II–IV, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1802, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by The National Foundation for Cancer Research, Bethesda, MD.

of oxidized insulin was prepared as outlined previously (18). The concentrations of B chain of insulin stock solution were determined from amino acid analysis. Human thrombin was a gift from Dr. Genesio Murano, Bureau of Biologics, Food and Drug Administration, Bethesda, MD. Monodansylcadaverine hydrochloride was from Sigma. Methods for the synthesis of the PTU derivatives of the α,ω-diaminoalkanes and [^{14}C]PPTU and analytical data are presented in the *Miniprint Supplement*.

Methods—Kinetic studies were carried out at 37 °C in 0.1 M Tris acetate buffer (pH 7.5) containing 0.1 mM EDTA, 20 mM $CaCl_2$, and 30 mM NaCl. Apparent Michaelis constants for PPTU were measured for both guinea pig liver and plasma transglutaminase systems. For the liver enzyme, 0.02–0.10 mM [^{14}C]PPTU was used with 0.2 mM B chain of oxidized insulin and 0.2–2.0 mM [^{14}C]PPTU was used with 4.0 mM Z-Gln-Gly. For the plasma enzyme, 0.01 mM fibrinogen, 2 NIH units of thrombin, 1 mM dithiothreitol, and 0.2–2.0 mM [^{14}C] PPTU were used. Measurement of [^{14}C]PPTU incorporation into fibrin and into the B chain of oxidized insulin was made by trichloroacetic acid precipitation of the proteins as described for factor XIIIa assay (18). The rate of incorporation of [^{14}C]PPTU into Z-Gln-Gly was measured by thin layer chromatography on polyamide sheets (20). Measurement of incorporation of [^{14}C]methylamine into Z-Gln-Gly was made by a method using DEAE-cellulose paper strips (21). Inhibition studies of liver transglutaminase-catalyzed methylamine incorporation into the B chain of oxidized insulin and into Z-Gln-Gly were performed in 100 μl of reaction mixtures containing 0.1 M Tris acetate (pH 7.5), 0.1 mM EDTA, 20 mM $CaCl_2$, 0.15 mg/ml enzyme, 0.37–2.0 mM [^{14}C]methylamine, 0.1 mM B chain of oxidized insulin or 1.0 mM Z-Gln-Gly , and up to 1 mM inhibitors. Inhibition studies of plasma transglutaminase-catalyzed reactions were carried out in reaction mixtures of 100 μl composed of 0.1 M Tris acetate buffer (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 20 mM $CaCl_2$, 0.01 mg/ml enzyme, 1.0–5.0 mM [^{14}C]methylamine, 0.01 mM fibrinogen, 2 units of thrombin, and up to 1 mM inhibitors. Inhibition of fibrin clot stabilization was studied in 0.01 M Tris acetate buffer containing 2 mM $CaCl_2$, 0.15 M NaCl, and 5 mM dithiothreitol at various inhibitor concentrations. The concentrations of fibrinogen and plasma transglutaminase were 2.5 and 0.015 mg/ml, respectively. Clot formation was initiated by the addition of 1 unit of thrombin. The clot was washed three times with cold 0.01 M Tris acetate buffer containing 2 mM EDTA and 0.15 M NaCl. The washed clot was placed in 6 M urea, and the soluble protein was estimated by the method of Bradford (22). All kinetic studies were carried out under conditions where no more than 10% of the substrate at the lowest concentration was consumed within the reaction periods. Reciprocal velocities were plotted against the reciprocals of substrate concentration. The data was fitted to the Michaelis-Menten equation (Equation 1) where the terms have their usual meaning:

$$v = \frac{VA}{K_m + A} \quad (1)$$

All fits were performed by means of an interactive curve-fitting program, MLAB, developed at the National Institutes of Health and run on a DEC-10 computer (23). Data for linear competitive inhibition were fitted to Equation 2 as follows.

$$v = \frac{VA}{K_m\,(1 + I/K_i) + A} \quad (2)$$

Inhibitory activity of fibrin clot stabilization is expressed as per cent solubility in 6 M urea. Per cent solubility was estimated from the fraction (Su − Su,I)/(Su,Ca − Su,I) where Su represents soluble proteins in 6 M urea, *Su,I* represents soluble proteins in 6 M urea in the presence of inhibitor, and *Su,Ca* represents soluble protein in 6 M urea in the presence of Ca^{2+}. The comparison of the inhibitory activity of each inhibitor is expressed as that relative to the inhibitor activity of PPTU. PPTU caused a 50% inhibition of clot solubilization at 1 mM.

Lethal dose effects of PPTU, EPTU, and MDC were measured in mice by intraperitoneal administration of the inhibitors dissolved in sterile saline solution. A group of 10 mice were utilized for each dose level of the compound, and survival rates were estimated from the percentage of animals that survived 7 days post-administration. A full tabulation of kinetic and inhibitor constants are presented in the Miniprint Supplement.

RESULTS

The liver transglutaminase is one of the most extensively studied transglutaminases with regard to its substrate specificity, structural properties, and cellular regulation (18, 24, 25). Liver transglutaminase has been shown to have much broader substrate specificity than other transglutaminases (18, 24). We selected two different glutamine-containing substrates for inhibitor studies: one, Z-Gln-Gly , is a simple, very small substrate for liver transglutaminase, whereas the other, the B chain of oxidized insulin, is a more complex peptide that contains a single glutamine residue and that also serves as a substrate for both liver transglutaminase and factor XIIIa. The mode of inhibition by PTU derivatives of α,ω-diaminoalkanes was examined by kinetic analysis. A double reciprocal plot of reaction velocities against methylamine concentration in the presence and absence of PPTU is shown in Fig. 1. Saturating levels of the oxidized B chain of insulin were used as the glutamine substrate. The results show a typical competitive inhibition profile where only the slope $(K_a/V)(1 + I/K_i)$ is affected, without effect on the intercept. When the slopes from the double reciprocal plot were plotted against inhibitor concentrations, a linear fit was obtained (Fig. 1, *inset*), indicative of linear competitive inhibition as represented in Equation 2. The inhibition constants for the various chain length arylamines obtained using Z-Gln-Gly and the oxidized B chain of insulin were compared as shown in Fig. 2; the arylamine with the pentamethylene chain between the terminal amino group and the PTU group showed the greatest inhibitory activity, while that with the ethylene chain showed almost no activity. The arylamine with the hexamethylamine side chain showed slightly less inhibitor activity than that with the pentamethylamine group. A similar pattern of inhibitor specificity between different alkylamine side chains of PTU was observed using the two glutamine substrates. However, the K_i value for each of the inhibitors obtained with the use of Z-Gln-Gly was significantly higher than that of the same inhibitor obtained with the oxidized B chain of insulin.

Plasma transglutaminase (factor XIIIa) present in plasma

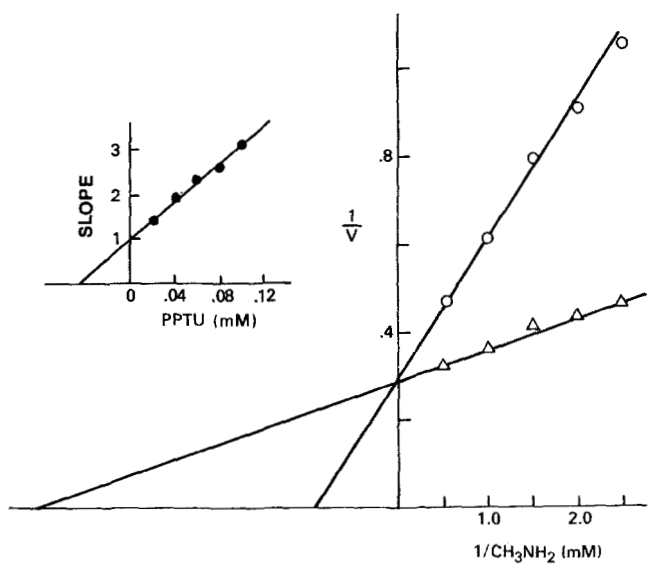


FIG. 1. **Lineweaver-Burk plots for guinea pig liver transglutaminase-catalyzed CH_3NH_2 incorporation into the B chain of oxidized insulin in the presence (○) and absence (△) of 0.1 mM PPTU.** Velocity is given in nanomoles of CH_3NH_2 incorporated per min/nmol of enzyme. Each point represents the average of duplicate determinations. The *inset* shows a plot of the slope against the inhibitor concentrations.

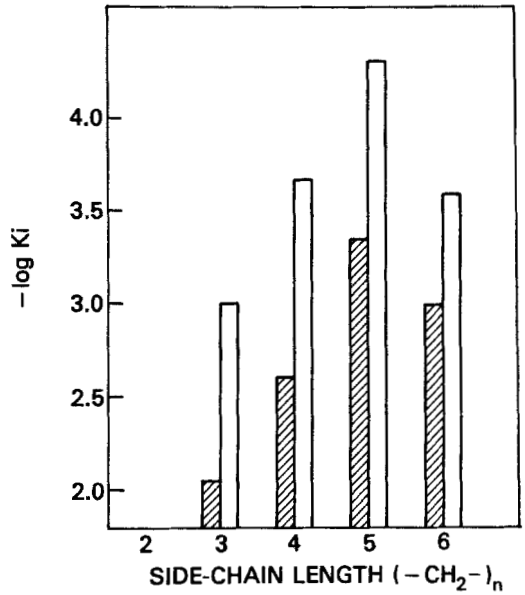


FIG. 2. **Correlation between the methylene $(-CH_2-)_n$ side chain length of alkylamine derivatives of PTU and the K_i values obtained with these inhibitors in guinea pig liver transglutaminase-catalyzed CH_3NH_2 incorporation into the B chain of oxidized insulin (□) or into Z-Gln-Gly (▨).**

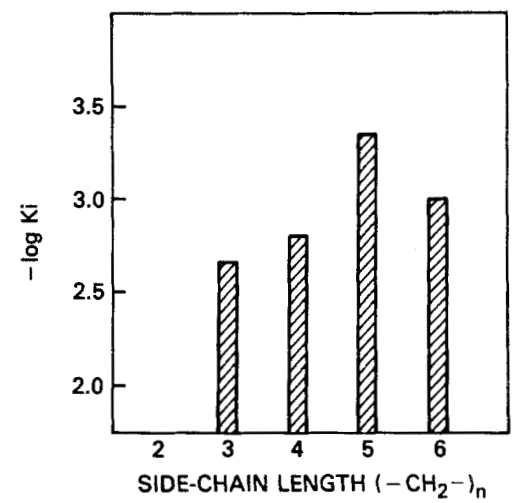


FIG. 3. **Correlation between methylene $(-CH_2-)_n$ side chain length of alkylamine derivatives of PTU and the K_i values obtained with these inhibitors in plasma transglutaminase-catalyzed CH_3NH_2 incorporation into fibrinogen.**

and platelets is the most abundant form of transglutaminase, but it has a more limited substrate specificity. The dipeptide, Z-Gln-Gly , which has been used as an effective substrate for liver transglutaminase, is a very poor substrate for plasma transglutaminase. In plasma, this enzyme catalyzes the interchain cross-linking of fibrin through ϵ-(γ-glutamyl)lysine bond formation. For these inhibition studies, fibrin was used as a glutamine substrate. Fig. 3 shows the $-\log K_i$ values obtained with the varying chain length arylamines. Again, the arylamine with the pentylamine side chain showed the highest inhibitor activity and that with the ethylamine side chain displayed practically no inhibition. The arylamine with the hexylamine side chain was less inhibitory than that with the pentylamine group.

The inhibition of fibrin cross-linking was examined by clot solubility in a chaotropic reagent (6 M urea). The fully cross-linked fibrin polymer is the least soluble, whereas the non-cross-linked fibrin polymer is completely soluble in 6 M urea. In Fig. 4, the inhibitory effects of PPTU and EPTU on fibrin clot solubility in 6 M urea are shown. EPTU showed almost no effect, while in the presence of PPTU, there is a concentration-dependent increase of fibrin solubilization. A comparison of the relative inhibition of fibrin cross-linking by each inhibitor (Fig. 5) showed that the arylamine with the pentylamine side chain was the most effective. Thus, with both the liver and the plasma transglutaminases, the structural requirement for alkylamine side chain appears to be the same.

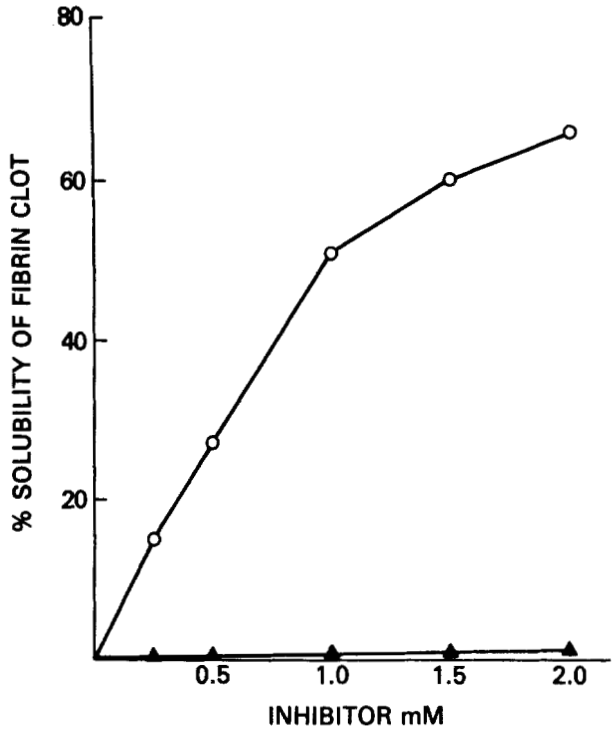


FIG. 4. **Inhibition of fibrin clot stabilization by PPTU (○) and EPTU (▲).**

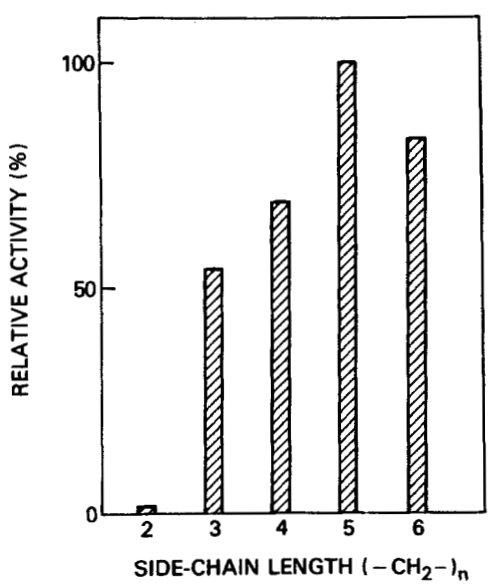


FIG. 5. **Correlation between methylene $(-CH_2-)_n$ side chain length of alkylamine derivatives of PTU and the relative inhibitory activities with these inhibitors in plasma transglutaminase-catalyzed fibrin clot stabilization (fibrin cross-linking).** Relative activity was estimated from the per cent solubility of fibrin clot in the presence of 1.0 mM inhibitor divided by the per cent solubility of fibrin clot in the presence of 1.0 mM PPTU.

TABLE I

Kinetic constants obtained with [^{14}C]PPTU and various glutamine substrates in the liver and plasma transglutaminases-catalyzed reaction

Trans-glutaminase	Glutamine substrate[a]	Constants	
		K_m(app)	V^b
		mM	*min*$^{-1}$
Liver	B chain of insulin	0.084 ± 0.005	2.59 ± 0.11
	Z-Gln-Gly	0.740 ± 0.08	17.60 ± 1.1
Plasma	Fibrinogen	0.330 ± 0.028	19.60 ± 0.61

[a] The concentrations of glutamine substrates used were as follows: B chain of insulin, 0.2 mM; Z-Gln-Gly, 4.0 mM; and fibrinogen, 0.01 mM.

[b] Values for V are given in nanomoles of [^{14}C]PPTU incorporated per min/nmol of enzyme.

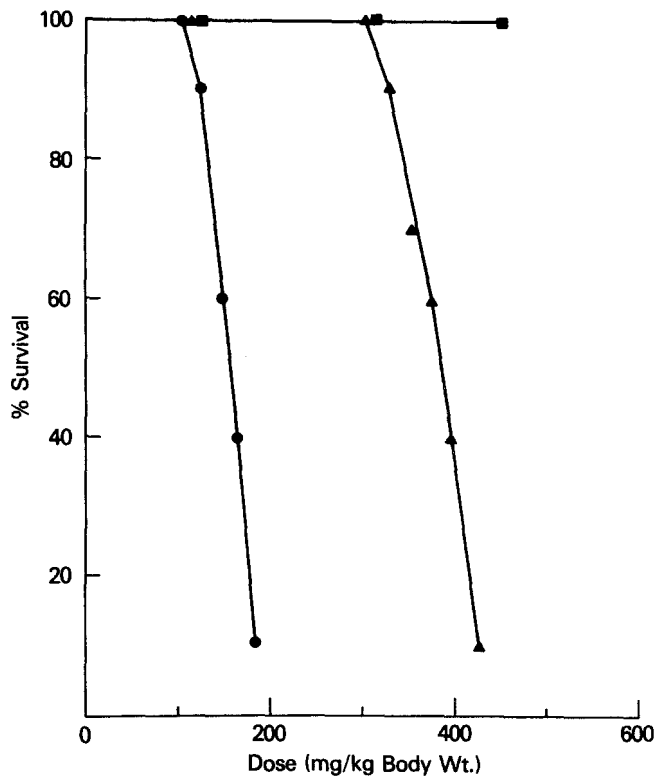


FIG. 6. **LD_{50} of PPTU, EPTU, and MDC.** The percentages of survival from the groups of 10 mice following intraperitoneal administration of various dose levels of PPTU (▲), EPTU (■), and MDC (●) are shown.

The inhibition expected by the arylamines probably is a result of their role as substrates. Thus, the Michaelis constant obtained with the use of PPTU as second substrate should be in close agreement with its K_i value as an inhibitor. Table I shows the kinetic constants obtained from the direct incorporation of [^{14}C]PPTU into various glutamine substrates by both liver and plasma transglutaminase. The K_m(app) values obtained are within the range of experimental error for the comparable K_i values (see Tables II–IV).

In order to evaluate the physiological tolerance of PPTU, EPTU, and MDC, various dose levels of the inhibitors were administered to mice by intraperitoneal injection and the lethal dose response was examined over a 7-day period. Those animals which were affected died within a 24-h period. As shown in Fig. 6, MDC is the most toxic (LD_{50} = 160 mg/kg), whereas PPTU was tolerated at three times higher levels. EPTU showed no lethal effect at these dose levels.

DISCUSSION

A series of phenylthiourea derivatives of α,ω-diaminoalkanes were synthesized to be examined as possible inhibitors of factor XIII and tissue transglutaminase-mediated reactions in pathogenesis (27). These derivatives were designed to have three structural features: 1) a primary amino group which should compete with second substrate by means of nucleophilic substitution at the acyl-enzyme intermediate during catalysis; 2) a hydrophobic phenyl group to enhance affinity of the inhibitor for subsites near the catalytic site of the enzyme and to promote better diffusion into cells; and 3) a thiourea group to increase solubility in physiological media. The kinetic analysis of PPTU inhibition of methylamine incorporation into the B chain of oxidized insulin showed linear competitive inhibition as defined by Equation 2 (Fig. 1). In order to evaluate and compare the inhibition constants presented here, separate experiments were done to measure the apparent K_m value by direct incorporation of [^{14}C]PPTU using the same glutamine substrate. Under these conditions, the K_m(app) value obtained was equal to the K_i values for this amine, evidence that inhibition is, indeed, a consequence of the substrate property of the arylamine. For the various arylamines tested, greater specificity is reflected in the higher $-\log K_i$ values presented in Figs. 2, 3, and 5. Examination of the data presented in these figures reveals some interesting features of the amine inhibitor specificity of the acyl-enzyme intermediates formed with liver and plasma enzymes. As the number of methylene groups between PTU and the amino group is increased up to a certain number, progressively better inhibitor activity is observed. The peak inhibitor activity is noted with the pentylamine side chain, and an increase in chain length of one methylene group caused a decline of inhibitor activity. The PTU derivative of diaminopentane is a better inhibitor (2 orders of magnitude) than the best of the aliphatic amines studied (26), suggesting that orientation of PPTU in the acyl-enzyme intermediate may involve additional binding sites for the PTU group. A similar suggestion has been made regarding the binding of monodansylcadaverine (11). The amino group in each of the inhibitors used here would be likely to have about the same pK_a, 10.6 ± 0.1. The ϵ-amino group of a lysine residue in a peptide possesses approximately the same pK_a (28, 29). It is interesting to examine the effectiveness of these inhibitors on the basis that only the non-ionized form of an amine acts as a second substrate (30, 31). If Michaelis constants can be expressed relative to the effective concentration of unprotonated amine species existing at the pH of the experiments (pH 7.5) rather than on the basis of the total concentration of the amine substrate, K_m for PPTU can be estimated as 6.7×10^{-8}, which indicates that it is indeed an excellent substrate.

Preliminary studies[2] on the uptake and distribution of [^{14}C]PPTU showed rapid diffusion into Chinese hamster ovary cells. At concentrations near its K_i values, no inhibitory activity was observed on the calmodulin-dependent nucleotide phosphodiesterase, and no effect on cell proliferation of Chinese hamster ovary and B16 melanoma cells *in vitro* was observed. In the animal experiment, PPTU was shown to be effective in retardation of Lewis lung carcinoma cell metastasis in mice.[2] These results suggest that the phenylthiourea derivative of pentylamine is an excellent transglutaminase inhibitor and may be useful in the study of transglutaminase function in cellular systems.

REFERENCES

1. Chung, S. I. (1972) *Ann. N. Y. Acad. Sci.* **202,** 240–255
2. Chung, S. I. (1975) in *Isozymes* (Markert, C. L., ed) Vol. I, pp.

259–273, Academic Press, New York
3. Folk, J. E. (1980) *Annu. Rev. Biochem.* **49,** 517–531
4. Novogrodsky, A., Quittner, S., Rubin, A. L., and Stenzel, K. H. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1157–1161
5. Leu, R. W., Herriott, M. J., Moore, P. E., Orr, G. R., and Birchbrichler, P. J. (1982) *Exp. Cell Res.* **141,** 191–199
6. Murtaugh, M. P., Mehta, K., Johanson, J., Myers, M., Juliano, R. L., and Davies, P. J. A. (1983) *J. Biol. Chem.* **258,** 11074–11081
7. Murtaugh, M. P., Arend, W. P., and Davies, P. J. A. (1984) *J. Exp. Med.* **159,** 114–125
8. Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N., and Bruner-Lorand, J. (1968) *Biochemistry* **7,** 1214–1223
9. Stenberg, P., Nilsson, L., Erickson, O., and Lunden, R. (1971) *Acta Pharm. Suec.* **8,** 415–422
10. Gross, M., Whetzel, N. K., and Folk, J. E. (1975) *J. Biol. Chem.* **250,** 7693–7699
11. Lorand, L., Parameswara, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jonsson, N. A., Mikiver, L., and Moses, P. (1979) *Biochemistry* **18,** 1756–1765
12. Moger, W. H. (1982) *Can. J. Physiol. Pharmacol.* **60,** 858–861
13. Mato, J. S., Pencer, D., Vasanthakumar, G., Schiffmann, E., and Pastan, I. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1929–1932
14. Cornwell, M. M., Juliano, R. L., and Davies, P. J. A. (1983) *Biochim. Biophys. Acta* **762,** 414–419
15. Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M., and Folk, J. E. (1971) *J. Biol. Chem.* **246,** 1093–1098
16. Folk, J. E., and Cole, P. W. (1966) *J. Biol. Chem.* **241,** 5518–5525
17. Chung, S. I., Lewis, M. S., and Folk, J. E. (1974) *J. Biol. Chem.* **249,** 940–950
18. Chung, S. I., and Folk, J. E. (1972) *J. Biol. Chem.* **247,** 2798–2807
19. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) *J. Biol. Chem.* **248,** 1395–1470
20. Lorand, L., and Campbell, L. K. (1971) *Anal. Biochem.* **44,** 207–220
21. Abe, T., Chung, S. I., DiAugustine, R. P., and Folk, J. E. (1977) *Biochemistry* **16,** 5495–5501
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Knott, G. D., and Reece, D. K. (1971) *Modellab Users Documentation,* Division of Computer Research and Technology Report, National Institutes of Health, Bethesda, MD
24. Folk, J. E. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* **54,** 1–55
25. Davies, P. J. A., and Murtaugh, M. P. (1984) *Mol. Cell. Biochem.* **58,** 69–77
26. Gross, M., Whetzel, N. K., and Folk, J. E. (1977) *J. Biol. Chem.* **252,** 3752–3759
27. Folk, J. E., and Finlayson, J. S. (1977) *Adv. Protein Chem.* **31,** 1–133
28. Ellenbogen, E., (1952) *J. Am. Chem. Soc.* **74,** 5198–5202
29. Tanford, C. (1962) *Adv. Protein Chem.* **17,** 69–87
30. Clark, D. D., Mycek, M. J., Neidle, A., and Waelsh, H. (1959) *Arch. Biochem. Biophys.* **79,** 338–354
31. Folk, J. E., and Cole, P. W. (1966) *Biochim. Biophys. Acta* **122,** 244–264

SUPPLEMENTARY MATERIAL
to
Development of Selective Inhibitors of Transglutaminase:
Phenylthiourea Derivatives

K. N. Lee, L. Fesus, S. T. Yancey, J. E. Girard, and S. I. Chung

Supplement 1

Preparation and Properties of Transglutaminase Inhibitors

Synthesis of phenylthiourea derivatives of α,ω-diaminoalkanes

The following general procedure for the synthesis of the phenylthiourea derivatives is a modification of the procedure described by Stoutland et al. (1).

A solution of 0.1 mole of phenylisothiocyanate in 25 ml of absolute ether was added dropwise to a stirred solution of 0.2 mole of the appropriate bifunctional amine in 150 ml of isopropyl alcohol while maintaining the reaction temperature below 15°. The mixture was stirred until a white precipitate separated, then diluted with water to about 400 ml, and allowed to stand overnight at ambient temperature. The mixture was stirred and acidified to pH 2.6 by the dropwise addition of concentrated hydrochloric acid, and then heated in a 70° water bath for 30 minutes while stirring. The remaining precipitate in the mixture was removed by filtration and was washed with warm water. This precipitate is a disubstituted by-product. This material (N,N'-bisphenylthiocarbamylpentamethylenediamine) formed using 1,5-diaminopentane was recrystallized from acetic acid. m.p. 145–146°. It showed the following analysis; for $C_{19}H_{24}N_4S_2$: calculated; C, 61.29; H, 6.45; N, 15.05. Found: C, 61.17; H, 6.20; N, 14.82.

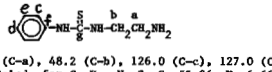
The filtrate was evaporated in vacuo, producing a light yellow solid residue which was then dissolved in water. The aqueous solution was cooled and made basic with saturated sodium hydroxide solution. The mono-phenylthiourea derivative of α,ω-diaminoalkane was then precipitated. The product was collected by filtration and washed with ice-cold water. 1-(2-aminoethyl)-3-phenylthiourea and 1-(3-aminopropyl)-3-phenylthiourea were recrystallized from water; 1-(4-aminobutyl)-3-phenylthiourea, 1-(5-aminopentyl)-3-phenylthiourea, and 1-(6-aminohexyl)-3-phenylthiourea from ethyl acetate.

Analytical Methods

Melting points were determined with a Thomas-Hoover capillary melting point apparatus. Infrared spectra were recorded on a Perkin-Elmer Model 397 spectrophotometer. ^{13}C NMR spectra were recorded on a Bruker WP 80 Fourier transform NMR system using tetramethylsilane as an internal standard. Mass spectra were determined using a Hewlett Packard 5995A GC/MS system. Elemental analyses were carried out by Galbraith Laboratories, Knoxville, Tennessee. Spectral (IR, MS, ^{13}C NMR) data were compatable with the assigned structures in all cases.

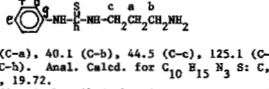
Analytical Data on Phenylthiourea Derivatives

1-(2-aminoethyl)-3-phenylthiourea - m.p. 135–136°(lit. 1, m.p. 136–137). IR (KBr)$_3$348 (N-H), 1240 (C=S)cm^{-1}. Molecular ion calcd for $C_9H_{13}N_3S$: 195.0831, Found: 195.1. ^{13}C NMR (CD_3OD)



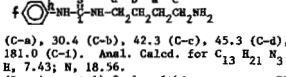
δ 41.9 (C-a), 48.2 (C-b), 126.0 (C-c), 127.0 (C-d), 130.4 (C-e), 139.7 (C-f), 183.0 (C-g). Anal. Calcd. for $C_9H_{13}N_3S$: C, 55.36; H, 6.66; N, 21.31. Found: C, 55.07; H, 6.52; N, 21.53.

1-(3-aminopropyl)-3-phenylthiourea - m.p. 106–107°. Molecular ion calcd for $C_{10}H_{15}N_3S$: 209.0988, Found: 209.1. ^{13}C NMR ($CDCl_3$)



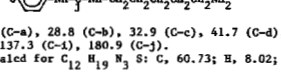
δ 31.2 (C-a), 40.1 (C-b), 44.5 (C-c), 125.1 (C-d), 126.4 (C-e), 129.5 (C-f), 137.0 (C-g), 180.5 (C-h). Anal. Calcd. for $C_{10}H_{15}N_3S$: C, 57.39; H, 7.17; N, 20.09. Found: C, 57.62; H, 6.93; N, 19.72.

1-(4-aminobutyl)-3-phenylthiourea - m.p. 86–87°. Molecular ion calcd for $C_{11}H_{17}N_3S$: 223.1144, Found: 223.1. ^{13}C NMR ($CDCl_3$)



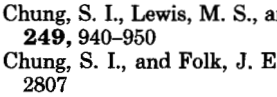
δ 26.6 (C-a), 30.4 (C-b), 42.3 (C-c), 45.3 (C-d), 125.3 (C-e), 126.7 (C-f), 129.7 (C-g), 137.0 (C-h), 181.0 (C-i). Anal. Calcd. for $C_{13}H_{21}N_3S$: C, 59.17; H, 7.62; N, 18.83. Found. C, 59.22; H, 7.43; N, 18.56.

1-(5-aminopentyl)-3-phenylthiourea - m.p. 77–78°. IR (KBr)$_3$345 (N-H), 1240 (C=S) Cm^{-1}. Molecular ion calcd for $C_{12}H_{19}N_3S$: 237.1301, Found: 237.1. ^{13}C NMR ($CDCl_3$)



δ 24.1 (C-a), 28.8 (C-b), 32.9 (C-c), 41.7 (C-d), 45.1 (C-e), 125.1 (C-f), 126.7 (C-g), 130.0 (C-h), 137.3 (C-i), 180.9 (C-j).
Anal. Calcd for $C_{12}H_{19}N_3S$: C, 60.73; H, 8.02; N, 17.71. Found: C, 60.28; H, 8.08; N, 17.17.

1-(6-aminohexyl)-3-phenylthiourea - m.p. 84–85°. Molecular ion calcd for $C_{13}H_{21}N_3S$: 251.1457, Found: 251.2. ^{13}C NMR ($CDCl_3$)



δ 26.5 (C-a), 26.7 (C-b), 29.0 (C-c), 33.6 (C-d), 42.0 (C-e), 45.4 (C-f), 125.1 (C-g), 126.9 (C-h), 129.9 (C-i), 136.8 (C-j), 181.0 (C-k). Anal. Calcd for $C_{13}H_{21}N_3S$: C, 62.13; H, 8.36; N, 16.73. Found: C, 62.06; H 8.59; N, 16.46.

Synthesis of ^{14}C-Labeled 1-(5-Aminopentyl)-3-Phenylthiourea

In a conical vial, 2.5 ml of 0.01 N HCl solution containing 250 μCi (0.24 mg) of [1,5-^{14}C] cadaverine hydrochloride (specific activity: 106.3 μCi/μmol)(New England Nuclear) was lyophilized. The residue was dissolved 12 μl of 0.2 N Tris(hydroxymethyl)aminomethane, and 68 μl of isopropanol was added. A solution of 0.4 mg of phenyl isothiocyanate in 19.7 μl of absolute ether was added in a small portions. After each addition, the vial was shaken gently. At the end of the addition, the mixture was allowed to stand for 8 hours at ambient temperature. The mixture was separated on a TLC plate (Silica Gel 60 0.25 mm, EM Laboratories, Inc., Associate of E. Merck, Darmstadt, Germany) using the solvent system n-butanol-acetic acid-water (10:3:2). Four bands appeared on the TLC plate; cadaverine (Rf 0.15); 1-(5-aminopentyl)-3-phenyl-thiourea (Rf 0.65); phenylisothiocyanate (Rf 0.77); and N,N'-bisphenyl-thiocarbamylpentamethylenediamine (Rf 0.94). The first two bands were detected by ninhydrin reaction and the second two bands were detected using short wave UV light (254 nm). The identity of each band was confirmed by the Rf value of each pure compound. At Rf 0.65, the band was extracted by 0.01 N HCl [yield: 39.58 μCi(15.8%)].

Analysis of [^{14}C]-PPTU by TLC in two different solvent systems; n-butanol-glacial acetic acid-water (10:3:2) and anhydrous ethanol-58% ammonium hydroxide (7:3) gave a single ninhydrin positive spot which contained more than 97% of the applied radioactivity.

References to Supplement 1

1. Stoutland, O., Helgen, L. and Agre, C.L. (1959). J. Org. Chem. 24, 818–820.

Supplement 2

Kinetic and Inhibitor Constants of PTU derivatives of Alkylamines in the transglutaminase catalyzed reactions:

Tables II and III list kinetic and inhibitor constants obtained by fits to equation 2 of data for synthetic amine inhibition of [^{14}C]-methylamine incorporation into the B chain of oxidized insulin, and into Z-Gln-Gly by liver transglutaminase. In Table IV are recorded constants for human plasma factor XIIIa obtained by fits to equation 2 of data for synthetic amine inhibition of [^{14}C]-methylamine incorporation into human plasma fibrin. In each case double reciprocal plots of initial velocities of [^{14}C]-methylamine incorporation against methylamine concentrations (five concentrations between 0.4 and 2.0 mM for the liver enzyme, or between 1.0 and 5.0 mM for the plasma enzyme) at various levels of inhibitor showed patterns of intersection on the vertical axis typical of competitive inhibition.

Table II
Kinetic Constants of PTU derivatives of Alkylamines in the Liver Transglutaminase catalyzed Methylamine incorporation into the B chain of oxidized Insulin

Inhibitor	Constant[a/]		
	Ki (mM) *	K (mM)	V[b/] (min^{-1})
PTU-$(CH_2)_2$-NH_2	N.I.		
PTU-$(CH_2)_3$-NH_2	0.99 ± 0.06	0.24 ± 0.01	3.09 ± 0.04
PTU-$(CH_2)_4$-NH_2	0.22 ± 0.02	0.23 ± 0.02	3.04 ± 0.05
PTU-$(CH_2)_5$-NH_2	0.049± 0.004	0.25 ± 0.02	2.49 ± 0.13
PTU-$(CH_2)_6$-NH_2	0.27 ± 0.02	0.26 ± 0.03	3.10 ± 0.16
Monodansylcadaverine	0.025± 0.002	0.24 ± 0.02	3.07 ± 0.09

a/ The values for the constants were obtained by fits to Equation 2. Ki, the inhibitor constant; K, the apparent Michaelis constant for methylamine; V, the apparent maximum velocity of methylamine incorporation.
b/ Values for V are given in nmoles of CH_3NH_2 incorporated/min/nmole of enzyme.
* N.I.: no inhibition.

Table III
Kinetic Constants of PTU derivatives of Alkylamines in the Liver Transglutaminase catalyzed Methylamine Incorporation into Z-Gln-Gly

Inhibitor	Constant[a/]		
	Ki (mM)	K (mM)	V (min^{-1})
PTU-$(CH_2)_2$-NH_2	N.I.*		
PTU-$(CH_2)_3$-NH_2	9.17 ± 0.8	0.36 ± 0.03	14.9 ± 0.8
PTU-$(CH_2)_4$-NH_2	2.54 ± 0.2	0.35 ± 0.02	15.6 ± 1.3
PTU-$(CH_2)_5$-NH_2	0.46 ± 0.04	0.40 ± 0.04	14.8 ± 0.1
PTU-$(CH_2)_6$-NH_2	1.08 ± 0.08	0.39 ± 0.03	15.9 ± 1.4
Monodansylcadaverine	0.25 ± 0.03	0.41 ± 0.04	14.6 ± 1.2

a/ The values for the constants were obtained by fits to Equation 2. Ki, the inhibitor constant; K, the apparent Michaelis constant for methylamine; V, the apparent maximum velocity of methylamine incorporation.
* N.I.: no inhibition.

Table IV
Kinetic Constants of PTU derivatives of Alkylamines in the Plasma Factor XIIIa catalyzed Methylamine Incorporation into Fibrinogen

Inhibitor	Constant[a/]		
	Ki (mM)	K (mM)	V (min^{-1})
PTU-$(CH_2)_2$-NH_2	N.I.*		
PTU-$(CH_2)_3$-NH_2	2.11 ± 0.2	1.25 ± 0.12	18.65 ± 0.99
PTU-$(CH_2)_4$-NH_2	1.45 ± 0.2	1.14 ± 0.11	17.56 ± 0.46
PTU-$(CH_2)_5$-NH_2	0.44 ± 0.04	1.38 ± 0.14	18.34 ± 0.72
PTU-$(CH_2)_6$-NH_2	1.06 ± 0.1	1.30 ± 0.12	19.09 ± 0.18
Monodansylcadaverine	0.21 ± 0.01	1.32 ± 0.10	19.01 ± 0.16

a/ The values for the constants were obtained by fits to Equation 2. Ki, the inhibitor constant: K, the apparent Michaelis constant for methylamine; V, the apparent maximum velocity of methylamine incorporation.
* N.I.: no inhibition.